Chapter 7

Effect of Alloxan-Diabetes and Subsequent Treatment with Insulin on Kinetic Properties of Cytochrome Oxidase Activity from Rat Brain Mitochondria

Introduction

Earlier studies from our laboratory have shown that, Streptozotocin (STZ)-diabetes significantly altered the oxidative energy metabolism in the rat brain mitochondria (1). Content of mitochondrial cytochromes decreased in STZ-diabetic animals; insulin treatment restored these values almost near normality (1). Also, the lipid/phospholipid profile of brain mitochondria changed significantly in alloxan-diabetes and after insulin treatment (2). Especially, treatment with insulin significantly increased the DPG component besides changes of small magnitude in PI, PS and SPM. Additionally, significant changes were also noted on kinetic properties of FoF_1 ATPase (complex V) in brain mitochondria (Chapter 4 of the Thesis).

Cytochrome oxidase (complex IV) is the terminal sink of electrons in the electron transport chain and the rate of respiration in mitochondria depends on cytochrome oxidase content (3). The membrane-embedded enzyme is surrounded by core lipids: mainly phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (4-6). DPG is absolutely essential for the enzyme activity (4-6). It was therefore of interest to find out if the aforementioned changes in cytochromes and lipid/phospholipid profile (1, 2) influenced the temperature kinetic properties of cytochrome oxidase at early and late stages of alloxan-diabetes. In parallel studies effects of insulin treatment were also examined. The results are described below.

Materials and Methods

N, N, N', N'-tetramethyl-*p*-phenylenediamine (TMPD) was purchased from Sigma Chemical Co., St. Louis, MO, U. S. A. Sources of other chemicals are detailed in Chapter 2 of the Thesis.

Detailed procedure for induction of diabetes, insulin treatment, isolation of mitochondria, and data analysis including regression analysis are as described in Chapter 2 of the Thesis.

Assay of cytochrome oxidase activity

Measurement of cytochrome oxidase activity was carried out polorographically 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 $CaCl_2$, 0.4 mM $AlCl_3$ and 10 mM sodium ascorbate (3). Concentration of TMPD was 100 μM. For the temperature kinetics studies, the temperature was varied from 5 to 53 °C with an increment of 4 °C at each step.

The cytochrome oxidase activity, v, is expressed as n mole O_2/min/mg protein.

Results

In the preliminary studies, cytochrome oxidase activity was determined at room temperature (25 °C) and at physiological temperature (37 °C). These data are given in Table 1. As can be noted, early diabetic state did not have any effect on the enzyme

activity whereas at the late stage the enzyme activity decreased by 12 and 22 % at the two temperatures. This may relate to the decreased cytochromes contents which we have reported earlier (1). Insulin treatment in one week diabetic animals significantly increased the enzyme activity (86 and 80 % increase at the two temperatures compared to control) while in one month diabetic animals insulin treatment was ineffective in restoring the activity to normality. The activity ratios were comparable under all experimental conditions (Table 1).

Since the insulin status differentially affected the enzyme activity at early and late stage, in the next set of experiment the temperature dependence of the enzyme activity was examined. The typical temperature curves and corresponding Arrhenius plots for the one week and one month groups are shown in Fig. 1 and 2 respectively. Under all the experimental conditions maximum activity was recorded at 53 °C. However, the temperature versus activity plots differed and the differences are self evident (Fig. 1 and 2, Panels A-C). The Arrhenius plot for the controls was chair shaped with two phase transition temperature around 15 and 36 °C and three energies of activation E_H, E_I and E_L for high, intermediate and low temperature ranges respectively. At the early stage of diabetes, enzyme activity displayed biphasic Arrhenius plot (Fig. 1 Panel E) and insulin treatment restored the plot to normality i.e. chair shaped (Fig 1, Panel F). However, the pattern was reversed compared to the control i.e. the value of E_I was higher than that for E_H (e.g. see Table 2). At the late stage, the pattern of Arrhenius plots for diabetic and insulin treated diabetic groups resembled to those for control (Fig. 2 Panels D-F).

Table 1. Effect of alloxan-diabetes and insulin treatment on cytochrome oxidase activity of rat brain mitochondria

Group	Treatment	Activity (nmole O_2 / min / mg protein)		Activity Ratio
		25 °C	37 °C	
One week	Control	33.57 ± 1.15	88.32 ± 6.56	2.63 ± 0.11
	Diabetic	35.60 ± 1.75	91.30 ± 4.22	2.56 ± 0.12
	Diabetic + Insulin	62.40 ± 0.79[b,§]	158.91 ± 7.96[b,§]	2.55 ± 0.09
One month	Control	34.10 ± 2.53	86.40 ± 5.16	2.53 ± 0.10
	Diabetic	30.10 ± 1.25	67.60 ± 1.72[a]	2.25 ± 0.12
	Diabetic + Insulin	27.50 ± 1.82	65.00 ± 3.23[a]	2.36 ± 0.11

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent observations in each group.

Activity ratio was calculated as: activity at 37 °C/activity at °C

a, $p<0.01$ and b, $p<0.001$ compared to the corresponding control.
§, $p<0.001$ compared to the corresponding diabetic.

Figure 1

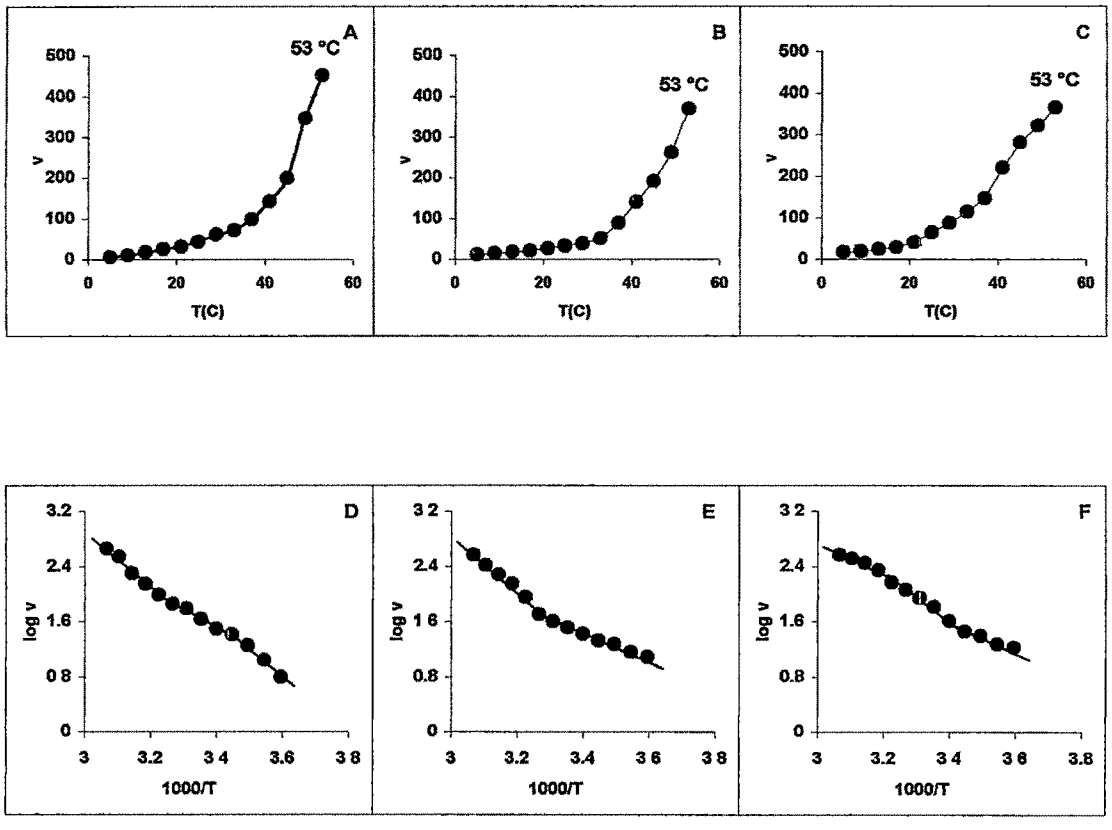
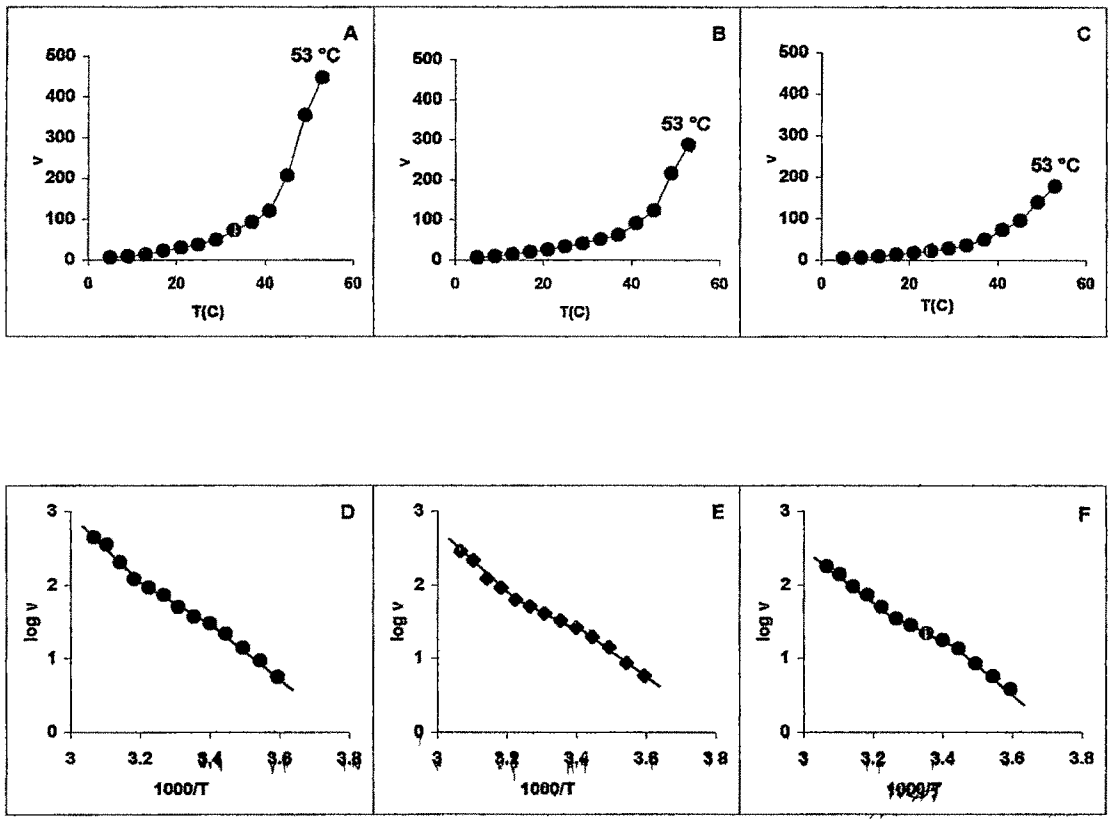


Figure 2


A
53 °C
v
T(C)
B
53 °C
v
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C
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D
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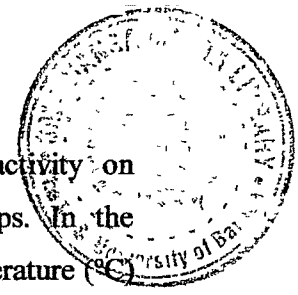

Figure 1. Typical plots depicting dependence of cytochrome oxidase activity on temperature and the corresponding Arrhenius plots for one week groups. In the temperature curves, the enzyme activity v, on ordinate is plotted versus temperature (°C) on abscissa. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots, log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celsius + 273.18). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 2. Typical plots depicting dependence of cytochrome oxidase activity on temperature and the corresponding Arrhenius plots for one month groups. In the temperature curves, the enzyme activity v, on ordinate is plotted versus temperature (°C) on abscissa. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Arrhenius plots, log of v on ordinate is plotted against 1000/T on abscissa where v and T represent respectively, the activity at corresponding absolute temperature T (temperature in ° Celsius + 273.18). D, E and F represent to the control, diabetic and insulin treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Table 2. Effect of alloxan-diabetes and insulin treatment on Arrhenius kinetics property of rat brain mitochondrial cytochrome oxidase

		Energy of activation KJ/mole			Phase transition temperature Tt (°C)	
Group	Treatment	E_H	E_I	E_L	T_{t1}	T_{t2}
One week	Control	95.30 ± 2.22	46.58 ± 1.65	87.13 ± 1.37	15.32 ± 0.68	36.78 ± 0.92
	Diabetic	76.81 ± 1.30[b]	—	39.40 ± 1.89[b]	29.45 ± 0.83[b]	—
	Diabetic + Insulin	43.75 ± 2.19[b,§]	89.60 ± 4.36[b]	33.20 ± 2.37[b]	19.18 ± 0.44[b,§]	34.12 ± 1.33
One month	Control	88.89 ± 4.03	41.45 ± 2.04	83.34 ± 3.48	15.03 ± 0.65	36.98 ± 1.42
	Diabetic	93.84 ± 2.62	45.03 ± 1.54	81.79 ± 2.27	13.01 ± 0.83	39.60 ± 1.75
	Diabetic + Insulin	82.95 ± 2.46	50.23 ± 2.69[a,]	76.00 ± 2.28	16.11 ± 0.97[§]	38.81 ± 1.43

The experimental details are given in the text. The results are given as mean ± SEM of 6-8 independent observations in each group.

a, $p<0.05$ and b, $p<0.001$ compared to the corresponding control.
§, $p<0.001$ compared to the corresponding diabetic.

The data on energies of activation and phase transition temperature(s) are given in Table 2. Thus, in the control animals the values for E_H and E_L were comparable whereas the value for E_I was almost half compared to the values for E_H and E_L. In early state of diabetes, the value for E_H decreased to some extent but that of E_L decreased considerably (55 % decrease). In the insulin treated animals a reversed picture was noted; the values for E_H and E_L decreased to less than half compared to the control whereas value of E_I became almost double. E_H, E_I and E_L were unchanged in one month diabetic and insulin treated diabetic animals. In early stage of diabetes the phase transition temperature (T_{t1}) reached an intermediate value of T_{t1} and T_{t2} of control. Following insulin treatment T_{t1} and T_{t2} became comparable to control. In one month group, diabetic state or insulin treatment had only marginal effects on T_{t1} and T_{t2} (Table 2).

Discussion

It is clear from the data presented that cytochrome oxidase activity did not change in early diabetic state whereas it decreased at the late stage. Insulin treatment caused hyper-stimulation in one week diabetic animals whereas in one month diabetic animals treatment with insulin was ineffective (Table 1).

Earlier studies from our laboratory have shown that in the liver mitochondria from diabetic rats after insulin treatment the contents of cytochromes aa_3 and $c + c_1$ increased significantly only in one month group (7). In the kidneys there was marginal increased in the content of cytochrome aa_3 under these conditions (8). Even in the brain mitochondria insulin treatment in one month diabetic animals tended to normalize cytochrome contents

(1). Viewed in this context it is unlikely that hyper-stimulation of the activity in early diabetic group following insulin treatment which observed here (Table 1) could be attributed to increased cytochromes content. On the other hand, the ineffectiveness of insulin in bringing to normality the cytochrome oxidase activity in one month diabetic animals may relate to the decreased in content of cytochromes which we have reported earlier (1).

One other factor underlying the observed differences could be the changes in the lipid core of the enzyme (4, 5) and also the membrane fluidity which could influence the electron transfer. That the temperature versus activity curves and Arrhenius plot were altered significantly and differentially at early and late stage in an insulin-status-dependent would lend support to such an assumption. Regression analysis therefore was carried out across the group to find out correlation between the enzyme activity, energies of activation and phase transition temperature(s). For the regression analysis the lipid/phospholipid and fluidity data from Chapter 4 of the Thesis were used. The data on regression coefficient r values are given in Table 3.

As can be noted, the enzyme activity at 25 and 37 °C correlated negatively with florescence polarization (p), thus it is directly correlated with the membrane fluidity since florescence polarization (p) is an inverse function of membrane fluidity (Table 3). The E_H correlated positively with cholesterol (CHL) content and showed negative correlation with membrane fluidity. The E_L showed positive correlation with total phospholipids and CHL contents whereas correlated negatively with membrane fluidity. The T_{tl} showed a

Table 3. Correlation between activity and kinetics parameters of cytochrome oxidase and membrane lipid/phospholipid composition and fluorescence polarization, p.

Parameter	Correlation with			
	Lipid / Phospholipid Class		Fluorescence polarization, p	
	Positive	Negative	Positive	Negative
Activity 25 °C	—	—		(- 0.870)
Activity 37 °C	—	—		(- 0.820)
E_H	CHL (+0.607)		(+ 0.772)	
E_L	TPL (+0.672) CHL (+0.538)		(+ 0.732)	
Tt_1	PC (+0.753) PE (+0.713)	TPL (-0.685) SPM (-0.770) PI (-0.763) PS (-0.645)		

The experimental details are given in the text.

Values given in the parentheses indicate r, regression coefficient which is based on 6-8 independent experiments in each group.

SPM: sphingomyelin; PC: phosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; TPL: total phospholipids; CHL: cholesterol.

strong positive correlation with PC and PE and correlated negatively with TPL, sphingomyelin (SPM), phosphatidylinositol (PI) and phosphatidylserine (PS) (Table 3). Thus, the core phospholipids PC and PE seem, to the some extent seem to have regulatory role in the temperature kinetics while DPG does not seem to play any regulatory role. In related studies we have shown that although content of DPG in the brain mitochondria is relatively much lower than in the other tissues, it is not rate limiting factor for the cytochrome oxidase activity (6).

One interesting feature of the present study is that in early diabetic state energies of activation in both high as well as low temperature ranges decreased significantly with the phase transition temperature assuming an intermediate value. This may possibly reflect a compensatory mechanism for maintaining the catalytic efficiency of the enzyme in early stage of diabetes. At the later stage of the disease the only significant change from corresponding control was a slight decrease in value of E_I. The results would imply that possibly at the late stage of the disease the membrane lipid milieu may realign itself to provide a semblance of normal lipid environment in order to maintain the catalytic efficiency of the enzyme. The fact that, insulin treatment did not significantly influence the Arrhenius pattern and energies of activation and phase transition temperature(s) (Table 2) would substantiate such assumption. It is possible that realignment of lipid microdomains would be an essential requirement, since the cytochrome oxidase is the terminal electron sink and its efficient functioning is vital to the cell. Although the content of DPG increased following insulin treatment, this may represent only influence

of DPG biosynthesis by mitochondria. Since regression analysis did not show involvement of DPG in any of parameters (Table 3).

Summary

The enzyme activity was unchanged in one week diabetic group whereas in one month diabetic animals the activity decreased by 22 %. Insulin treatment increased the activity almost by 2 fold in one week diabetic group but was ineffective in one month diabetic groups.

The Arrhenius pattern revealed that, like brain mitochondrial ATPase, the cytochrome oxidase activity displayed two phase transition temperatures and three values for energies of activation.

In controls, the values for E_H was highest followed by E_L and the values of E_I was lowest. The pattern changed only in one week diabetic group where only E_H and E_L were present; insulin treatment restored the Arrhenius pattern to normality but the values for E_I was highest followed by E_H and the values of E_L was lowest.

References

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