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Characterization of two D- β -hydroxybutyrate dehydrogenase populations in heavy and light mitochondria from jerboa (*Jaculus orientalis*) liver

Driss Mountassif¹, Mostafa Kabine¹, Norbert Latruffe² and M'Hammed Saïd El Kebbaj¹

¹ *Laboratoire de Biochimie, Faculté des Sciences, Université Hassan II-Aïn Chock, km 8 route d'El Jadida BP. 5366, Casablanca, Morocco.*

² *LBMC (GDR-CNRS n° 2583), Faculté des Sciences, 6 Bd Gabriel, Université de Bourgogne, 21000 Dijon cedex, France.*

Abstract:

Mitochondrial membrane bound and phospholipid dependent D- β -hydroxybutyrate dehydrogenase (BDH) (EC 1.1.1.30), a ketone body converting enzyme in mitochondria, has been studied in two populations of mitochondria (heavy and light) of jerboa (*Jaculus orientalis*) liver. The results reveal significant differences between the BDH of the two mitochondrial populations in term of protein expression, kinetic parameters and physico-chemical properties. These results suggest the existence of two distinct BDH populations, one located in the heavy mitochondria and the other one in the light fraction. This different BDH distribution could be the consequence of the lipidic composition changes of the inner mitochondrial membrane from heavy and light mitochondria which would modify both BDH insertion and BDH lipid dependent catalytic properties.

Keywords:

D- β -hydroxybutyrate dehydrogenase, heavy and light mitochondria, *Jaculus orientalis*.

Abbreviations:

BDH, D- β -Hydroxybutyrate dehydrogenase; DL-BOH, DL- β -hydroxybutyrate ; EDTA, Ethylenediamine tetraacetic acid ; ELISA, Enzyme-Linked Immunosorbent Assay; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid ; Mes, 4-N-morpholinoethanesulfonic acid ; NEM, N-ethylmaleimide; NAD(H), Nicotinamide adenine dinucleotide oxidized / (reduced) forms ; PGO, Phenylglyoxal; SDS-PAGE, Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis; Tris, Trihydroxymethyl-aminomethane.

Introduction:

Mitochondria represent the main energy producer of the cell. They are also the site of the ketone bodies production. These compounds play a role in the energy metabolism of extrahepatic tissues, too as end products of the fatty acids degradation. The interconversion of ketone bodies is made by D- β -hydroxybutyrate dehydrogenase (BDH) (EC 1.1.1.30) first described in 1909 by Wakeman and Dakin in dog liver tissue [1]. In eukaryotic cells, BDH is an inner mitochondrial membrane bound enzyme, tightly associated with the NAD-linked electron transport chain, where its active site is located on matrix side of mitochondria [2-6]. BDH is synthesized in the cytoplasm as a precursor with a larger size and post translationally imported into mitochondria involving the processing of its N-terminus presequence [7]. The catalytic activity of the enzyme is lecithin-dependent [8-9]. As reported by Williamson and al [10], in liver, the enzyme catalyses the transformation of acetoacetate into D- β -hydroxybutyrate in the presence of NADH, which is transported through the blood stream to peripheral tissues, i.e. brain, heart, kidney, etc. In extrahepatic tissues, D- β -hydroxybutyrate is converted into acetoacetate, in the presence of NAD⁺, which is used, after its conversion to acetyl-CoA, by respiratory chain as fuel of ATP production, or after formation of acetoacetyl-CoA, for fatty acid synthesis. A catalytic mechanism of the interconversion of D-3-hydroxybutyrate and acetoacetate in both liver and peripheral tissues has been previously proposed by our group [11].

The jerboa (*Jaculus orientalis*), a nocturnal herbivorous rodent living in the subdesert highland of Morocco has been chosen for our study for the following reasons. This rodent is an appropriate organism to study metabolic regulation due to its remarkable tolerance to heat and dry diet and also because it is one of the small mammals that can undergo hibernation [12].

The present work deals with the characterization and the comparison between the D- β -hydroxybutyrate dehydrogenase of the two mitochondrial populations, i.e. mitochondria of big size (heavy) and mitochondria of small size (light) from jerboa liver.

In previous studies, heavy and light mitochondria were isolated from rat liver and combined to get an high content of mitochondria in order to purify a significant amount of BDH [4, 5, 11]. Here for the first time we isolated separately the two mitochondria populations from the jerboa liver in order to study there individual properties including BDH activity, content and properties.

Materials and methods:

Animals:

Adults jerboas (120-150g), 4-6 months, were captured in the area of Engil Aït Lahcen (in subdesert East Moroccan highland). They were adapted to the laboratory conditions during 3 weeks at a temperature of 22°C with food (salad and rat diet), and water ad libitum before sacrifice. The light cycle in the entire experiment was set to 14 hours light and 10 hours dark.

Liver mitochondria and mitoplasts isolation:

The jerboas were decapitated and liver was rapidly removed for mitochondrial extraction according to Fleischer and al. [13]. This method lead to the preparation of two populations of mitochondria so called heavy and light.

The mitoplasts (outer mitochondrial membrane-free mitochondria) preparation was done according to the method described by Kielley and Bronk [14]. Liver mitochondria were swelled in 20 mM KH_2PO_4 buffer at 0.5 ml/mg of protein during 30 minutes at 0°C. The medium was then centrifuged at 15300 g during 30 minutes. The mitoplasts were collected in the pellet.

Protein assay:

Protein content was measured according to the Bradford procedure, using bovine serum albumin (BSA) as standard [15].

Thiol determination:

Thiol assay was done according to the technique of Ellman [16]. 200 μl of 5% SDS and 795 μl of 20 mM potassium phosphate was added to 5 μl of sample. 15 minutes later, 100 μl of 2 mM DTNB were added. After 30 minutes of incubation at 25°C, the absorbance was measured at 405 nm against a blank containing all the reagents except mitochondria. A standard range going from 0 to 125 nmol of thiol was carried out with 2 mM of reduced glutathion.

Phospholipids extraction and composition:

Phospholipids extraction was done according to the technique of Rouser and Fleischer [17]. One volume of mitoplasts was added to 13.3 volumes of chloroform, 6.7 volumes of methanol and 4.2 volumes of 0.8% KCl. The mixture was crushed by Ultraturax at 7500 rpm per minute during 3 minutes. After sedimentation, the organic phase was recovered. At the latter were added 47 volumes of 0.8% KCl, 48 volumes of methanol and 3 volumes of chloroform. Then, the chloroformic phase was concentrated by rotavapor (Heidolph).

The amount of phospholipids was determined by measuring the phosphorus concentration according to Chen and al. [18]. Ten μ l of sample were mineralized with 450 μ l of 70% perchloric acid for one hour. After 10 minutes, one added respectively 4.5 ml of water, 500 μ l of 2.5% ammonium molybdate and 500 μ l of 10% ascorbic acid. The mixture was then incubated for 5 minutes in boiling water for coloring development. After 10 minutes of cooling, the absorbance was measured at 820 nm against a blank containing all the reagents. A standard range from 0 to 5 μ g of phosphorus was carried out with KH_2PO_4 at 2 μ g/ml.

The thin layer chromatography was carried out on dried silicagel plate (20 cm x 20 cm). After deposit of 40 μ g of phospholipids phosphorus, the migration was done with a medium containing chloroform/methanol/water (65/25/4;v/v/v). After the end of the migration, the spots were revealed with the 2,7-dichlorofluoresceine. The appeared bands were scraped, mineralized and estimated for their phosphorus content.

Enzyme assays:

Subcellular marker enzymes were assayed according to the following methods: succinate dehydrogenase [19] for mitochondria; palmitoyl-CoA oxidase [20] for peroxisomes; NADPH-cytochrome c reductase [21] for microsomes and glyceraldehyde 3-phosphate dehydrogenase [22] for cytosol.

BDH activity was measured at 37°C as described in [23] by following NADH production at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 100 μg of protein in a medium containing: 6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol, 0.3 mM dithiothreitol, in the presence of 2 mM NAD^+ (Sigma) and 2.5 μg rotenone (final addition) to prevent NADH reoxidation by the respiratory chain. The activity was started by the addition of DL- β -hydroxybutyrate (Sigma) to 10 mM final concentration.

BDH Kinetic studies:

Initial velocities of the enzymatic reaction were performed by varying the concentration of the substrates, BOH (from 2.5 to 10 mM) or NAD^+ (from 0.5 to 2 mM). Values of the Michaelis constants (K_m), dissociation constants (K_D) and maximal velocity for the oxidation of BOH and reduction of NAD^+ by the BDH were made from mathematical analysis according to the method of Cleland [24].

BDH chemical modification:

Two chemical modifiers were used in this study: N-Ethylmaleimide (NEM) (Sigma) and Phenylglyoxal (PGO) (Aldrich-chemie) which are known to react specifically with cysteyl [25] and arginyl residues [26] respectively. Hundred μg of thawed liver mitochondria protein were preincubated for 5 minutes at 25 °C in the buffered medium (6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol). The inhibitor was added at zero time of incubation. Aliquots were removed at different times of incubation for enzymatic activity measurements.

Determination of optimal pH and temperature dependent BDH activity:

- The effect of pH on the BDH activity was studied in the range of pH varying from 4 to 10 using a mixture of different buffers (Tris, Mes, Hepes, potassium phosphate and sodium acetate).

- Temperature effects were characterized by activation and denaturation processes:

- For activation, the buffered medium containing 6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol was incubated for 2 minutes at various temperatures (from 5 to 80°C). Then, 2.5 µg of rotenone, 2 mM of NAD⁺ and 100 µg of protein were added. After 2 minutes at temperature studied, the reaction was started immediately by addition of 20 mM of BOH.
- For denaturation, 100 µg of protein liver mitochondria were incubated at different temperatures (from 5 to 80°C) for 2 minutes in the buffered medium containing 6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol. Then, 2.5 µg of rotenone and 2 mM of NAD⁺ were added and the enzymatic activities were measured by later addition of 20 mM of BOH after 2 minutes of incubation at 37°C.

For kinetic BDH thermal stability study, several mitochondria aliquots (100 µg) from the same proteic extract were incubated at 37°C and the BDH activity was measured at various times of incubation.

BDH Arrhenius-plot was obtained by measuring the enzymatic activity at various temperatures from 5 to 40°C and analyzed as described by Raison [27].

Western-blotting:

After SDS-PAGE (12%) [28] and subsequent transfer in nitrocellulose [29], the mitochondrial proteins (50µg) were exposed to 1/100 dilution of monospecific polyclonal anti-BDH antibody and detected with the secondary antibody of anti-rabbit, IgG peroxidase conjugate (diluted to 1/2500) (Promega).

Enzyme-Linked Immunosorbent Assay:

The ELISA method was done according to the technique of Kemeny [30].

Zymogram gel:

It was carried out in 5% (w/v) acrylamide gel under non-denaturing conditions. The gel was stained for in situ location of NAD⁺ linked BDH activity bands by incubation for one hour in the standard reaction mixture for the oxidation reaction (NAD⁺ 2 mM supplemented with 20 μ M phenazine methosulfonate and 1 mM p-nitroblue tetrazolium chloride). The enzyme activity band (50 μ g) was located by the appearance of a deep-purple band of the resulting insoluble formazan salt.

Statistical data analysis:

In each assay, the experimental data represent the mean of three independent assays. Student t-test was used; A p-value lower than 0.05 was considered significant.

RESULTS:

Biochemical characterization of mitochondrial populations of jerboa:

We isolated two mitochondrial populations from liver of active jerboa, heavy and light fractions. In table I, we report the relative activities of subcellular marker enzymes in the two mitochondrial fractions. As one can see, the two fractions present a high content on mitochondria with a low one of peroxisomes, microsomes and cytosol. From the data, the purity is estimated at 72% and 80% for heavy and light mitochondria respectively.

In table II, we report the data related to the protein and phosphorus content of these mitochondria. One can see, the protein yield is different between the two mitochondrial populations. This is corroborated with variations in the content of thiol groups titration which is considered as a good method of determination of protein content. No difference was observed concerning phosphorus content.

The content of inner membrane phospholipids of mitochondria is different between the two mitochondria. The amount of phosphatidylcholine is significantly greater in heavy mitochondria than in light ones. No differences were observed about phosphatidylinositol, phosphatidylserine and cardiolipin.

Determination of BDH physicochemical parameters from the heavy and light mitochondria:

The optimal pH of the BDH activity on the level of the two mitochondrial populations is identical and equal to 8 (not shown).

Figure 1 shows that the optimal temperature of the BDH activity is near 35°C for heavy mitochondria (A) and 40°C for light ones (B).

The incubation of the heavy and light mitochondria at 37°C (figure 1C) leads to a high stability in light mitochondria as shown by the thermal inactivation kinetic constant ratio

K_H/K_L of 0.15. This effect has been also observed at other temperatures tested, i.e. stored at -20°C, 4°C and 28°C (results not shown). Interestingly, the Arrhenius-plots (figure 2) show a break near 30°C only for BDH from heavy mitochondria.

BDH activity was also measured with two other position isomers of D-β-hydroxybutyrate, i.e. D-α-hydroxybutyrate and D-γ-hydroxybutyrate at 10 mM final concentration (Data not shown). The results obtained reveals that BDH activity is 40% lower for the γ-hydroxybutyrate in heavy mitochondria than the light ones, but for the α-hydroxybutyrate, no significant differences were observed.

Figure 3 reports comparative BDH sensitivity of the heavy and light mitochondria towards two well known chemical aminoacids reagents (NEM and PGO) [25, 26]. As one can see, the BDH is strongly and rapidly inactivated by both reagents and the inactivation constants (n =stoichiometry, k_2 = inactivation kinetic constant of second order) depend on the time of incubation and on the reagent concentrations. They are not significantly different for the two BDH mitochondrial origins: i.e. for the BDH from the heavy mitochondria inactivated with NEM, $n = 1.09$, $K_2 = 40 \text{ min}^{-1}\text{mM}^{-1}$ and $n = 1.38$, $K_2 = 25 \text{ min}^{-1}\text{mM}^{-1}$ for inactivated with PGO. For the BDH from the light ones, $n = 1.13$, $K_2 = 43.5 \text{ min}^{-1}\text{mM}^{-1}$ and $n = 1.13$, $K_2 = 28 \text{ min}^{-1}\text{mM}^{-1}$ for NEM and PGO respectively.

BDH kinetic parameters from the heavy and light mitochondria, V_{\max} , $K_m\text{BOH}$, $K_m\text{NAD}^+$ and $K_m\text{NAD}^+$ are reported in the table III. Interestingly, they show that the specific activity of the BDH from the heavy mitochondria is higher than that from the light ones ($\times 2.77$). Moreover, the kinetic constants with respect to the NAD^+ and BOH are larger from the heavy mitochondria than from light ones ($\times 1.8$ for the $K_m\text{NAD}^+$, $\times 1.4$ for the $K_m\text{NAD}^+$, $\times 2.1$ for $K_m\text{BOH}$). In order to explain these differences, SDS-PAGE was made (Figure 4A). Its analysis revealed the absence of the BDH band. Thus, the amount of BDH polypeptide of the two mitochondrial populations was estimated both by western-blotting (figure 4B) and by

ELISA (figure 4C). As one can see, this amount is always higher in heavy mitochondria than in the light ones. These above results are in accordance with there observed when BDH activity was measured in non denaturing conditions electrophoresis (Zymogram gel) (figure 4D).

DISCUSSION:

In order to purify mitochondrial BDH from eucaryotic cell with high yield, we previously used a mixed mitochondrial population (heavy and light) [4, 5, 11, 25, 26, 31]. In our knowledge, no study of BDH were performed from each of isolated populations was done until now.

In this work, we report a comparative study of BDH from heavy and light mitochondria, in terms of physicochemical properties, kinetic parameters and content. The interest to work on jerboa (*Jaculus orientalis*) is due to the fact that this animal is a true hibernator animal [32] developing obesity by accumulating the fat during prehibernating period. Its fat is eliminated during hibernation period leading to high production via BDH of D- β -hydroxybutyrate to serve as an energetic fuel in addition to carbohydrates [33]. This study on BDH focuses on jerboa in its active state.

The data report interesting differences between BDH from heavy and light liver mitochondria. We found that BDH activity is higher in heavy mitochondria than in light ones (table III). This can be due either to the amount of BDH which is different between the mitochondrial populations or that the organelle phospholipid composition change induce variation in BDH activity which is lipid-dependent enzyme. Indeed, the amount of BDH in heavy mitochondria was also found to be higher than in light ones. This was confirmed by western, ELISA and zymogram gel (figures 4B, C and D). Secondly, the phospholipid composition of heavy and

light mitoplasts (table II) is significantly different namely concerning phosphatidylcholine which is the activator of BDH [31, 34].

Thus, the differences of BDH activity between the two populations of mitochondria appear due to both the amount of BDH present and to differences in phospholipid composition.

Previous studies on the interactions of BDH with phospholipids [6, 9, 31, 33, 34, 35] have demonstrated that the composition of membrane phospholipid induced to structural modification of BDH. Taking this into account, we have estimated the physicochemical and kinetic characteristics of BDH from heavy and light liver mitochondria. BDH of these mitochondria shows an identical optimal pH of 8 (data not shown). Thermal parameters reveal interesting differences; 1) the optimal temperature is about 35°C for the BDH from the heavy mitochondria while 40°C from the light ones (figure 1); 2) the BDH from the heavy mitochondria is less stable to the temperature than that from the light ones; 3) Arrhenius-plots (figure 2) show a break near 30°C for only heavy mitochondria BDH. These over all observations suggest that the two mitochondrial origins BDH are different in term of conformation, possibly resulting from different insertion. This conformational change is also confirmed by the study of BDH stereospecificity differences towards 3 isomers of D-hydroxybutyrate i.e. α , β and γ -hydroxybutyrate (Data not shown).

Comparable effects of two chemical modifying reagents (NEM and PGO) suggest that the two BDH have similar catalytic site (figure 3).

The determination of kinetics parameters indicated that the heavy and the light mitochondrial BDH behave differently (table III). Indeed, the affinity of the heavy mitochondria with respect to the NAD⁺ and BOH is stronger than that of the light ones. Beside this the high content of BDH in heavy mitochondria partially explains that the specific activity of BDH is twice more higher for the heavy mitochondria than that for the light ones (table III).

In a recent paper [36] we have reported the existence of two BDH isoforms from brain and liver mitochondria from active and cold adapted jerboas.

In conclusion, the results reveal the possible existence of two different BDH populations in jerboa liver associated to either light or heavy mitochondria: One would be associated in great quantity to the heavy mitochondria and the second in small quantity to the light ones. The cause of these distributions may be a difference in the lipid composition of the inner mitochondrial membrane between these mitochondria. This latter would have two consequences; 1) a modulation in the targeting of the BDH precursor where the mitochondrial import this would be higher in heavy mitochondria than in light ones; 2) a modulation of the phospholipid dependent BDH activation and BDH membrane insertion. Differences in the thermal stability and thermal behavior as well as changes in the kinetic parameters are a clear illustration. The cellular and physiological roles of the two liver mitochondrial populations associating two BDH subdistribution are unknown.

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		Homogenate	Heavy mitochondrial fraction	Light mitochondrial fraction
Mitochondria	Succinate dehydrogenase (absorbance/min/mg de proteins)	1.04 ± 0.12	2.25 ± 0.18 [x 2.16]*	1.83 ± 0.21 [x 1.76]*
Peroxisomes	Palmitoyl-CoA oxydase (nmol/min/mg de proteins)	7.52 ± 0.47	0.58 ± 0.04 [x 0.07]*	1.48 ± 0.12 [x 0.2]*
Microsomes	NADPH-Cytochrome C reductase (nmol/min/mg de proteins)	79.3 ± 9.44	15.74 ± 3.25 [x 0.2]*	None
Cytosol	Glyceraldehyde-3- phosphate dehydrogenase (µmol/min/mg de proteins)	0.89 ± 0.05	None	None
Mitochondrial purity		-	72%	80%

Table I : Subcellular marker enzyme activities in two mitochondrial isolated fractions from jerboa liver compared to liver homogenate. Values are given in specific activities. Numbers in brackets correspond to the variation compared to homogenate. Values are given as means of four separated experiments. * Variations statistically significant at $p < 0.01$. For experimental conditions, see Materials and Methods.

	Total protein/liver weight (mg/g)	Total thiol groups/protein (nmol/mg)	Total phospholipid phosphorus/liver weight (mg/g)	Proteins/phospholipid phosphorus ratio	Phospholipids (%)			
					<u>PE</u>	<u>PI/PS</u>	<u>CL</u>	<u>PC</u>
Heavy mitochondria	92.88 ±5.76	100 ±7	10.8 ±0.9	8.6	28 ±6	32 ±5	17 ±2	23 ±3
Light mitochondria	83.2 ±4.8	56* ±8	11 ±1.2	7.56**	33 ±5	38 ±4	15 ±3	14* ±1

Table II : Characterization of the liver heavy and light mitochondria and lipid composition analysis by thin layer chromatography of the heavy and light mitoplasts. PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; PC, Phosphatidylcholine and CL, Cardiolipin. After deposit of 40µg lipid phosphorus of phospholipids, migration and revelation, the amount of phosphorus were measured in band each as described in Materials and Methods. Values are given as means ± standard deviations of three independent experiments and have been taken into account of the mitochondrial fractions purities (table I). * p<0.01 and ** p<0.05 (Student t-test).

BDH parameters from	K_m NAD⁺ (mM)	K_m BOH (mM)	K_D NAD⁺ (mM)	V_{max} (nmol NADH/min/mg of protein)
Heavy mitochondria	0.21 ±0.01	1.60 ±0.22	1.16 ±0.50	0.61 ±0.05
Light mitochondria	0.39* ±0.03	3.36* ±0.33	1.66 ±0.33	0.22* ±0.01

Table III : Determination of the kinetic parameters of the liver BDH from heavy and light mitochondria. Experiments were varying NAD⁺ concentration (0.5; 1; 1.5 and 2 mM) or BOH concentration (2.5; 5; 7.5 and 10 mM). Values are given as means ± standard deviations of three independent experiments. V_{max} values have been calculated according to the mitochondrial fraction purities (table I). * p<0.01 (Student t-test).

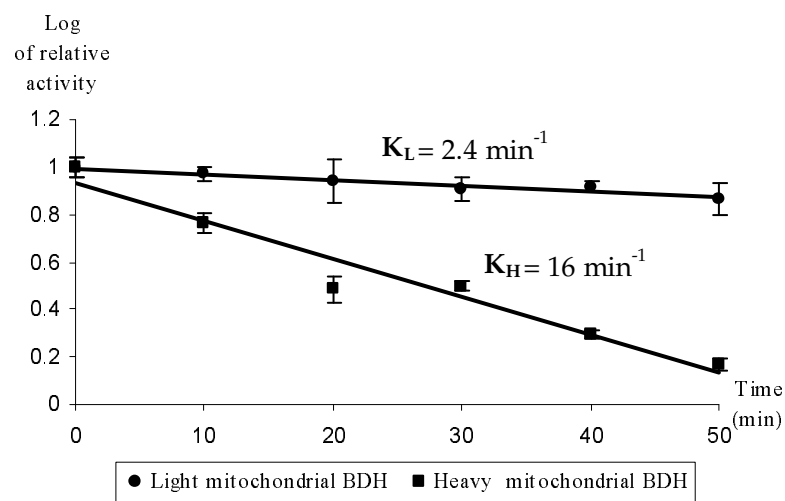
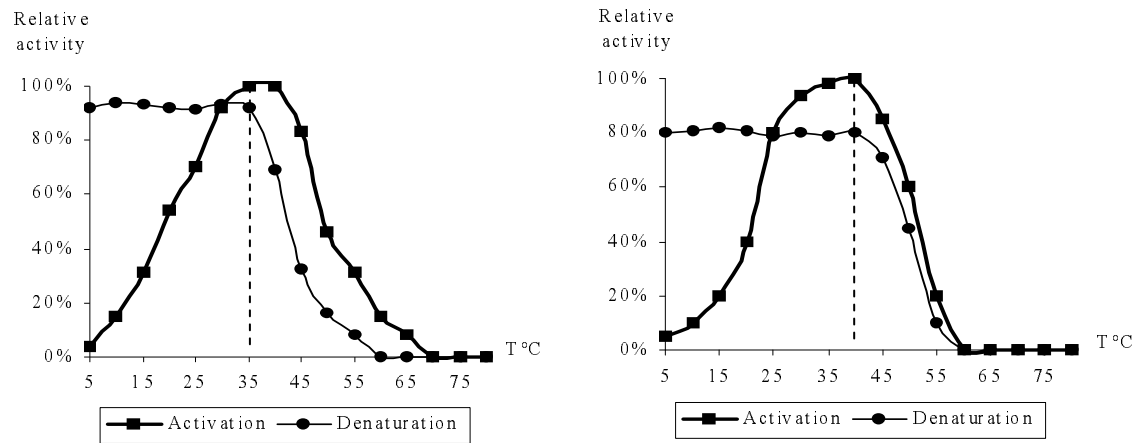
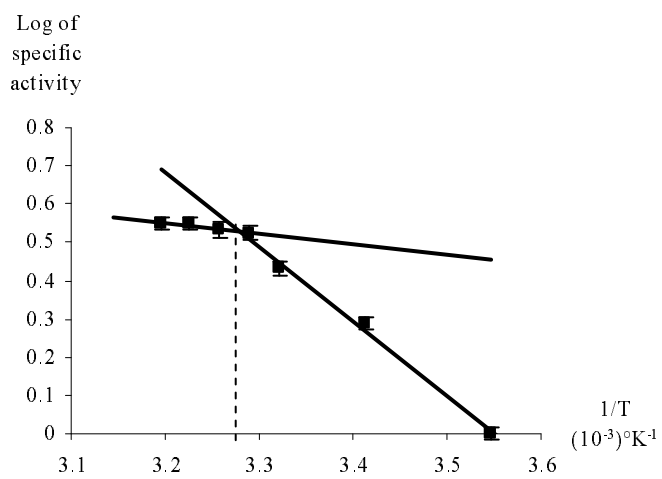
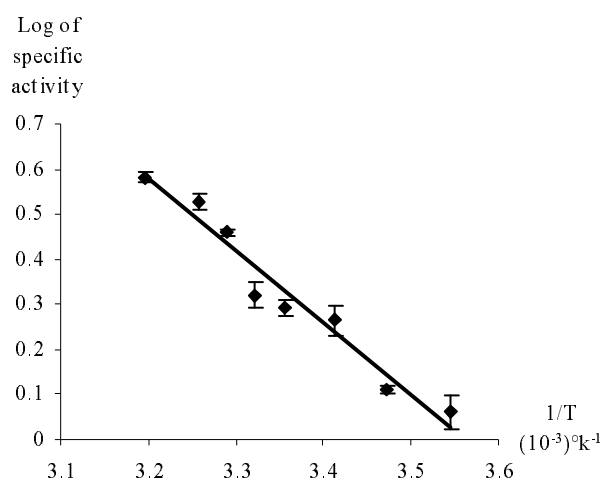


Figure 1



A
Heavy mitochondrial BDH



B
Light mitochondrial BDH

Figure 2

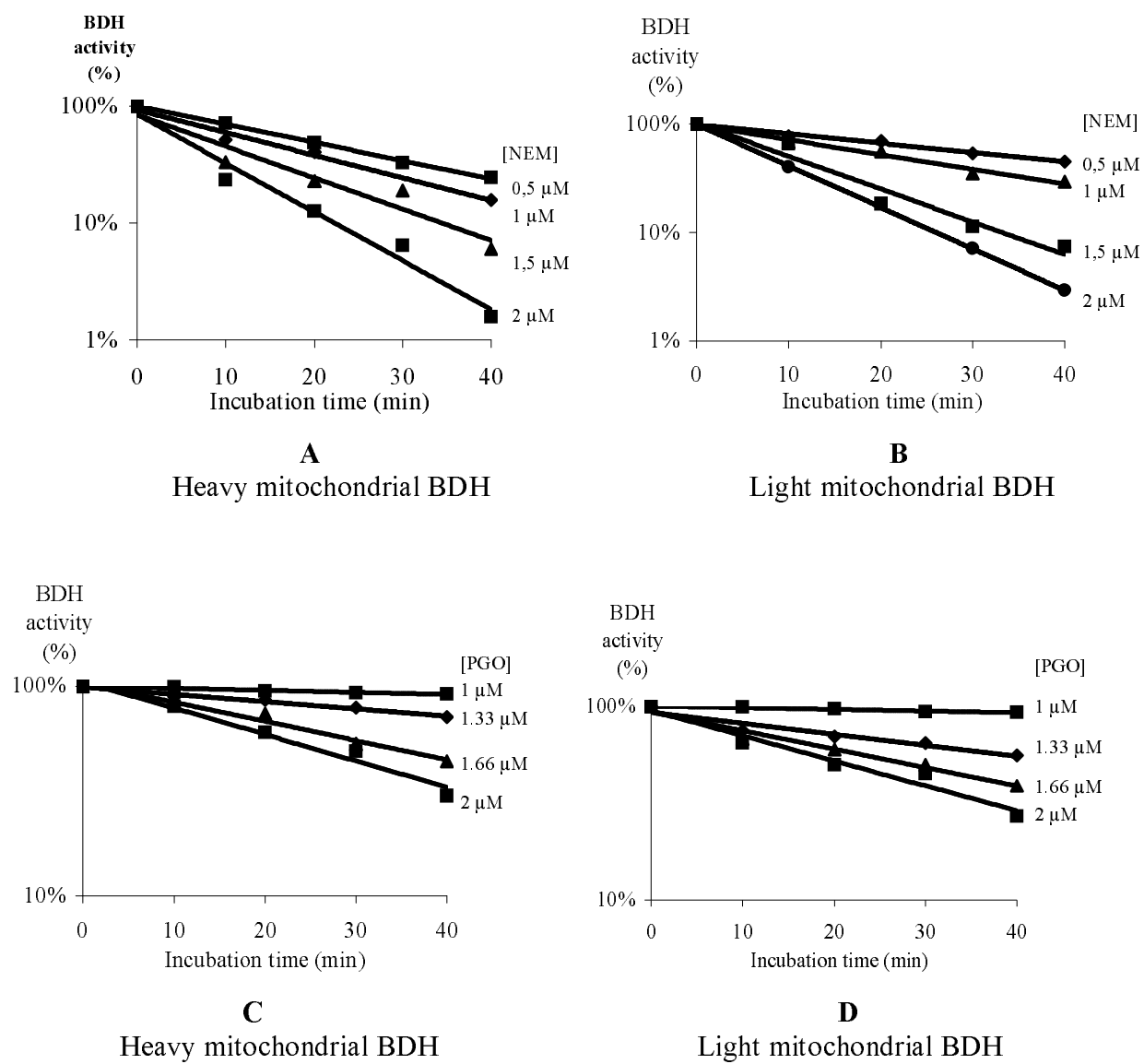
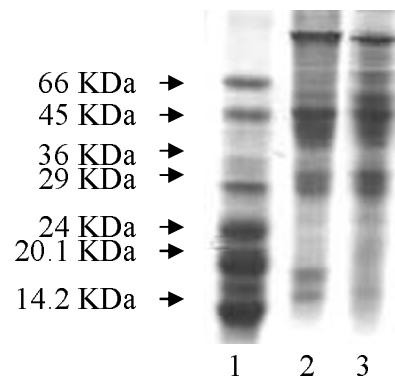
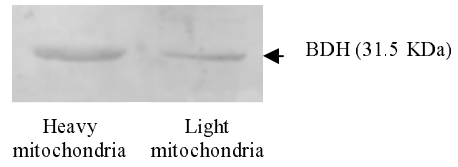


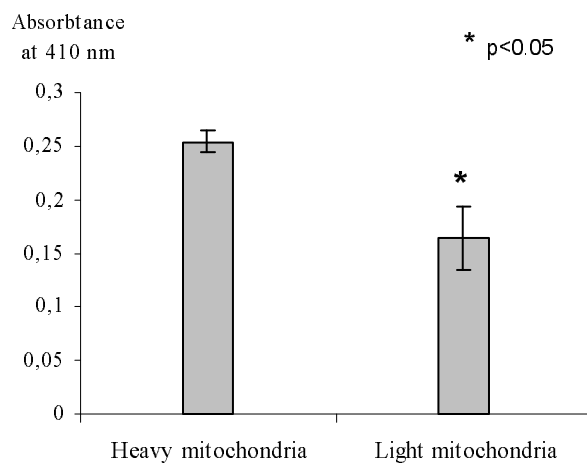
Figure 3



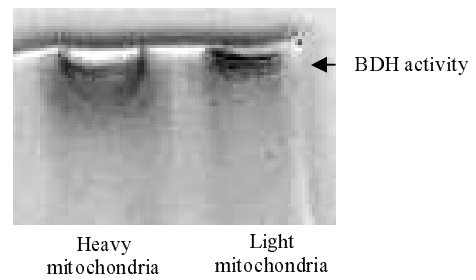
A
SDS-PAGE



B
Western-blot



C
ELISA



D
Zymogram gel

Figure 4

Legends of figures

Figure 1: Effect of the temperature on the activity of the liver BDH from heavy (A) and light (B) mitochondria. This was followed by activation and denaturation processes at different temperatures (from 5 to 80°C).

Thermal stability of liver BDH activity from heavy and light mitochondria at 37°C (C). Liver mitochondrial proteins (100 µg) were incubated at 37°C and the BDH activity was measured at various incubation times. Values are given as means of three separated experiments.

Figure 2: Arrhenius-plots of the liver BDH from heavy (A) and light (B) mitochondria were obtained by measuring the enzymatic activity using 100 µg of liver mitochondrial protein at various temperatures (from 5 to 40°C). Values are given as means of three separated experiments.

Figure 3: Time course of the liver BDH inactivation from heavy and light mitochondria by various concentrations of N-ethylmaleimide [NEM] (A,B) or phenylglyoxal [PGO] (C,D) (semi log plot). Liver mitochondrial proteins (100 µg) were preincubated 5 minutes at 25°C in phosphate buffer pH 8. PGO or NEM was added at zero time of incubation. Aliquots were removed at different times for enzymatic activity measurement. Aliquots of the control assay were removed at the same time in order to calculate the percentage of residual BDH activity. Values are given as means of three separated experiments.

Figure 4: SDS-PAGE (A), Western-blotting (B), ELISA (C) and Zymogram gel (D) of the liver BDH from heavy and light mitochondria of the jerboa. A p-value lower than 0.05 was considered significant (Student t-test). SDS-PAGE, Western-blotting and Zymogram gel were assayed with 50µg of proteins and ELISA with 10µg of protein. Lane 1, 2 and 3 represent successively markers, heavy and light mitochondrial protein fractions. For experimental conditions, see Materials and Methods.