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PREHIBERNATION AND HIBERNATION EFFECTS ON THE D-3-HYDROXYBUTYRATE DEHYDROGENASE OF THE HEAVY AND LIGHT MITOCHONDRIA FROM LIVER JERBOA (*JACULUS ORIENTALIS*) AND RELATED METABOLISM

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Abstract:

The D-3-hydroxybutyrate dehydrogenase (BDH) (EC 1.1.1.30) from liver jerboa (*Jaculus orientalis*), a ketone body converting enzyme in mitochondria, in two populations of mitochondria (heavy and light) has been studied in different jerboa states (euthermic, prehibernating and hibernating). The results reveal: (1) important variations between states in terms of ketones bodies, glucose and lipid levels; (2) significant differences between the BDH of the two mitochondrial populations in term of protein expression and kinetic properties. These results suggest that BDH leads an important conformational change depending on the physiological state of jerboa. This BDH structural change could be the consequence of the lipid composition modifications in inner mitochondrial membrane leading to changes in BDH catalytic properties.

Keywords:

D-3-hydroxybutyrate dehydrogenase, Jerboa (*Jaculus orientalis*), euthermic, prehibernation and hibernation states, heavy and light mitochondria, isoforms.

Abbreviations:

AcAc, Acetoacetate; BDH, D- β -Hydroxybutyrate dehydrogenase; DL-BOH, DL-3-hydroxybutyrate; CPK, Creatine phosphokinase; EDTA, Ethylenediamine tetraacetic acid ; ELISA, Enzyme-Linked Immunosorbent Assay; GOT, Glutamate Oxalate Transaminase; GPT, Glutamate Pyruvate Transaminase; HDL, High Density Lipoprotein; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid ; LDL, Low Density Lipoprotein; Mes, 4-N-morpholinoethanesulfonic acid ; NAD(H), Nicotinamide adenine dinucleotide oxidized / (reduced) forms ; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TCA, Trichloroacetic acid; TMB, Tetramethyl benzidine; Tris, Trihydroxymethyl-aminomethane.

Introduction:

Hibernation is a strategy adopted by several mammals in order to survive the lack of food and the low ambient temperatures. This state requires a special adaptation of cell metabolism with physiological change that necessitates a strict control of lipid metabolism particularly, since lipids play a critical role in supplying the energy requirements. The mitochondria play an important role in energy production namely during hibernation. They are also the site of the ketone bodies production. These compounds, which result from the degradation of fatty acids, play a crucial role in the energy metabolism of extrahepatic tissues. The interconversion of ketone bodies is made by D-3-hydroxybutyrate dehydrogenase (BDH) (EC 1.1.1.30) first described in dog liver tissue (Wakeman and Dakin, 1909). 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 the matrix side of mitochondria (Wise and Lehninger, 1962; Nielsen et al., 1973; Latruffe and Gaudemer, 1974; Gaudemer and Latruffe, 1975; McIntyre et al., 1978). 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 (Kante et al., 1987). BDH was largely studied in several organisms: *Rhodopseudomonas spheroides* (Bergmeyer et al., 1967), beef heart (Nielsen et al., 1973), rat liver (Latruffe et al., 1974), ruminant's heart and liver (Cherkaoui-Malki et al., 1992) and dromedary liver (Nasser et al., 2002). The molecular mass of the subunit size of the purified BDH was 31.5 KDa for bovine heart, rat liver, and rat brain mitochondria (Bock and Fleischer, 1975; Vidal et al., 1977; McIntyre et al., 1988; Zhang and Churchill, 1990) and about 67 KDa for dromedary liver (Nasser et al., 2002). Purified BDH is devoid of lipid and can insert spontaneously and unidirectionally into performed phospholipid vesicles or natural membranes (McIntyre et al., 1979). It has previously been proposed that activation of BDH by Phosphatidyl-Choline (PC) containing liposomes involves an allosteric mechanism (Sander mann et al. 1986), whereby PC enhances coenzyme binding (Rudy et al., 1989). The primary sequence of BDH was initially determined for the enzyme from rat liver (Churchill et al., 1992) and for the enzyme from human heart (Marks et al., 1992) by cDNA cloning. The mature form of this enzyme consists of 297 amino acids and the Northern blot analysis identifies a 1.3-kilobase mRNA (Marks et al., 1992). A comparison of the BDH amino acid sequences with other reported sequences reveals a homology with the superfamily of short-chain alcohol dehydrogenases. The N-terminal of the enzyme include the coenzyme binding domain and putative active site conserved residues. The C-terminal of BDH and other family members show little sequence homology and this region likely contains elements responsible for the binding of BOH. The catalytic activity of the enzyme is lecithin-dependent (Sekuzu et al., 1961; Gazzoti et al., 1964). As reported by Williamson et al., 1971, in liver, the enzyme, in the presence of NADH, catalyses the conversion of acetoacetate into D-3-hydroxybutyrate which is transported through the blood stream to peripheral tissues, i.e. brain, heart, kidney, etc. In extrahepatic tissues, D-3-hydroxybutyrate is converted into acetoacetate in the presence of NAD⁺. Acetoacetate is then consumed, after its conversion to acetyl-CoA, by the respiratory chain as fuel for 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 (El kebbaj and Latruffe, 1997).

The jerboa (*Jaculus orientalis*), a nocturnal herbivorous rodent living in the subdesert highland of Morocco, was chosen for our study due to the following reasons: This rodent is an appropriate organism to study metabolic regulation due to its remarkable resistance to heat, arid conditions and especially to cold. Also, this rodent is a true hibernator (El Hilali and Veillat, 1979) developing obesity by accumulating fat during its prehibernation period. Its fat is eliminated during hibernation leading to a high production via BDH of D-3-hydroxybutyrate to serve as an energy source in addition to carbohydrates (Kante et al., 1990).

Recently, we demonstrated the existence of two mitochondrial populations (Mountassif et al., 2006). The population of large mitochondria referred to as "heavy" (0.85 μ m in length and 0.5

µm in width) and the population of small mitochondria referred to as “light” (0.35 µm in length and 0.25 µm in width); also, we reported that BDH of the two mitochondrial populations were different in terms of activities, content and physico-chemical properties. Their differences can, be correlated with the lipid composition of the mitochondrial membrane, which would modify both the BDH structure and activity, as lecithin requiring enzyme and the pre-BDH import and its insertion into the inner mitochondrial membrane.

To explore the mechanisms of the regulatory processes associated with prehibernation and hibernation states, we performed a comparative study of these physiological states in terms of plasma analyses and the comparison of BDH expression from both heavy and light liver mitochondria in euthermic (active), prehibernating and hibernating jerboas.

Materials and methods:

Animals:

Adult greater Egyptian jerboas (*Jaculus orientalis*, Rodentia, Dipodidae) (120-150 g, 4-6 months old), were captured in the area of Engil Aït Lahcen (in subdesert East Moroccan highland). They were adapted to laboratory conditions during 3 weeks at a temperature of 22°C with food (salad and rat chow), and water *ad libitum* before they were killed. The light cycle during the experiment was set to 14 h light and 10 h dark.

Induced hibernation:

Prehibernating (PH) and hibernating (H) states were performed as described by Montoya et al., 1979 and Baddouri et al., 1986: A group of 8 animals called (PH) (4 per cage) was kept with food in a cold room (6 °C) for 3 weeks. A second group (H) (n=4) was housed as PH group but at the end of 3 weeks of the prehibernation period, the food was removed leading to hibernation established for 1 week. After 6 days of hibernation, animals were sacrificed. A third group of animals considered as euthermic (n=4) was remained at 22 °C and called active (A).

Plasma analyses:

Immediately after jerboa decapitation, the blood was collected and centrifuged for 10 min at 600g and the plasma portion was removed and frozen at -20°C until assays. Before assay, the plasma samples were centrifuged at XXX g for 5 min.

The ketonemia (BOH level) was estimated by the technique described by Williamson et al., (1962) slightly adapted and modified by our laboratory as follow; the serum was deproteinised with 1N perchloric acid mixed with serum (v/v); the precipitate of proteins was eliminated by centrifugation (1200 x g for 10 min). The supernatant was neutralized with KOH 1N and centrifuged. The medium of BDH measurement was: 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) and 25.38% Hydrazine. The absorbance of reduced NAD⁺ (NADH) was measured at 340 nm at time 0 immediately after that 250µl of supernatant were added. After one hour of incubation at 37°C, the absorbance was again measured. One can consider that after 60 min, BOH is completely oxidized, and that AcAc produced is combined with hydrazine leading the reaction irreversible.

$$[\text{BOH}] = [(\text{absorbance at 60 min} - \text{absorbance at 0 min}) / 6220] \times (\text{total volume of measure} / \text{volume of serum}) \times \text{factor of dilution of serum}.$$

The determination of glycemia, urea, total bilirubine, creatinine, total cholesterol, HDL, LDL, triglycerides, CPK and aminotransaminases (GOT and GPT) was carried out by Laboratoire des Analyses Médicales (Centre National de Transfusion Sanguine, Casablanca).

Liver mitochondria and mitoplast isolation:

The jerboas were decapitated and the liver was rapidly removed for mitochondrial extraction according to Fleischer et al. (1979). This method allows the preparation of the two mitochondrial populations.

The mitoplasts (outer mitochondrial membrane-free mitochondria) preparation was done according to the method described by Kielley and Bronk (1958). Liver mitochondria were swelled in a 20 mM phosphate buffer at 0.5 ml/mg of protein for 30 min at 0°C. The medium was then centrifuged at 12 000 g for 30 min. The mitoplasts were collected in the pellet.

Protein assay:

Protein content was measured according to the Bradford (1976) procedure, using bovine serum albumin (BSA) as standard.

Phospholipid extraction and composition:

Phospholipids were extracted according to the technique of Rouser and Fleischer (1967). One volume of mitoplasts was added to chloroform/methanol/0.8% KCl (13.3/6.7/4.2; v/v/v). The mixture was homogenized with an Ultraturrax at 7,500 rpm for 3 min. After sedimentation, the organic phase was recovered and 0.8% KCl/methanol/chloroform (47/48/3; v/v/v) was added. The chloroform phase was then concentrated in a rotary evaporator. The amount of phospholipids was determined by measuring the phosphorus concentration according to Chen et al. (1956).

Ten microlitres of sample were mineralized with 450 μ L of 70% perchloric acid for 60 min. After 10 min, 4.5 mL of water, 500 μ L of 2.5% ammonium molybdate and 500 μ L of 10% ascorbic acid were added. The mixture was then incubated for 5 min in boiling water for color development. After 10 min 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 established with KH_2PO_4 at 2 μ g/mL.

Thin layer chromatography was carried out on dried silicagel plates (20 cm x 20 cm). Phospholipid phosphorus (40 μ g) was loaded, and plates were developed with chloroform/methanol/water (65/25/4; v/v/v). Spots were revealed with 2, 7-dichlorofluoresceine. The bands were scraped off, mineralized and their phosphorus content was measured.

Enzyme assays:

Subcellular marker enzymes were assayed according to the following methods: succinate dehydrogenase (King, 1967) for mitochondria; palmitoyl-CoA oxidase (Lazarow and De Duve, 1976) for peroxisomes; NADPH-cytochrome c reductase (Beaufay et al., 1974) for microsomes and glyceraldehyde 3-phosphate dehydrogenase (Serrano et al., 1991) for cytosol.

BDH activity was measured at 37°C as described by Lehninger et al. (1960), by monitoring NADH production at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 100 μ g of protein in a medium containing: 6 mM potassium phosphate at pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol, 0.3 mM dithiothreitol, in the presence of 2 mM NAD^+ (Sigma-Aldrich) and 2.5 μ g rotenone (final addition). The reaction was started by the addition of DL-3-hydroxybutyrate (Sigma) to 10 mM final concentration.

BDH kinetic studies:

Initial velocities were measured by varying the concentration of BOH (from 2.5 to 10 mM) or NAD^+ (from 0.5 to 2 mM). Michaelis constants (K_m), dissociation constants (K_D) and maximal velocity for the oxidation of BOH and the reduction of NAD^+ by the BDH were obtained by mathematical analysis following Cleland (1963).

Determination of optimal pH and temperature dependent BDH activity:

The effect of pH on BDH activity was studied in range from pH 4 to pH 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 min at temperatures from 5 to 80°C. Then, 2.5 µg of rotenone, 2 mM of NAD⁺ and 100 µg of protein were added. The reaction was started immediately by the addition of 20 mM of BOH.

For denaturation, 100 µg of liver mitochondrial protein were incubated at temperatures from 5 to 80°C for 2 min in 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 activity was measured by the later addition of 20 mM of BOH after 2 min of incubation at 37°C.

A BDH Arrhenius-plot was obtained by measuring the enzymatic activity at temperatures from 5 to 40°C and analyzed as described by Raison (1973).

Western-blotting:

After SDS-PAGE (12%) (Laemmli, 1970) and subsequent transfer to nitrocellulose (Towbin et al., 1991), the mitochondrial proteins (50µg) were exposed to a 1:100 dilution of monospecific polyclonal anti-BDH antibody (BDH rat liver) and detected with the secondary antibody of anti-rabbit, IgG peroxidase conjugate (diluted 1:2500) (Promega).

Enzyme-Linked immunosorbent assay:

The ELISA method was performed according to Kemeny (1991). Mitochondrial proteins (10 µg) were exposed to 1:100 dilution of monospecific polyclonal anti-BDH antibody (BDH rat liver) and detected with the secondary antibody of anti-rabbit, IgG peroxidase conjugate (diluted 1:2500) (Promega) and the absorbance at 410 nm was measured with an ELISA reader after addition of tetramethyl benzidine (TMB) (Sigma).

Immunofluorescence staining:

The jerboas were decapitated and the livers were immediately removed, fixed in Bouin liquid and embedded in paraffin. 4 µm sections were rinsed with PBS, and then blocked with 1% bovine serum albumin (BSA) in PBS (1% BSA–PBS). Sections were then incubated in the primary antibody (1/100) (rabbit anti-BDH of rat) at room temperature for 60 minutes, washed for 5 min with three changes of PBS, and incubated in the fluorescence-labeled goat secondary antibody anti rabbit IgG solution (Sigma) 1/100 for 60 minutes. Sections were then washed for 5 min with three changes of PBS. Fluorescence-stained sections were examined under an epifluorescence microscope (Olympus). Evans blue was used as against dye, it appears in red. BDH appears in yellow.

Statistical analysis:

In each assay, the experimental data represent the mean of four independent assays ± SEM. Means were compared using the Student *t*-test. Differences were considered significant at $p < 0.05$ and very significant at the level $p < 0.01$.

Results:

Plasma analysis:

Table 1 reports the results of plasma analysis from the three physiological states of jerboa, euthermic, prehibernating and hibernating. We see a significant increase at the jerboa hibernating state compared to euthermic ones for the following parameters: bilirubine level (x 5.1), ketonemia (D-3-hydroxybutyrate level) (x 3.7), urea level (x 1.7) and HDL cholesterol (x 1.27). In contrast, there is a significant decrease in glycemia (x 0.64) and triglycerides level (x 0.56) were found. No changes were observed for GOT, GPT, CPK activities and total cholesterol, LDL and creatinine levels.

Biochemical characterization of mitochondrial populations of jerboa at different states:

The two mitochondrial populations, heavy and light fractions were isolated from liver at different jerboa states (euthermic (A), prehibernating (PH) and hibernating (H)). In Table 2, the relative activities of subcellular marker enzymes in the two mitochondrial fractions were reported. As one can see, the two fractions present high mitochondria content with a low contamination by peroxisomes, microsomes and cytosol. From the data, the purity is estimated at 72%, 80%, 78%, and 86%, 78%, 80% for heavy and light mitochondria at euthermic, prehibernating and hibernating jerboa states respectively.

The table 2 shows also a significant decrease of three markers at the hibernating state compared to the euthermic ones: succinate dehydrogenase (x 0.69), NADPH cytochrome c reductase (x 0.69) and glyceraldehyde-3-phosphate dehydrogenase (x 0.76). But, a significant increase was found in peroxisomal palmitoyl CoA oxidase activity (x 1.26) at the hibernating state compared to the euthermic ones.

Determination of BDH physicochemical parameters of mitochondrial populations at different jerboa states:

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

The optimal temperature for BDH activity is 35°C for heavy mitochondria and 40°C for light ones at euthermic state and it is equal to 40°C for the two mitochondrial populations at prehibernating and hibernating states (not shown).

BDH kinetic parameters from the heavy and light mitochondria (V_{max} , K_mBOH , K_mNAD^+ and K_DNAD^+) at different jerboa states were reported in Table 3. Interestingly, the specific activity of the BDH from the heavy mitochondria is 2.77-fold higher than that from the light mitochondria at euthermic state. But, in prehibernating state, the BDH activity from light mitochondria is 1.56-fold higher than that from the heavy ones. In hibernating state, the both of BDH activities are identical. The table 3 also shows that the BDH activity from heavy mitochondria is 0.40-fold lower and 2.68-fold higher at prehibernating and hibernating states respectively than that at euthermic state. For the BDH activity from the light mitochondria, it's 1.77 and 8.40-fold higher at prehibernating and hibernating states respectively than that at euthermic state.

Moreover, the kinetic constants with respect to the NAD^+ and BOH are higher in the light mitochondria than in the heavy ones (x 1.8 for the K_mNAD^+ , x 1.4 for the K_DNAD^+ , x 2.1 for K_mBOH). The table 3 also shows that in prehibernating state, the K_mNAD^+ from light mitochondria is 1.62-fold higher than that in euthermic state and the K_DNAD^+ from heavy and light mitochondria respectively at hibernating state is 4.29 and 5.18-fold higher than that at euthermic state.

Arrhenius-plots were carried out for the two mitochondrial populations at different jerboa states (figure 1). Interestingly, the plots show a break at 30°C for BDH from heavy mitochondria at euthermic and hibernating states and at 25°C for BDH from light mitochondria at prehibernating state. For other BDH mitochondrial populations no changes were found.

BDH protein expression

The BDH protein expression was approached in the three physiological states of jerboa both in heavy and light mitochondria by western-blotting (Fig. 2B A) and ELISA (Fig. 2C B). This amount of BDH is higher in the heavy mitochondria than that in the light ones at euthermic state. At prehibernating state, it is identical between the two mitochondrial populations but lower in the heavy mitochondria compared to euthermic state. At hibernating state, the BDH amount is higher for both mitochondrial populations compared to euthermic and prehibernating states.

In order to confirm *in situ* the differences observed in BDH protein expression (figure 2), jerboa liver sections (4 μ m) were revealed by the anti-BDH and fluorescence-labeled secondary antibody. The BDH appears yellow in sections. As one can see in figure 3, the yellow points decrease at the prehibernating state (B) and increase at the hibernating state (C) compared to

euthermic ones (A).

Phospholipids composition of heavy and light mitoplasts

In order to correlate the causes of differences observed in BDH kinetic parameters and Arrhenius-plots, the content of inner membrane phospholipids of different mitochondrial populations was determined. Table 4 shows that at euthermic state (reported in Mountassif et al., 2006), the two mitochondrial fractions with the amount of phosphatidylcholine being significantly greater in heavy mitochondria than in light ones (x 1.64). No differences were observed for phosphatidylinositol, phosphatidylserine and cardiolipin. At prehibernating state, the amount of phosphatidylethanolamine is much lower (x 0.39) and cardiolipin is largely higher (x 2.18) in heavy mitochondria than in light mitochondria at hibernating state. Table 4 shows also that phosphatidylethanolamine of heavy mitochondria decreases (x 0.47) significantly at prehibernating state compared to euthermic ones but cardiolipin and phosphatidylcholine increase (x 0.71 and 0.77) respectively. At hibernating state, cardiolipin of heavy mitochondria decreases (x 0.30) compared to euthermic state. Also, phosphatidylcholine of light mitochondria increases at prehibernating state (x 0.50) and at hibernating state (x 0.70) compared to euthermic ones.

Interestingly, the amounts of phosphatidylinositol and phosphatidylserine are quite high and were unchanged for all the isolated mitochondrial.

Comparison of impact of prehibernation and hibernation on BDH activities in jerboa and rat

In order to show that variations in BDH activity found in different physiological states of jerboa were particular to hibernator animals, we compared the response of rat (not hibernating animal) submitted at the same conditions of jerboa in term of BDH liver activity. The results reported in table 5 show a low changes in rat BDH activity compared to jerboa while passing from one state to another. Indeed, at euthermic state, BDH activity from the heavy mitochondria is 2.77-fold higher than that from the light ones for jerboa and 1.87-fold for rat. For jerboa, BDH activity from heavy mitochondria decreases (x 0.4) and increases (x 2.68) at prehibernating and hibernating states respectively, but for rat, no changes were observed. Also, BDH activity from light mitochondria increases (x 2.41 and x 2.21) at prehibernating and hibernating states respectively compared to euthermic ones for rat while it increases for jerboa (x 1.77 and x 8.40).

Discussion:

Hibernation is an adaptive strategy for some mammalian species to conserve energy in cold or unfavourable environments related to food availability. These mammals can reduce dramatically their basal metabolic, heart, and respiratory rates as well as their body temperature during hibernation (Magnus and Henderson, 1988; Weekley, 1995). Among mammalian hibernators, *Jaculus orientalis* is an excellent model for hibernation research due to its small body size and distinct behavioural pattern in relation to hibernation. Also, it's a true hibernator (El Hilali and Veillat, 1979) developing obesity by accumulating fat during its prehibernation period. Its fat is eliminated during hibernation leading to a high production of D-3-hydroxybutyrate to serve as an energy source in addition to carbohydrates (Kante et al., 1990). Its annual cycle consists of two phases, the summer season during which squirrels reproduce and accumulate fat, and the hibernating phase which is composed of alternating dormancy and arousal bouts (El Hilali and Veillat, 1979).

The changes involved in hibernation are precisely controlled and can only be reverted by internally-driven mechanisms, which suggests a specific biochemical regulation.

In this work, we report a comparative study of plasma metabolites on jerboa at different states (euthermic or active, prehibernating and hibernating) and the comparison of the BDH from the heavy and light mitochondria, in terms of kinetic parameters and content at these states.

The plasma analysis (table 1) indicates a significant increase at the jerboa hibernating state compared to euthermic ones in ketonemia and a significant decrease in glycemia and triglycerides

level. This could be with the consumption of glucose (first energy source) and stimulation of lipolysis leading to an increase in liver ketone bodies production and their passage into the blood. A raise of lipolysis has been previously proposed as a consequence of starvation in squirrels (Krulowicz, 1985). The table 1 shows a highly increase in urea and bilirubine levels with can results of the fact that jerboa does not urinate and defecate during hibernation, so the urea and bilirubine were concentrated in blood. Also, we found a significant increase in HDL cholesterol can protect jerboa life since HDL is the best protector agent against atherosclerosis which can be developed during hibernation and caused by the weak blood circulation due to the low body temperature and low number of cardiac beats.

The isolation of two mitochondrial populations, heavy and light fractions, from liver at different jerboa states (euthermic (A), prehibernating (PH) and hibernating (H) reveals a significant decrease of three subcellular markers at the hibernating state compared to the euthermic ones: succinate dehydrogenase, NADPH cytochrome c reductase and glyceraldehyde-3-phosphate dehydrogenase. For this latter, similar results were found in jerboa skeletal muscle (Soukri et al., 1995, 1996). This decrease can be explained by the fact that hibernation causes a reduction in body activities, including metabolic rate and body temperature is maintained within a few degrees above ambient temperatures. On the other hand, we found a significant increase in peroxisomal palmitoyl CoA oxidase activity at the hibernating state compared to the euthermic ones. This increase can be linked to the stimulation of lipolysis of fat accumulated during prehibernation period in order to oxidize fatty acids and subsequently produce ketone bodies (principal fuel consumed during hibernation).

Table 3 reveals interesting differences between BDH from heavy and light liver mitochondria at different jerboa states. We found that BDH activity is higher in heavy mitochondria than in light ones at euthermic state. In the other hand, at prehibernating and hibernating states, BDH activity is higher in light mitochondria than in heavy ones. We can suggest that cold exposure influences and increases light mitochondrial BDH biogenesis because it is more stable than heavy mitochondria. In previous study, we showed that BDH from the light mitochondria were more thermostable at different temperatures than the heavy ones (Mountassif et al., 2006). Table 3 indicate that BDH activity from heavy mitochondria decreases at prehibernating state and increases at hibernating state compared to euthermic ones while BDH activity from light mitochondria slightly increases lowly at prehibernating state but highly at hibernating conditions.

The determination of kinetic parameters indicated that the heavy and the light mitochondrial BDH at different jerboa states behave differently (Table 3). Indeed, the affinity of the BDH from heavy mitochondria with respect to the NAD^+ and BOH is stronger than from the light ones at euthermic state (Mountassif et al., 2006). In the prehibernating state, its affinity decreases (i.e. K_m , K_D increases) with respect to the NAD^+ and are higher in the light mitochondria. In the hibernation, the two BDH have similar affinities to NAD^+ and to BOH and were better compared to the euthermic state.

The differences in kinetic parameters can be the consequences of BDH conformational structure changes. The results of figure 3 support the existence of different BDH conformation. Indeed, Arrhenius-plots show a break at 30°C for BDH from heavy mitochondria at euthermic and hibernating states and at 25°C for BDH from light mitochondria at prehibernating state. For other situations no changes were found.

The differences observed might be caused either by the amount of BDH-which is different between the mitochondrial populations-or by the fact that the organelle phospholipid composition change since BDH is a lipid-dependent enzyme. In order to explain the differences, western blotting, ELISA (figure 2) and BDH immunofluorescence staining (figure 3) were done. The results obtained reveal a low decrease at prehibernating state and a high increase in BDH expression at hibernating ones. BDH activity is necessary during hibernation to produce ketone bodies used by all jerboa tissues to generate energy. Thus, the differences of BDH activity between the two populations of mitochondria at different jerboa states appear to be due to the amount of BDH present.

The analysis of BDH mRNA level by northern blotting for different states showed no significant variation (Kabine et al., 2003). So, we can postulate that variations in BDH expression observed would be due to a post-transcriptional regulation.

The phospholipid composition of heavy and light mitoplasts at different jerboa states were significantly different especially concerning phosphatidylcholine, an activator of BDH (El Kebbij et al., 1985; Loeb-Hennard and McIntyre, 2000), phosphatidylethanolamine and cardiolipin (table 4). Previous studies on the interactions of BDH with phospholipids (Gazzoti et al., 1964; McIntyre et al., 1978; Berrez et al., 1984; Kante et al., 1990) have demonstrated that the composition of membrane phospholipids induced structural modifications of BDH. The local environment of the protein may result in an important kinetic change. A change of physical properties of the mitochondrial membrane related to the hibernation process has been also reported in ground squirrel (Raison and Lyons, 1971). Thus, the differences observed in BDH kinetic parameters (table 3) and BDH temperature-dependent activation between mitochondrial populations appear to be due to the differences in phospholipid composition of the inner mitochondrial membrane.

In order to show that all variations found in different physiological states of jerboa were particular to hibernator animals, we compared the liver BDH activity in rat (not hibernating animal) submitted at the same conditions of jerboa. The results obtained (table 5) show only a low change in rat BDH activity compared to jerboa while passing from one state to another. While rats die after 7 days of hibernating conditions (e.g. cold and starved), in contrast jerboas can resist, probably because they activate lipogenesis during prehibernation in order to accumulate fat and stimulate lipolysis during hibernation in order to generate ketone bodies. In addition, jerboas have a temperature regulation system and adapt their heart beating which allows him to resist and support the cold conditions contrary to the rat (Hooper and El Hilali, 1972).

In conclusion, prehibernation and hibernation of jerboa lead to regulation of liver mitochondrial BDH. The enzyme activity as well as kinetic parameters are varying according to physiological states of jerboa (euthermic, prehibernating and hibernating). This regulation of BDH activity would lead to a change in plasma D-3-hydroxybutyrate level in order to adapt energy metabolism in peripheral tissues. Our results reveal that the distinct enzymatic forms of liver BDH in jerboa are subjected to differential regulation depending on the state. This regulation could be a result of protein expression and modifications of mitochondrial membrane composition.

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TABLES & FIGURES

Table 1
Jerboa plasma analysis at euthermic, prehibernating and hibernating states

| | Euthermic state | Prehibernating state | Hibernating state |
|--|-----------------|----------------------------------|------------------------------------|
| D-3-Hydroxybutyrate (mM) | 0.17 ± 0.03 | 0.15 ± 0.05 | 0.63 ± 0.09 (×3.7) ^{a,b} |
| Glucose (g l ⁻¹) | 1.25 ± 0.12 | 1.3 ± 0.11 | 0.80 ± 0.09 (×0.64) ^{a,b} |
| Total cholesterol (g l ⁻¹) | 1.11 ± 0.22 | 1.17 ± 0.18 | 1.68 ± 0.25 (×1.51) ^a |
| HDL cholesterol (g l ⁻¹) | 0.74 ± 0.06 | 0.80 ± 0.07 | 0.94 ± 0.08 (×1.27) ^a |
| LDL cholesterol (g l ⁻¹) | 0.18 ± 0.07 | 0.21 ± 0.1 | 0.28 ± 0.11 |
| Triglycerides (g l ⁻¹) | 1.44 ± 0.46 | 1.66 ± 0.23 | 0.81 ± 0.17 (×0.56) ^{a,b} |
| GOT (U l ⁻¹) | 159 ± 5.65 | 145 ± 9.62 | 143 ± 6.71 |
| GPT (U l ⁻¹) | 6 ± 2.24 | 8.75 ± 1.33 | 6.08 ± 1.92 |
| CPK (U l ⁻¹) | 1143 ± 280 | 698 ± 421 | 826 ± 335 |
| Urea (U l ⁻¹) | 0.38 ± 0.08 | 0.36 ± 0.07 | 0.65 ± 0.11 (×1.7) ^{a,b} |
| Creatinine (U l ⁻¹) | 6.5 ± 0.07 | 6.03 ± 0.05 (×0.92) ^a | 7.96 ± 0.13 (×1.22) ^{a,b} |
| Total bilirubin (U l ⁻¹) | 8 ± 3 | 17.6 ± 6 | 41 ± 11 (×5.1) ^{a,b} |

Values are given as means of four separated experiments ± standard deviations. Numbers in brackets correspond to the variation compared to the euthermic state.

^a Variations statistically significant at $p < 0.01$ compared to the euthermic state (ANOVA).

^b Variations statistically significant at $p < 0.01$ compared to the prehibernating state (ANOVA).

Table 2
Subcellular marker enzyme activities in the heavy and light mitochondrial fractions from jerboa liver compared to liver homogenate at euthermic, prehibernating and hibernating jerboa states

| | | Mitochondria | Peroxisomes | Microsomes | Cytosol | Mitochondrial |
|------------------------------|------------------------------|--|--|---|---|---------------|
| | | Succinate dehydrogenase (absorbance min ⁻¹ (mg protein) ⁻¹) | Palmitoyl-CoA oxidase (nmol min ⁻¹ (mg protein) ⁻¹) | NADPH-cytochrome c reductase (nmol min ⁻¹ (mg protein) ⁻¹) | Glyceraldehyde-3-phosphate dehydrogenase (μmol min ⁻¹ (mg protein) ⁻¹) | purity (%) |
| Euthermic state ^c | Homogenate | 1.04 ± 0.12 | 7.52 ± 0.47 | 79.3 ± 9.44 | 0.89 ± 0.05 | — |
| | Heavy mitochondrial fraction | 2.25 ± 0.18 (×2.16) ^b | 0.58 ± 0.04 (×0.08) ^b | 15.74 ± 3.25 (×0.2) ^b | None | 72 |
| | Light mitochondrial fraction | 1.83 ± 0.21 (×1.76) ^b | 1.48 ± 0.12 (×0.2) ^b | None | None | 80 |
| Prehibernating state | Homogenate | 0.67 ± 0.09 ^a | 9.53 ± 0.52 | 67.5 ± 8.32 | 0.95 ± 0.07 | — |
| | Heavy mitochondrial fraction | 1.70 ± 0.23 (×2.53) ^b | 0.37 ± 0.09 (×0.04) ^b | 12.46 ± 2.89 (×0.18) ^b | None | 78 |
| | Light mitochondrial fraction | 1.27 ± 0.21 (×1.89) ^b | 1.32 ± 0.09 (×0.14) ^b | None | None | 86 |
| Hibernating state | Homogenate | 0.71 ± 0.08 ^a | 11.12 ± 0.76 ^a | 54.2 ± 5.53 ^a | 0.67 ± 0.06 ^a | — |
| | Heavy mitochondrial fraction | 1.88 ± 0.17 (×2.64) ^b | 0.41 ± 0.07 (×0.03) ^b | 10.39 ± 3.84 (×0.19) ^b | None | 78 |
| | Light mitochondrial fraction | 1.40 ± 0.21 (×1.97) ^b | 2.21 ± 0.14 (×0.2) ^b | None | None | 80 |

Values are given in specific activities. Values are given as means ± SD of four separate experiments. Numbers in brackets correspond to the variation compared to homogenate. For experimental conditions, see Section 2.

^a Variations statistically significant at $p < 0.01$ compared to euthermic state (ANOVA).

^b Variations statistically significant at $p < 0.01$ compared to homogenate (ANOVA).

^c Published in Mountassif et al. [10].

Table 3

Determination of the kinetic parameters of the liver BDH from heavy and light mitochondria at euthermic, prehibernating and hibernating jerboa states

| | | $K_m \text{ NAD}^+$ (mM) | $K_m \text{ BOH}$ (mM) | $K_D \text{ NAD}^+$ (mM) | V_{\max} (nmol NADH min ⁻¹ (mg protein) ⁻¹) |
|------------------------------|--------------------|----------------------------|--------------------------|--------------------------|--|
| Euthermic state ^d | Heavy mitochondria | 0.21 ± 0.01 | 1.60 ± 0.22 | 1.16 ± 0.50 | 0.61 ± 0.05 |
| | Light mitochondria | 0.39 ± 0.03 ^c | 3.36 ± 0.33 ^c | 1.66 ± 0.33 | 0.22 ± 0.01 ^c |
| Prehibernation state | Heavy mitochondria | 0.31 ± 0.03 | 3.78 ± 0.21 ^a | 4.75 ± 0.75 ^a | 0.25 ± 0.04 ^a |
| | Light mitochondria | 0.24 ± 0.01 ^{b,c} | 4.90 ± 0.36 | 2.25 ± 0.25 ^c | 0.39 ± 0.06 ^{b,c} |
| Hibernation state | Heavy mitochondria | 0.25 ± 0.02 | 2.77 ± 0.5 | 0.32 ± 0.27 ^a | 1.64 ± 0.11 ^a |
| | Light mitochondria | 0.35 ± 0.12 | 4 ± 0.94 | 0.41 ± 0.32 ^b | 1.85 ± 0.13 ^{b,c} |

Experiments with varying NAD concentrations (0.5, 1, 1.5 and 2 mM) or BOH concentrations (2.5, 5, 7.5 and 10 mM). Values are given as means ± SD of three independent experiments. V_{\max} values have been calculated according to the purity of the mitochondrial fractions (Table 1).

^a Variations statistically significant at $p < 0.01$ compared to heavy mitochondria of the euthermic state (ANOVA).

^b Variations statistically significant at $p < 0.01$ compared to light mitochondria of the euthermic state (ANOVA).

^c Variations statistically significant at $p < 0.01$ between heavy and light mitochondria at the same state.

^d Published in Mountassif et al. [10].

Table 4

Lipid composition analysis by thin layer chromatography of the heavy and light mitoplasts at euthermic, prehibernating and hibernating jerboa states

| | | Phospholipids (%) | | | |
|------------------------------|--------------------|---------------------|--------|-----------------------|-----------------------|
| | | PE | PI/PS | CL | PC |
| Euthermic state ^d | Heavy mitochondria | 28 ± 6 | 32 ± 5 | 17 ± 2 | 23 ± 3 |
| | Light mitochondria | 33 ± 5 | 38 ± 4 | 15 ± 3 | 14 ± 1 ^c |
| Prehibernation state | Heavy mitochondria | 13 ± 3 ^a | 33 ± 1 | 24 ± 2 ^a | 30 ± 2 ^a |
| | Light mitochondria | 33 ± 6 ^c | 28 ± 7 | 11 ± 2 ^c | 28 ± 6 ^b |
| Hibernation state | Heavy mitochondria | 30 ± 4 | 34 ± 6 | 5 ± 1 ^a | 31 ± 6 |
| | Light mitochondria | 25 ± 3 | 34 ± 4 | 21 ± 3 ^{b,c} | 20 ± 2 ^{b,c} |

PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; CL, cardiolipin. After deposit of 40 µg lipid phosphorus of phospholipids, migration and revelation, the amount of phosphorus was measured in each band as described in Section 2. Values are given as means ± SD of three independent experiments.

^a Variations statistically significant at $p < 0.01$ compared to heavy mitochondria of the euthermic state (ANOVA).

^b Variations statistically significant at $p < 0.01$ compared to light mitochondria of the euthermic state (ANOVA).

^c Variations statistically significant at $p < 0.01$ between heavy and light mitochondria at the same state.

^d Published in Mountassif et al. [10].

Table 5

BDH activities in the heavy and light mitochondrial fractions from jerboa and rat livers at euthermic, prehibernating and hibernating states

| | | Jerboa | Rat |
|---|------------------------------|----------------------------|---------------------------|
| Euthermic state ^d | Heavy mitochondrial fraction | 0.61 ± 0.05 | 9.76 ± 0.4 |
| | Light mitochondrial fraction | 0.22 ± 0.01 ^c | 5.21 ± 0.56 ^c |
| Prehibernating state for jerboas and cold with food for rats | Heavy mitochondrial fraction | 0.25 ± 0.04 ^a | 11.7 ± 0.46 |
| | Light mitochondrial fraction | 0.39 ± 0.06 ^{b,c} | 12.57 ± 0.46 ^b |
| Hibernating state for jerboas and cold with starvation for rats | Heavy mitochondrial fraction | 1.64 ± 0.11 ^a | 9.24 ± 0.2 |
| | Light mitochondrial fraction | 1.85 ± 0.13 ^{b,c} | 11.54 ± 0.19 ^b |

Values are given in specific activity (nmol min⁻¹ (mg protein)⁻¹). Values are given as means ± SD of four separate experiments.

^a Variations statistically significant at $p < 0.01$ compared to heavy mitochondria of the euthermic state (ANOVA).

^b Variations statistically significant at $p < 0.01$ compared to light mitochondria of the euthermic state (ANOVA).

^c Variations statistically significant at $p < 0.01$ between heavy and light mitochondria at the same state.

^d Published in Mountassif et al. [10].

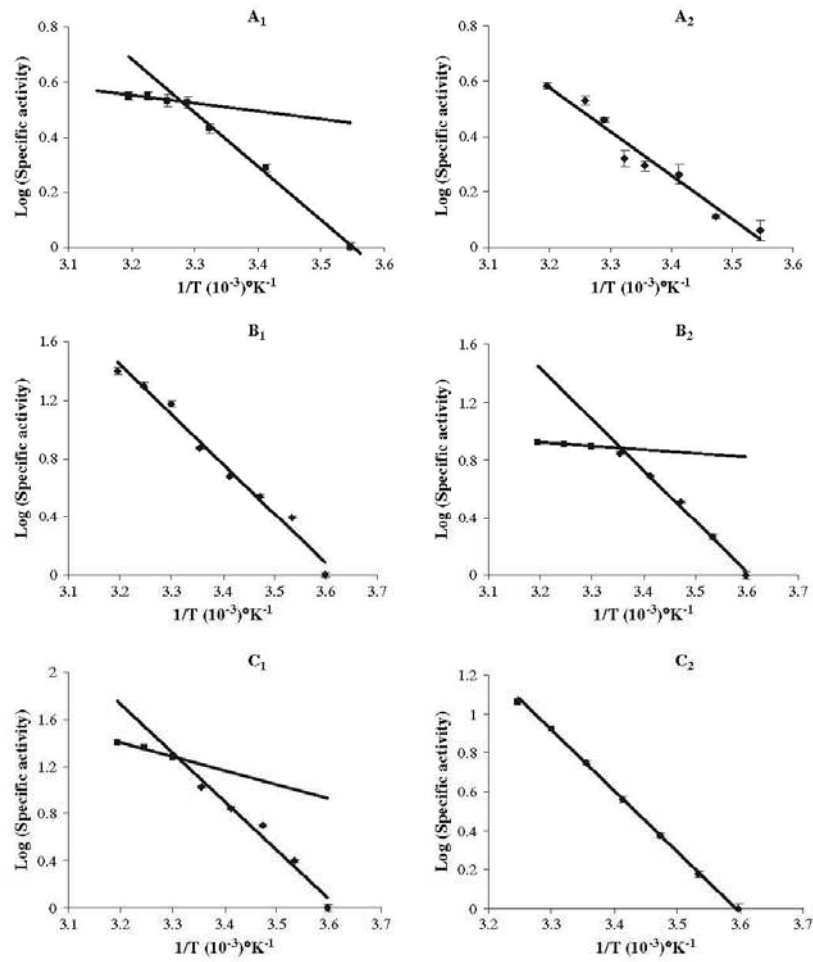


Fig. 1. Arrhenius-plots of the liver BDH from heavy (1) and light (2) mitochondria at euthermic (A) (cf. Mountassif et al. [10]), prehibernating (B) and hibernating (C) jerboa states. Plots were obtained by measuring the enzymatic activity using 100 μ g of liver mitochondrial protein at various temperatures (from 5 to 40°C). Values are given as mean \pm S.D. of three separate experiments.

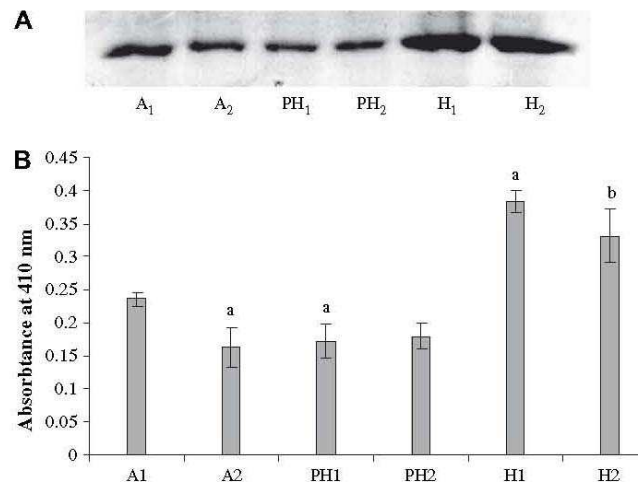


Fig. 2. Western-blotting (A) and ELISA (B) of the liver BDH from heavy and light mitochondria at euthermic (A), prehibernating (PH) and hibernating (H) jerboa states. Western-blotting was assayed with 50 μ g of proteins and ELISA with 10 μ g of protein. Lanes represent heavy (1) and light (2) mitochondrial protein fractions from euthermic (A), prehibernating (PH) and hibernating (H) states, respectively. a and b represent significant variations compared to heavy and light mitochondrial protein fractions respectively at euthermic state ($p < 0.05$, Student *t*-test). For experimental conditions, see Section 2.

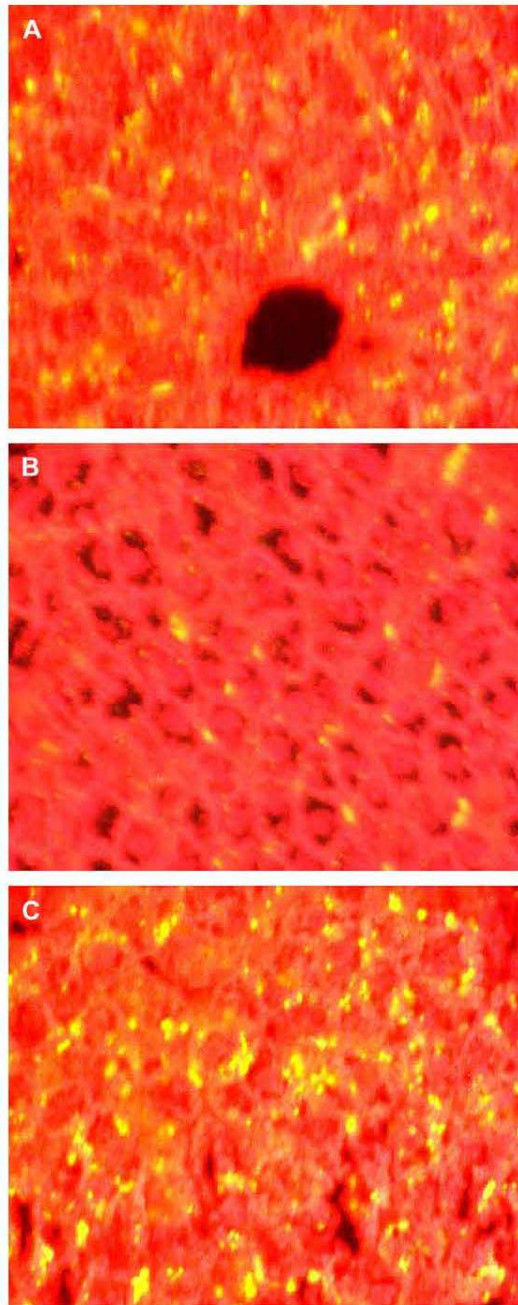


Fig. 3. Immunofluorescence staining and subsequent microscopy of the liver BDH from euthermic (A), prehibernating (B) and hibernating (C) jerboa states. For experimental conditions, see Section 2.