Chapter 2

Insulin-Status-Dependant Modulation of FoF_1 ATPase Activity in Rat Liver Mitochondria

Introduction

Diabetes mellitus is the condition characterized by defective glucose metabolism (1-4). However, it is also recognized that insulin also regulates practically all anabolic processes. These include transport of monosaccharide and amino acids, lipid synthesis, protein synthesis, turnover of RNA and phosphorylation of several metabolic intermediates (2). Thus, it has been reported that the plasma phospholipids profile changes in Type 1 and Type 2 diabetic patients and in rat model of diabetes (5-8). It has also been reported that in human diabetics the plasma phospholipid transport protein (PLTP) decreased significantly (6,7,9). Changes in phospholipid composition and cholesterol content of reticulocytes and erythrocytes in human diabetics have been reported (10-12). These changes are believed to be responsible for acceleration of maturation process and/or decreased life span of RBCs (12). Likewise, it has been shown that in experimental diabetes the phospholipid and fatty acid composition changes significantly in the liver microsomal membrane as well as in the rat heart microsomes (13,14). Specifically, the activity of 18:3 and 22:6 desaturase decreases resulting in decrease in unsaturation index (13,15). The changes in lipid metabolism have been correlated with nerve Na^+, K^+ ATPase activity (15) as well Na^+, K^+ ATPase activity in human placenta and sarcolemmal membrane from rat heart (13,16). A few studies have been also carried out to examine the effects of diabetes induced disturbances in mitochondrial function (17-19) and lipid metabolism (20,21).

The FoF_1 ATPase (factor V) of mitochondria is localized in the inner membrane and has requirement for specific phospholipids for its activity (22). The enzyme *in situ* utilizes

transmembrane proton gradient and membrane potential $\Delta\psi$, generated during substrate oxidation to synthesize ATP. The enzyme is reversible proton translocating ATPase and can generate transmembrane proton gradient and $\Delta\psi$ by hydrolyzing ATP (24-26). Thus, the enzyme is crucial for energy transduction functions in mitochondria (23-26). Because of its location in the inner membrane and specific requirement for phospholipids, the enzyme becomes an ideal candidate to evaluate the modulatory effects of diabetes-induced changes in mitochondrial lipid/phospholipid composition.

In the light of the above, the effects of alloxan-induced diabetes at early and late stages on the kinetic properties of rat liver mitochondrial FoF_1 ATPase and lipid/phospholipid profile were examined. Effects of insulin treatment were also evaluated. These findings are summarized in the present Chapter.

Materials and methods

Chemicals

Bovine serum albumin (BSA) fraction V, 1, 6 diphenyl - 1, 3, 5-hexatriene (DPH) and Sodium salt of ethylenediaminetetraacetic acid (EDTA) were from Sigma Chemical Co., St. Louis, MO, U. S. A. Sodium salt of adenosine 5'- triphosphate (ATP) was from SRL, Mumbai. Silica gel G was from E. Meark, Germany. NPH insulin (40 U/ml) was obtained from Lilli, France S.A.S. All other chemicals were of analytical-reagent grade and were purchased locally.

Animals

Adult male albino rats of Charles-Foster strain (weighing between 200-250 g) were used. The animals were fasted overnight and the diabetic state was induced by injecting 12 mg alloxan/ 100 g bogy weight subcutaneously (s. c.) (26-28). Alloxan solutions were prepared freshly in saline. The control rats received an equivalent volume of the vehicle.

Experiments were carried out at the end of one week or one month of induction of diabetic state to ascertain the early onset and long-term effects (17-19). Animals falling in the one week diabetic group received insulin from the fifth day of induction of diabetes for three days and the rats in one month diabetic group received insulin starting from the forth week of induction of diabetes for seven days at a dose of 0.8 units of NPH insulin / 100 g body weight twice daily (around 7:00 AM and 6:00 PM) by s. c. route (27, 28).

Confirmation of diabetic state

The diabetic state was confirmed by the following testes:

Polyuria: Metabolic cages were used to determined urine volume. It is a special stainless steel arrangement which enables easy collection of urine and the volume can be measured by a measuring cylinder.

Urine glucose estimation by Benedict's test

10 ml benedict's quantitative solution and 3-4 g anhydrous Na_2CO_3 was added and boiled. During boiling, diluted urine is slowly run from the burette. The end point of the

titration is complete disappearance of blur color and formation of flocculent white precipitate (12, 26) and Serum glucose estimation by Anthrone Method (28).

Isolation of mitochondria

The animals were killed by decapitation and the tissues were quickly removed and placed in beakers containing chilled (0-4 °C) isolation medium. The isolation medium for liver, kidneys and brain mitochondria and cytosolic fractions contained 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA and 250 µg BSA/ml. The isolation medium for heart mitochondria and cytosolic fraction contained 0.3 M sucrose, 5 mM MOPS, pH 7.4, 1 mM EDTA, 250 µg BSA/ml. The tissues were minced if necessary and washed repeatedly with the isolation medium to remove adhering blood and 10% (w/v) homogenates were prepared using a Potter Elvehjem type glass – Teflon homogenizer. The nuclei and cell debris were sedimented by centrifugation at 650 x g for 10 min and discarded. The supernatant was subjected to a further centrifugation at 7,500 x g for 10 min. The resulting mitochondrial pellet was washed by suspending gently in the isolation medium and by resedimenting at 7,500 x g for 10 min. Finally the mitochondria were suspended in the isolation medium (29).

ATPase assay

ATPase activity was measured in the assay medium (total volume 0.1 ml) containing 50 mM Tris-HCl buffer pH 7.4, 75 mM KCl, 0.4 mM EDTA, 6 mM $MgCl_2$ and 100 µM 2, 4 – dinitrophenol (DNP). After pre-incubating the mitochondrial protein (30-50 µg) in the assay medium at 37 °C for 1 min, the reaction was initiated by the addition of ATP at a

final concentration of 5 mM (17, 30). The reaction was terminated after 10 min by addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution and the amount of librated inorganic phosphorus was estimated by the method of Katewa and Katyare (31).

For the substrate kinetics studies concentration of ATP was varied in the range from 0.1 mM to 5 mM.

For temperature kinetics studies, experiments were carried out with fixed ATP concentration (5 mM) and the temperature was varied from 5 to 53 °C with an increment of 4 °C at each step.

Reaction velocity v, is expressed as µmole of Pi liberated / hr / mg protein.

The data for substrate kinetics were analyzed using Sigma Plot version 6.1 by three methods i.e. the Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots for the determination of Km and Vmax (32). The values of Km and Vmax obtained by three methods were in close agreement and were averaged.

The Kcat or turnover number which represents the number of substrate molecules transformed S^{-1} was derived from corresponding Vmax values by using the formula

$$Kcat = \frac{Vmax\ (moles) \times N}{3600 \times 5.60 \times 10^5}$$

where N is Avogadro's Number and 5.60×10^5 is molecular weight of FoF_1 ATPase (24,25,33). Values of Kcat/Km were obtained as ratio of Kcat/Km [M] (34). Since the Vmax represents µmoles of ATP hydrolyzed / mg protein / hr, the Kcat/Km values thus calculated refer to unit mitochondrial protein.

The data on temperature kinetics were analyzed for determination of energies of activation in the high and low temperature ranges (E_H and E_L respectively) and phase transition temperature (T_t) according to the method described previously (33).

Lipid Analysis

Extraction of lipids

Aliquots of mitochondrial suspension containing 4-8 mg protein were extracted with 4 ml of freshly prepared chloroform: methanol mixture (2:1 v/v) as described earlier (35,36). The tubes were vortexed vigorously, allowed to stand at room temperature and the organic phase was carefully removed with the help of a broad gauge syringe. The samples were re-extracted with 3 ml of chloroform: methanol mixture as above and the resulting organic phases were pooled. The pooled chloroform: methanol extracts were treated with 0.1 volume of 0.017% $MgCl_2$, vortexed vigorously, allowed to stand at room temperature and organic phase was carefully removed with care being taken to avoid the proteolipid layer appearing between the organic and aqueous phases. The solvent was completely evaporated under the stream of nitrogen, after which the lipids were dissolved in known volume of chloroform: methanol mixture. Suitable aliquots were taken for the

estimation of cholesterol (37) and phospholipid phosphorous in the sample (38) and thin layer chromatography (TLC).

Separation of phospholipids by thin layer chromatography (TLC)

Separation of phospholipid classes was carried out by one dimetional thin layer chromatography (36,39) using silica gel G (6 g/13 ml distilled water per plate) was prepared by gentle mixing and spared on glass plates with the help of applicator with thickness of layer maintained to 0.25 mm. the layer was allowed to dry by leaving plates overnight at room temperature. Prior to use the plates were activated in an oven at 100 °C for 20-25 min.

Aliquots of the reconstituted samples containing 8-10 μg of phospholipid phosphorus were spotted on TLC plate in a way such that the diameter of the spot was minimum which ensure batter resolution. The conditions for preparation of TLC plates, chamber saturation etc were according to Stahl (40). The solvent used for the chamber saturation was chloroform: methanol: acetic acid: water (25:15:4:2 v/v). Before run, the plates were reactivate for 2 min at 110 °C. After the run was completed the plates were taken out and kept at room temperature for 3-4 hrs to remove the solvents.

After brief exposure of iodine vapor spots of individual phospholipid were marked and iodine was allowed to sublime off. After this the spots were carefully scraped and transferred to marked test tubes. To each tube 10 N of H_2SO_4 were added and the sample were heated on sand bath for 8-10 hrs. The tubes were allowed to cool after which 0.1 ml

of 70 % perchloric acid (PCA) was added. The tubes were then heated on sand bath for 3-4 hrs till the solution in tubes were clear and smell of chlorine was undetectable. The analysis of phosphorus content was according to the procedure of Bartlett (38).

The content of individual phospholipid classes were calculated by multiplying the values of TPL with % composition of individual phospholipid classes (36).

Determination of membrane fluidity

Membrane fluidity determination was carried out at 25 °C spectrophotoflourimetrically using DPH probe. Stock DPH solution (2 mM) was prepared in tetrahydrofuran and stored at 0-4 °C in an amber colored bottle. For measurement of fluorescence polarization, samples were taken in 3 ml of buffered sucrose solution (0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4) at a final protein concentration of 0.2 mg/ml, and an aliquot of stock DPH solution was added so that the molar ratio of probe to lipid was between 1:200 to 1:300 (41,42). The mixture was vortexed vigorously and left in dark for 30 min to permit equilibration of probe into membranes. Fluorescence polarization was measured in a Shimadzu RF 5000 spectrophotofluorimeter with a polarizer attachment. Excitation and emission wavelength were 360 nm and 430 nm; bandwidths were 5 nm and 10 nm respectively. Data were accumulated for 5 sec for each polarization setting: vertical (parallel) and horizontal (perpendicular) (43). The instrument has program for calculation of fluorescence polarization (p) from which value of r, r_α, and S can be calculated. The details of the methods have been described previously (43).

The regression analysis was carried out across the groups using Jandel Sigmastat Statistical Software, version 2.0.

The activity ratio was calculated by: Activity at 37 °C / Activity at 25 °C

Estimation of protein was by the procedure of Lowry et al. (44) using bovine serum albumin as the standard.

Statistical evaluation of data was by Students' t-test.

Results

Alloxan diabetes resulted in 14 and 34 % reduction in the body weight respectively at the end of one week and one month. Insulin treatment had marginal restorative effect in one week group while in the one month diabetic animal insulin treatment almost completely restored the body weight (Table 1).

The liver weight of the alloxan-diabetic rats decreased, by 20 % and 39 % respectively at the end of one week and one month. Treatment with insulin brought about significant increase in the liver weight not only compared to the diabetic groups (115 and 135 % increase respectively), but also beyond the corresponding values for control groups (73 and 44 % increase respectively) (Table 1). As a results of which, relative liver weight remained more or less same in both diabetic condition whereas increased significantly in insulin treated one week and month diabetic animals (Table 1).

Table 1. Effect of alloxan-diabetes and insulin treatment on body weight and liver weight of the rats

Group	Treatment	Final body weight, g	Liver weight	
			g	% Body weight
One week	Control	241.1 ± 5.7	8.05 ± 0.36	3.33 ± 0.14
	Diabetic	207.0 ± 5.8[b]	6.48 ± 0.30[a]	3.13 ± 0.12
	Diabetic + insulin	226.4 ± 8.8	13.98 ± 0.73[c,§]	6.17 ± 0.23[c,§]
One month	Control	273.2 ± 4.9	8.96 ± 0.51	3.28 ± 0.22
	Diabetic	181.4 ± 4.8[c]	5.48 ± 0.76[c]	3.02 ± 0.15
	Diabetic + insulin	290.1 ± 6.8[§]	12.89 ± 0.84[c,§]	4.44 ± 0.24[a,§]

The experimental details are given in the text. The results are given as mean ± SEM of 12-16 independent experiments in each group.

a, $p<0.01$; a, $p<0.002$ and a, $p<0.001$ compared to the corresponding control.
§, $p<0.001$ compared to the corresponding diabetic.

The levels of glucose in the serum increased by 3.6 and 4.6 fold in the two diabetic groups. Insulin treatments had partial restorative effect and in the insulin treated diabetic rats the serum glucose levels were always higher compared to those in the corresponding control groups (Table 2). Polyuria in the diabetic state was reflected in terms of 22 and 25 fold increase in the urine volume respectively in the two diabetic groups. Insulin treatments restored the urine volume to normality (2.0-2.5 ml/24 h). The urinary sugar excretion amounted to about 1.0-1.25 g / 24 h in the two diabetic groups; insulin treatment completely abolished the urinary sugar excretion (Table 2). These results are consistent with our previously reported observations (17-19).

In preliminary studies, the effect of alloxan-diabetes and subsequent insulin treatment on ATPase activity under different conditions was examined (Table 3). As can be noted, addition of Mg^{2+} stimulated the ATPase activity and addition of DNP showing maximum stimulation. Activity was intermediate in presence of both Mg^{2+} and DNP. Alloxan-diabetes stimulate the basal (-Mg^{2+} and – DNP) activity and the effect was more pronounce in early state whereas opposite effect was noted for Mg^{2+} and DNP stimulated activity and the decreased were more at the later state of diabetes. Insulin treatment was ineffective in restoring the ATPase activity to normality (Table 3).

Measurements of ATPase activity were carried out at 25 and 37 °C and the data are given in the Table 4. As can be noted, the enzyme activities increased with the temperature (Table 4). The enzyme activity was not affected in one week diabetic group and insulin

Table 2. Effect of alloxan-diabetes and insulin treatment on diabetes parameters.

Group	Treatment	Serum glucose level, mM	Urine volume, ml/24 h	Urine sugar, mg/ml	Sugar excretion, g/24 h
One week	Control	4.21 ± 0.35	2.53 ± 0.17	ND	ND
	Diabetic	15.02 ± 0.65[c]	45.63 ± 1.08[c]	22.44 ± 0.32	1.02 ± 0.03
	Diabetic + insulin	6.01 ± 0.35[a,§]	3.37 ± 0.12[b,§]	ND	ND
One month	Control	4.31 ± 0.33	2.71 ± 0.22	ND	ND
	Diabetic	19.69 ± 0.98[c]	54.61 ± 1.19[c]	22.87 ± 0.52	1.24 ± 0.03
	Diabetic + insulin	6.63 ± 0.39[c,§]	3.01 ± 0.15[§]	ND	ND

The experimental details are given in the text. The results are given as mean ± SEM of 12-16 independent experiments in each group.

a, $p<0.05$; b, $p<0.002$ and c, $p<0.001$ compared to the corresponding control.
§, $p<0.001$ compared to the corresponding diabetic.

Table 3. Effect of alloxan-diabetes and insulin treatment on rat liver mitochondrial FoF_1 ATPase.

		Activity, μmole of Pi liberated / hr / mg protein			
Group	Treatment	Basal	+ Mg	+DNP	+Mg, + DNP
One week	Control	0.79 ± 0.05	6.68 ± 0.31	18.21 ± 0.57	13.45 ± 0.54
	Diabetic	2.02 ± 0.11[c]	5.64 ± 0.23[a]	15.22 ± 0.47[b]	13.56 ± 0.54
	Diabetic + Insulin	1.40 ± 0.12[c,Ψ]	2.39 ± 0.20[c,§]	16.59 ± 0.55[b]	13.03 ± 0.44
One week	Control	0.76 ± 0.05	6.46 ± 0.30	17.61 ± 0.55	12.99 ± 0.52
	Diabetic	1.04 ± 0.08[b]	3.58 ± 0.17[c]	7.54 ± 0.30[c]	5.96 ± 0.13[c]
	Diabetic + Insulin	1.40 ± 0.07[c,Ψ]	4.91 ± 0.19[c,§]	20.79 ± 0.87[c,§]	13.88 ± 0.76[§]

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

a, $p<0.05$; b, $p<0.002$ and c, $p<0.001$ compared to the corresponding control.
Ψ, 0.01 and §, $p<0.001$ compared to the corresponding diabetic.

Table 4. Effect of alloxan-diabetes and insulin treatment on FoF_1 ATPase activity in rat liver mitochondria

Group	Treatment	Activity (μmole Pi liberated/hr/mg protein)		Activity Ratio
		25 °C	37 °C	
One week	Control	5.39 ± 0.42	13.44 ± 0.81	2.49 ± 0.12
	Diabetic	5.91 ± 0.46	13.24 ± 0.38	2.24 ± 0.11
	Diabetic + Insulin	6.15 ± 0.37	13.34 ± 0.60	2.17 ± 0.14
One month	Control	5.14 ± 0.40	11.96 ± 0.46	2.33 ± 0.15
	Diabetic	2.17 ± 0.17[a]	5.03 ± 0.36[a]	2.32 ± 0.13
	Diabetic + Insulin	7.46 ± 0.58[a,§]	14.31 ± 0.80[a,§]	1.92 ± 0.12

The experimental details are given in the text.

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

The results are given as mean ± SEM of 6-8 independent experiments in each group.

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

treatment had no effect. By contrast, in one month diabetic animals the activity decreased by 58 % and insulin treatment resulted in hyper-stimulation (64.6 % increase). The activity ratios remain more or less same under all the experimental conditions (Table 4).

In view of the differential changes in one week and one month groups further experiments were carried out to examine the kinetic behavior of the enzyme as a response to change in the substrate i.e. ATP concentration. Analysis in terms of Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden plots (32) revealed that under all experimental conditions, the enzyme resolved in three kinetically distinguishable components. The kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate. The typical substrate saturation curve (Fig. 1 and 2, Panels A, B, C) and corresponding Eadie-Hofstee plots (Fig. 1 and 2, Panels D, E, F) are shown. Data on Km and Vmax values of the three components as affected by insulin status are included in Table 5. Thus, in one week diabetic group, both Km and Vmax of component I increased while Km of component II decreased. Insulin treatment restored the Km of component I and decreased the Vmax of component I. For component II both Km and Vmax decreased. Long term diabetic state had a general Vmax lowering effects and the Km of component I and II decreased. Treatment with insulin restored Vmax values in general near normality while Km of component I increased by 2 fold and that of component II was restored.

In view of the observed changes in the Km and Vmax values under the different experimental conditions, the relative efficiency of the ATPase in terms of Kcat / Km

Table 5. Effect of alloxan-diabetes and insulin treatment on substrate kinetics properties of FoF_1 ATPase in rat liver mitochondria

Group	Treatment	Component I		Component II		Component III	
		Km	Vmax	Km	Vmax	Km	Vmax
One week	Control	0.17 ± 0.01	4.24 ± 0.12	1.13 ± 0.05	11.97 ± 0.33	2.78 ± 0.09	21.68 ± 0.50
	Diabetic	0.21 ± 0.01[c]	5.37 ± 0.27	0.95 ± 0.05[a]	11.11 ± 0.28	3.00 ± 0.17	21.59 ± 0.53
	Diabetic + Insulin	0.16 ± 0.01[ψ]	5.08 ± 0.14[d]	0.69 ± 0.04[d,§]	9.93 ± 0.23[d,ψ]	1.98 ± 0.09[d,§]	16.62 ± 0.58[d,§]
One month	Control	0.18 ± 0.01	4.08 ± 0.11	1.13 ± 0.05	11.97 ± 0.33	3.36 ± 0.20	22.40 ± 0.64
	Diabetic	0.12 ± 0.01[b]	2.03 ± 0.08[d]	0.82 ± 0.04[d]	4.57 ± 0.19[d]	3.02 ± 0.12	8.93 ± 0.29[d]
	Diabetic + Insulin	0.40 ± 0.02[d,§]	5.66 ± 0.17[d,§]	1.18 ± 0.07[§]	10.51 ± 0.47[a,§]	3.03 ± 0.16	18.01 ± 0.58[d,§]

The experimental details are given in the text. The Km (mM) and Vmax (μmole of Pi liberated / hr / mg protein) values were calculated as described in the text.

The results are given as mean ± SEM of 6-8 independent experiments in each group. As indicated in the text, the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate.

a, $p< 0.05$; b, $p< 0.01$; c, $p< 0.002$ and d, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.05$ and §, $p<0.001$ compared to the corresponding diabetic.

Figure 1. Typical substrate saturation curve (Panels A, B, C), corresponding Eadie-Hofstee (panels D, E, F) and Hill (panels G, H, I) plots for FoF_1 ATPase from liver mitochondria for one week group. In substrate saturation curve the enzyme activity v on abscissa is plotted versus [S] on ordinate v is the enzyme activity at the given ATP concentration [S]. A, B and C represent to the control, diabetic and insulin-treated diabetic groups. In Eadie-Hofstee plot the enzyme activity v on abscissa is plotted versus v/[S] on ordinate. D, E and F represent to the control, diabetic and insulin-treated diabetic groups. In Hill plot log (v/Vmax-v) on abscissa is plotted versus log [S] on ordinate. The Hill coefficients n_1 and n_2 depict number of ATP molecules bound for the given concentration range of ATP. G, H and I represent to the control, diabetic and insulin-treated diabetic groups. The plots are typical of 6-8 independent experiments in each group.

Figure 1

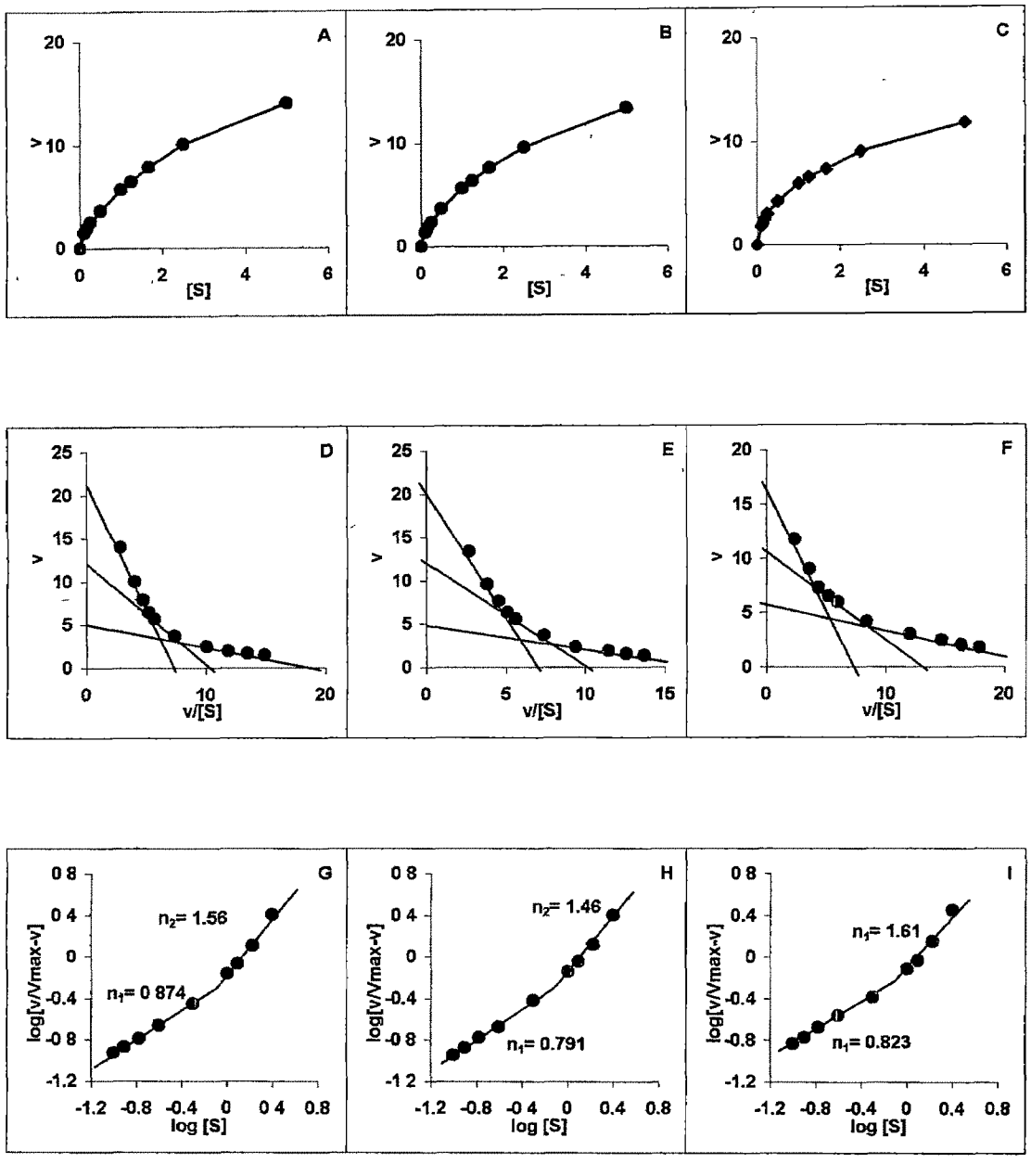
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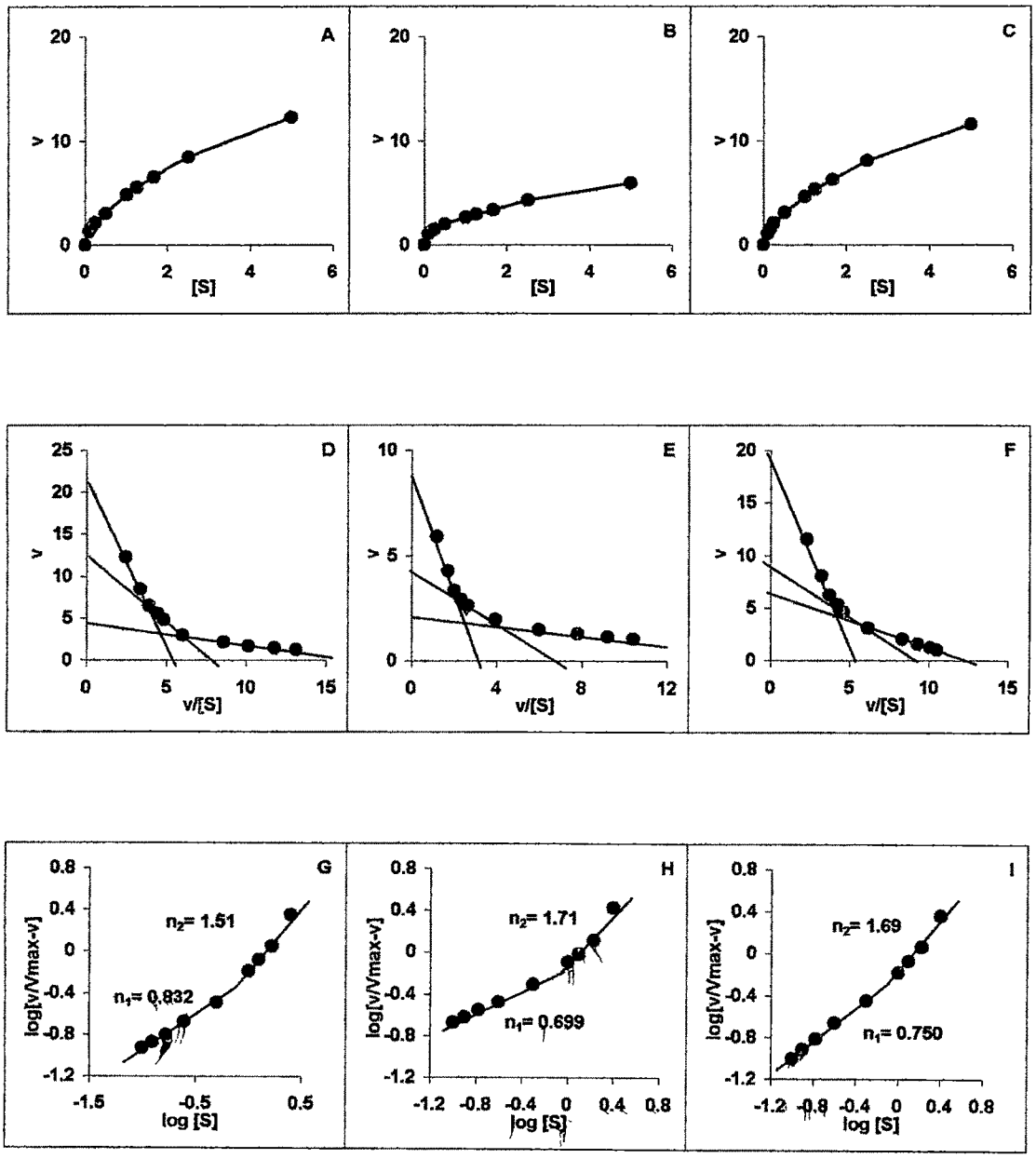
Figure 2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n_2= 1.71
n_1= 0.699
n_2= 1.69
n_1= 0.750

values was computed (34). As described in the text, Kcat represent the number of substrate molecule transformed per molecule of the enzyme per second (34). However, since it is not possible to know the number of enzyme molecules/mg of mitochondrial protein, we normalized the ^{App}Kcat / Km values as detailed in the 'Materials and Methods' section and designated these values as ^{App}Kcat / Km.

The data for ^{App}Kcat / Km are given in Table 3. As is evident, one week diabetic state did not affect the efficiency of any of the kinetic components of the enzyme. Treatment with insulin reduced the efficiency of component III. One month diabetic state increased the efficiency of components I and II; insulin treatment, in general, reduced the efficiency of all the three kinetic components; the effect being most pronounced on component I (Table 6)

Analysis of substrate kinetics data by Hill plots indicated that up to 1.0 mM ATP concentration one ATP molecule was bound while beyond this concentration of substrate two molecules of ATP were bound under all experimental conditions. Since the patterns were similar for all experimental groups only a typical Hill plots are shown in Fig. 1 and 2 Panels G, H and I.

In the next set of experiments, the effect of alloxan diabetes and insulin treatment on the temperature-dependant-change in the enzyme activity was examined. The data were then

Table 6. Effect of alloxan-diabetes and insulin treatment on Apparent Kcat/Km (^{App}Kcat/Km) values for the kinetic components of FoF_1 ATPase in rat liver mitochondria

Group	Treatment	^{App}Kcat/Km x 10^{11}		
		Component I	Component II	Component III
One week	Control	5.66 ± 0.21	2.44 ± 0.10	1.79 ± 0.12
	Diabetic	5.70 ± 0.25	2.77 ± 0.20	1.62 ± 0.01
	Diabetic + Insulin	5.18 ± 0.30	2.34 ± 0.10	1.35 ± 0.01[a,§]
One month	Control	5.98 ± 0.62	2.44 ± 0.25	1.82 ± 0.16
	Diabetic	10.55 ± 0.58[b]	3.57 ± 0.23[a]	1.87 ± 0.10
	Diabetic + Insulin	2.61 ± 0.13[b,§]	1.64 ± 0.07[a,§]	1.11 ± 0.01[b,§]

The experimental details are given in the text. The values of Kcat/ Km were computed as described in the text. The results are given as mean ± SEM of 6-8 independent experiments in each group.

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

Figure 3. Typical plots depicting dependence of enzyme activity on the temperature and corresponding Arrhenius plots for one week groups. In temperature curves, enzyme activity v on abscissa is plotted verses temperature (°C) on ordinate. 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 ° Celcius + 273.2). 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 3

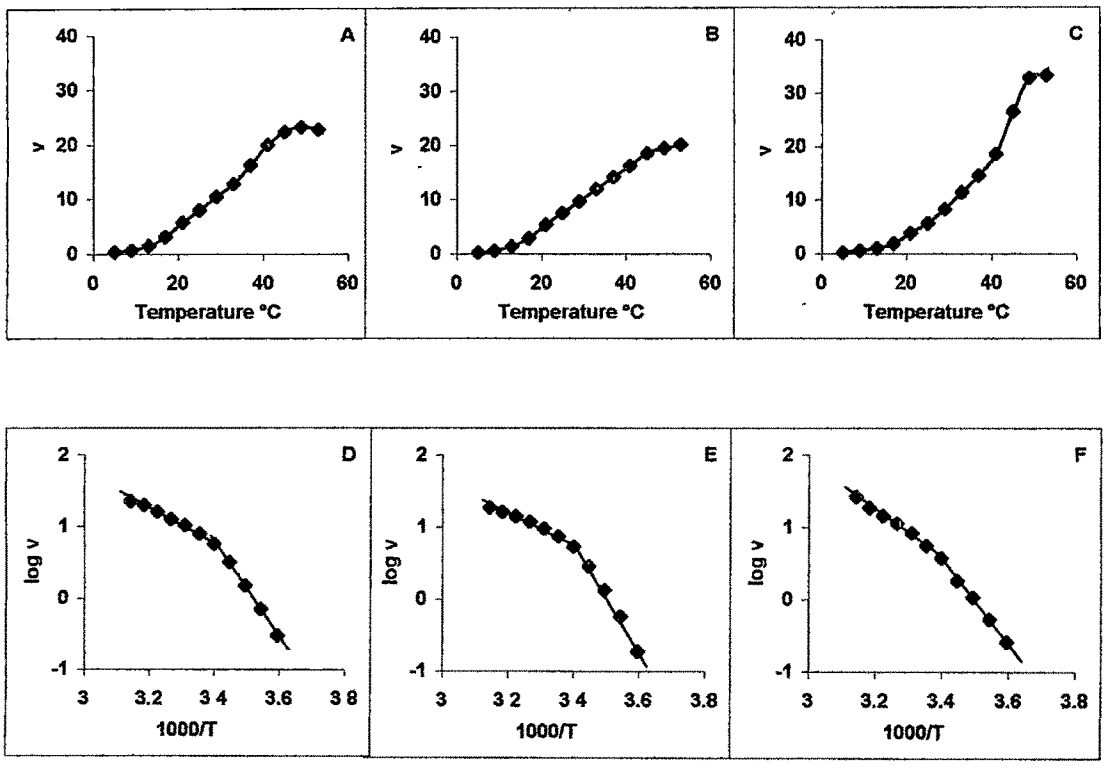
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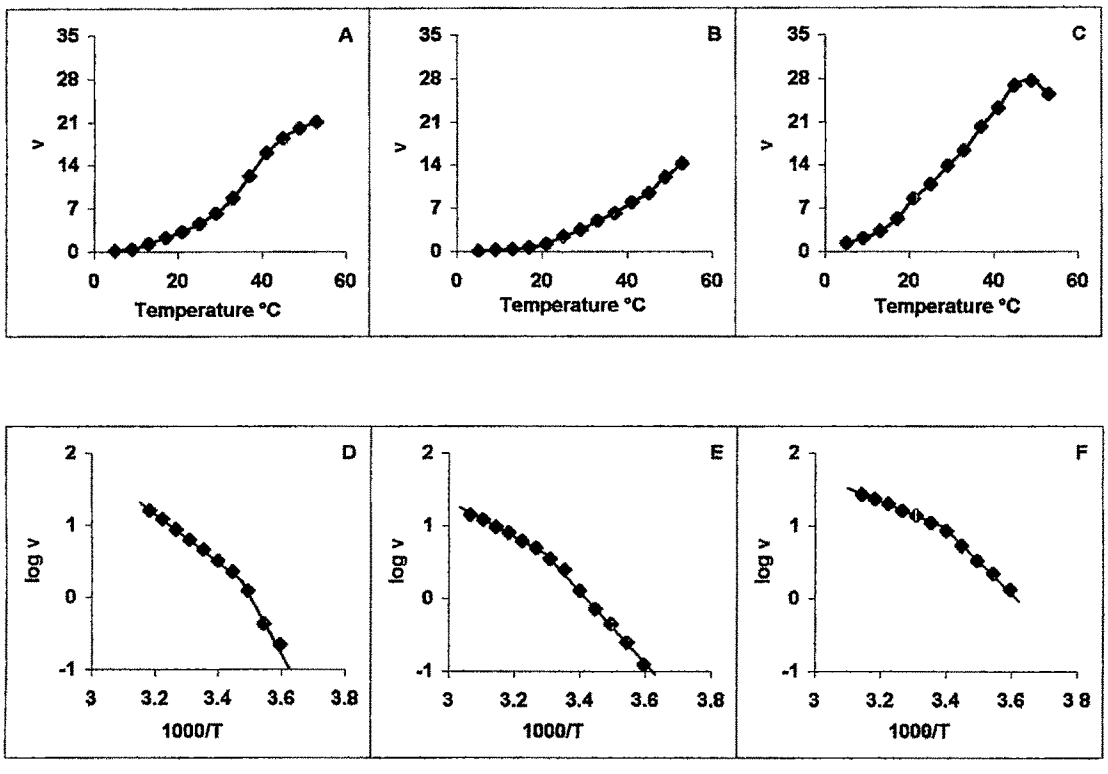
Figure 4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3.8

analyzed in terms of Arrhenius plots to find out energies of activation (E_H and E_L) and phase transition temperature (T_t). The typical activity versus temperature curves and corresponding Arrhenius plots are shown in Fig. 3 and 4 respectively for one week and one month groups. As can be noted in both the groups the pattern of activity versus temperature and the corresponding Arrhenius plots for diabetic and insulin treated diabetic animals differed considerably from the control. The differences were reflected in terms of the energies of activation in high and low temperature ranges (E_H and E_L respectively) and phase transition temperature (T_t). These data are summarized in Table 7. Thus, in one week diabetic animals the value of E_2 decreased. Insulin treatment lowered the value of E_1 (Table 7). In one month diabetic group the pattern was repeated and the decrease in E_2 was more accentuated. The generalized feature in both the groups was increased in T_t in diabetes which could not be corrected by insulin treatment in one month group (Table 7).

Since the insulin-status significantly altered the kinetic properties of ATPase, the effects of insulin-status on lipid/phospholipid profiles of the mitochondria were examined. These results are given in Tables 8-10. In one week diabetic group the content of total phospholipid (TPL) and cholesterol (CHL) did not change significantly and insulin treatment had no effect. By contrast, in one month diabetic group the TPL content decreased (32 % decreased) and CHL content increased by 51%. This was reflected in terms of decreased molar ratio of the two entities. Insulin treatment restored CHL content but had marginal effect on TPL content (Table 8).

Table 7. Effect of alloxan-diabetes and insulin treatment on Arrhenius kinetics properties of $FoF_1ATPase$ in rat liver mitochondria

		Energy of activation (KJ/mole)		Phase transition temperature T_t (°C)
Group	Treatment	E_H	E_L	T_t
One week	Control	57.54 ± 2.46	137.2 ± 5.14	18.83 ± 0.67
	Diabetic	51.48 ± 3.33	102.0 ± 5.93[c]	21.26 ± 0.22[b]
	Diabetic + Insulin	44.73 ± 2.04[b]	108.8 ± 5.31[b]	20.10 ± 0.78
One month	Control	57.20 ± 1.58	140.8 ± 4.99	17.71 ± 0.47
	Diabetic	52.80 ± 2.15	83.49 ± 5.21[c]	22.03 ± 1.35[a]
	Diabetic + Insulin	45.31 ± 3.10[b]	70.62 ± 4.70[c]	23.72 ± 1.68[b]

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

a, $p< 0.02$; b, $p< 0.01$ and c, $p<0.001$ compared to the corresponding control.

Table 8. Effects of alloxan diabetes on total phospholipids (TPL), cholesterol (CHL), content of rat liver mitochondria

Groups	Treatment	TPL (μg/mg protein)	CHL (μg/mg protein)	TPL/CHL (mole:mole)
One week	Control	176.21 ± 3.05	53.88 ± 1.73	1.66 ± 0.06
	Diabetic	179.42 ± 5.07	56.27 ± 2.13	1.60 ± 0.05
	Diabetic + Insulin	183.08 ± 7.40	52.23 ± 0.84	1.75 ± 0.07
One month	Control	172.83 ± 2.47	52.81 ± 1.41	1.65 ± 0.04
	Diabetic	118.14 ± 7.58^{c}	79.83 ± 1.88^{c}	0.74 ± 0.05^{c}
	Diabetic + Insulin	138.19 ± 5.27$^{c,\psi}$	47.55 ± 1.04^{b,§}	1.46 ± 0.07^{a,§}

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

a, $p< 0.05$; b, $p< 0.01$ and c, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.05$ and §, $p<0.001$ compared to the corresponding diabetic.

Analysis of phospholipid profile (Table 9) reveled that diabetic state had a generalized effect of increasing lysophospholipids (Lyso), sphingomylein (SPM), phosphatidylinositol (PI) and phosphatidylserine (PS) and a tendency to decrease phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Insulin treatment could correct the Lyso component and partially correct the PC and PE composition (Table 9). The computed contents of the individual phospholipid classes were generally consistent with the above data (Table 10).

Changes in the levels of cholesterol and altered phospholipid composition and contents could alter the fluidity of the membrane. This was ascertained by measuring the fluidity of the membranes. It is apparent form the data in Table 11 that the alloxan-diabetes resulted in decreased membrane fluidity. Insulin treatment in one week diabetic animals completely restored the membrane fluidity whereas in one month diabetic animals insulin had only marginal restorative effect (Table 11).

Discussion

As can be noted form the results, ATPase activity did not change in the early stage of alloxan diabetes as well as subsequent treatment with insulin whereas at the late stage the enzyme activity decreased to a great extent and insulin treatment caused hyper-stimulation (Table 3). These data supports our earlier findings on the oxidative phosphorylation in the liver mitochondria which was not affected by early diabetic stage

Table 9. Effects of alloxan-diabetes and insulin treatment on phospholipid composition of rat liver mitochondria.

Phospholipid Class	Composition (% of total)					
	One week			One month		
	Control	Diabetic	Diabetic + Insulin	Control	Diabetic	Diabetic + Insulin
Lyso	1.54 ± 0.04	4.70 ± 0.30^{e}	1.97 ± 0.11^{e,¶}	1.99 ± 0.06	4.98 ± 0.31^{e}	1.18 ± 0.08^{e,¶}
SPM	2.82 ± 0.15	5.21 ± 0.23^{e}	2.87 ± 0.12¶	2.92 ± 0.17	4.75 ± 0.15^{e}	5.25 ± 0.29^{e}
PC	45.09 ± 0.41	43.67 ± 0.53^{a}	45.03 ± 0.45	44.57 ± 0.61	41.41 ± 0.73^{c}	42.91 ± 0.50^{a}
PI	1.64 ± 0.09	2.19 ± 0.11^{d}	3.16 ± 0.20^{e,¶}	1.98 ± 0.09	4.66 ± 0.17^{e}	3.74 ± 0.22^{e,¶}
PS	1.65 ± 0.06	2.32 ± 0.10^{c}	2.93 ± 0.17^{b,ψ}	1.79 ± 0.08	5.53 ± 0.19^{e}	2.77 ± 0.18^{e,ψ}
PE	36.31 ± 0.39	30.91 ± 0.66^{e}	31.49 ± 0.59^{e}	35.11 ± 0.22	24.40 ± 0.42^{e}	32.19 ± 0.47^{e,¶}
DPG	10.91 ± 0.13	10.92 ± 0.34	12.56 ± 0.26^{e,¶}	12.01 ± 0.64	14.29 ± 0.62^{b}	12.02 ± 0.23§

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

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

a, $p< 0.05$; b, $p< 0.02$; c, $p< 0.01$; d, $p<0.002$ and e, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.01$; §, $p<0.002$ and ¶, $p<0.001$ compared to the corresponding diabetic.

Table 10. Effects of alloxan-diabetes and insulin treatment on content of individual phospholipids in rat liver mitochondria

Phospholipid Class	Content, μg / mg protein					
	One week			One month		
	Control	Diabetic	Diabetic + Insulin	Control	Diabetic	Diabetic + Insulin
Lyso	2.71 ± 0.09	8.44 ± 0.61^{d}	3.59 ± 0.22^{c,¶}	3.80 ± 0.15	5.59 ± 0.53^{d}	1.67 ± 0.11^{d,¶}
SPM	4.97 ± 0.28	9.36 ± 0.54^{d}	5.23 ± 0.26¶	4.95 ± 0.16	5.37 ± 0.46	7.19 ± 0.43^{d,ψ}
PC	79.61 ± 1.67	78.36 ± 2.27	82.51 ± 3.61	77.11 ± 1.23	47.57 ± 4.51^{d}	58.91 ± 1.84^{d,ψ}
PI	2.89 ± 0.16	3.93 ± 0.21^{c}	5.77 ± 0.44^{d,§}	3.22 ± 0.18	5.39 ± 0.58^{c}	5.12 ± 0.32^{d}
PS	2.91 ± 0.13	4.16 ± 0.24^{d}	5.39 ± 0.44^{d,ψ}	2.90 ± 0.11	6.37 ± 0.63^{d}	3.80 ± 0.27^{b,¶}
PE	64.02 ± 1.15	55.52 ± 1.89^{d}	57.63 ± 2.28^{a}	62.52 ± 1.04	27.82 ± 2.48^{d}	44.37 ± 0.18^{d,¶}
DPG	19.16 ± 0.40	19.71 ± 0.87	23.07 ± 1.20^{b,ψ}	20.07 ± 0.39	15.91 ± 1.12^{d}	16.41 ± 0.46^{d}

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

a, $p< 0.02$; b, $p< 0.01$; c, $p<0.002$ and d, $p<0.001$ compared to the corresponding control.
ψ, $p< 0.05$; §, $p<0.002$ and ¶, $p<0.001$ compared to the corresponding diabetic.

Table 11. Effects of alloxan-diabetes and insulin treatment on fluidity parameters of liver mitochondria.

Groups	Treatment	Fluidity Parameters			
		Fluorescence Polarization, p	Fluorescence anisotropy, r	Limited hindered anisotropy, rα	Order parameter, S
One week	Control	0.151 ± 0.006	0.107 ± 0.004	0.043 ± 0.003	0.313 ± 0.024
	Diabetic	0.205 ± 0.003[a]	0.147 ± 0.003[a]	0.096 ± 0.003[a]	0.489 ± 0.008[a]
	Diabetic + Insulin	0.146 ± 0.002[§]	0.103 ± 0.002[§]	0.037 ± 0.002[§]	0.301 ± 0.009[§]
One month	Control	0.153 ± 0.006	0.111 ± 0.006	0.046 ± 0.007	0.321 ± 0.009
	Diabetic	0.254 ± 0.005[a]	0.185 ± 0.004[a]	0.146 ± 0.005[a]	0.604 ± 0.010[a]
	Diabetic + Insulin	0.201 ± 0.002[a,§]	0.143 ± 0.002[a,§]	0.091 ± 0.002[a,§]	0.477 ± 0.006[a,§]

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

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

but at later stage the energy coupling efficiency decreased to a significant extent and insulin treatment more or less normalized the oxidative phosphorylation (17).

The important observations of the present studies were altered substrate and temperature kinetic properties. The observation that the diabetic state resulted in lowering of Km of component II in both the groups suggests this to an adaptive mechanism. However, this adaptive mechanism seems to have opposite effect on the Km of component I. A similar situation of opposite effects was obtained even with respect to insulin treatment. Thus, in one week diabetic group insulin treatment resulted in generalized decrease in Km whereas in one month diabetic group insulin treatment significantly elevated the Km values of component I and II. Interestingly, long term diabetes seemed to improve the catalytic efficiency of the enzyme (Table 6); insulin treatment of these animals drastically curtailed the efficiency (Table 6). Decreased in the values of E_2 in diabetic state is another interesting feature and may represent a modulatory response to improve catalytic efficiency (Table 7).

The above mentioned changes suggested that the changes in lipid/phospholipid profile of mitochondria may be responsible for these modulatory changes. Indeed significant changes occurred in the mitochondrial TPL and CHL contents, membrane fluidity and phospholipid composition and content. Since FoF_1 ATPase has known requirement of phospholipids, especially DPG (22) it was of interest to find out if the observed changes in kinetic properties across the groups could be correlated with lipid classes or their molar ratios. This possibility was evaluated by carrying out regression analysis across the

groups and the data are summarized in Table 12. As can be noted PE and total basic phospholipids (BPL) but not lecithin or SPM are positive modulator of $Vmax_2$ and $Vmax_3$ by contrast the acidic phospholipids (APL) PI, PS and DPG are negative modulators (Table 8). The molar ratio of TPL:CHL correlate positively with Vmax values of all the three kinetic components. Vmax values of component II and III correlated positively with molar ratio of TPL:PI, TPL:PS and TPL:PI+PS. A negative correlation of PC/PE, PI/BPL, PS/BPL and DPG/BPL with $Vmax_2$ and $Vmax_3$ was another interesting feature. E_2 correlated positively with PE, TPL/PI, TPL/PS and TPL/PS+PI whereas negative correlations were obtained with SPM, PI, PS and SPM/PC, PI/BPL, PS/BPL (Table 12). Based on these observations it may be suggested that besides changes in the phospholipid composition their charge distribution across the membrane and especially in the lipid micro domains in which FoF_1 ATPase is embedded and changes in membrane fluidity may be a significant modulatory role.

It has been recognized that the 18:3 and 22:6 desaturase activity decreases in diabetes (13,15), thus, lowering the unsaturation index. The observed increased in T_t which we note here (Table 7) is consistent with these observations.

Mutations in mitochondrial DNA leading to dysfunction of enzyme complexes such as PDH complex FoF_1 ATPase etc. have been proposed as underlying causes of diabetes (45-47). It is not clear at this stage whether a similar situation would prevail following exposure to alloxan. It may be suggested that changes in membrane lipid/phospholipid domains may play a regulatory role.

Table 12. Correlation between kinetics parameters of liver mitochondrial FoF_1 ATPase and membrane lipid/phospholipid composition

Parameter	Correlation with			
	Phospholipid Class		Phospholipid molar ratio	
	Positive	Negative	Positive	Negative
$Vmax_1$	—	—	TPL/CHL (+ 0.634)	—
$Vmax_2$	PE (+ 0.775) BPL (+ 0.817)	PI (- 0.650) PS (- 0.794) DPG (- 0.644)	TPL/CHL (+ 0.724) TPL/PI (+ 0.642) TPL/PS (+ 0.644) TPL/PS+PI (0.638)	PC/PE (- 0.706) PI/BPL (- 0.710) PS/BPL (- 0.734) DPG/BPL (- 0.731)
$Vmax_3$	PE (+ 0.805) BPL (+ 0.848)	PI (- 0.776) PS (- 0.848) DPG (- 0.698)	TPL/CHL (+ 0.696) TPL/PI (+ 0.732) TPL/PS (+ 0.728) TPL/PS+PI (+ 0.739)	PC/PE (- 0.728) PI/BPL (- 0.813) PS/BPL (- 0.828) DPG/BPL (- 0.767)
E_2	PE (+ 0.664)	SPM (- 0.735) PI (- 0.792) PS (- 0.656)	TPL/PI (+ 0.796) TPL/PS (+ 0.796) TPL/PS+PI (+ 0.807)	SPM/PC (- 0.730) PI/BPL (- 0.773) PS/BPL (- 0.779)

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.

Our results also show that the abberations introduced by alloxan diabetes could not be fully corrected by treatment with insulin. This is consistent with well acknowledged fact that the insulin does not correct all the maladies of diabetes (1,48,49). One therefore wonders if complete restoration to normalized could be achieved by combined treatment with insulin and c-peptide (48,49). This interesting possibility is worth exploring further.

Summary

Under all experimental conditions the enzyme displayed three kinetically distinguishable components. In one week diabetic animals the enzyme activity was unchanged, however, Km and Vmax of component I increased and Km of component II decreased. Insulin treatment resulted in lowering of Km and Vmax of component II and III.

One month diabetic state resulted in decreased enzyme activity while insulin treatment caused hyper-stimulation. Km of component I and II decreased together with decreased Vmax of all the components. Insulin treatment restored the Km and Vmax values. In late stage of diabetes the catalytic efficiency of components I and II increased; insulin treatment had drastic adverse effect. Binding pattern of ATP was unchanged under all experimental conditions.

Diabetic state resulted in progressive decrease in energy of activation in high temperature range (E_2). Insulin treatment lowered the energy of activation in low temperature range (E_1) without correcting the E_2 values.

The phase transition temperatures increased in diabetic state and were not corrected by insulin treatment.

Long term diabetes lowered the total phospholipid (TPL) content and elevated the cholesterol (CHL) content; insulin treatment had partial restorative effect. The membrane

fluidity decreased in general in diabetic condition and was not corrected by insulin treatment at late stage.

Regression analysis studies suggest that specific phospholipid classes and/or their ratios may play a role in modulation of the enzyme activity.

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