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D-3-hydroxybutyrate oxidation in mitochondria by D -3-Hydroxybutyrate dehydrogenase in *Tetrahymena pyriformis*

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Tetrahymena pyriformis **a ciliated protozoan, is considered as a good indicator of water pollution. However its energy supply is poorly understood. This work was focused on the metabolism of hydroxybutyrate through the study of the membrane bound mitochondrial NAD^+-dependent D-3-hydroxybutyrate dehydrogenase (EC. 1.1.1.30) (BDH), a ketone body catalysing enzyme involved in the interconversion of D-3- hydroxybutyrate to acetoacetate. Due to lack of informations, the physico-chemical properties and kinetic parameters of the enzyme were examined. The results are the following: 1) D-3-hydroxybutyrate is a good substrate for mitochondria. 2) The enzyme catalytic process follows a bi bi-ordered mechanism where the coenzyme binds first, then allowing the substrate linkage to the active site. 3) Two optimal pH values of 8 and 6.5 corresponding to D-3-hydroxybutyrate oxidation and to acetoacetate reduction respectively. On the other hand, pH changes affect the coenzyme binding to the active site. 4) The BDH activity was found strongly linked to submitochondrial vesicles indicating that the protozoan enzyme is membranous and could require lipids for its function as well as it is for the mammalian enzyme. Moreover, an optimal temperature (40°C) and a break appearing in the Arrhenius plot at 19°C were found. The break suggests a membrane lipid fluidity-dependency of BDH conformational change. 5) Several ligands of the active site including methylmalonate and succinate modulate the BDH activity and are competitive inhibitors toward D-3-hydroxybutyrate. 6) Divalent cations, Mg^{2+}, Mn^{2+} and Zn^{2+} protect BDH against thermal inactivation. The protection is the strongest in the presence of Zn^{2+}. Moreover, Ca^{2+} and Mg^{2+} are enzyme activators and modulate the substrate binding to the active site. On the other hand, EDTA, a chelating agent, inhibits the enzyme but prevents inhibition by substrate excess. This work provides new insights on the energy metabolism of *T. pyriformis* wild strain where D-3-hydroxybutyrate is a choice substrate where the properties of BDH have been established especially the activating role of non heavy divalent cations.**

Key words: D-3-hydroxybutyrate dehydrogenase, ketone body, mitochondria, *Tetrahymena pyriformis*.

INTRODUCTION

In mammalian, the NAD^+ -dependent D-3-hydroxybutyrate dehydrogenase (EC. 1.1.1.30) (BDH) is the ketone body converting enzyme located in the inner face of the mitochondrial membrane. Its physiological role appears to be dual, that is, in liver, to convert, acetoacetate into D-3-hydroxybutyrate, a reducing equivalent and a potential energetic fuel to be exported to extrahepatic tissues, especially in brain, heart, kidney and in muscle. In these latter tissues, BDH operates in the opposite way; secondly, to prevent acidification of high amount of acetoacetate, a strong acid form of ketone bodies which are overproduced during ketosis appearing during fasting, diabete mellitus, hyperlipidic diet. Concerning BDH properties, until now BDH was largely studied in several organisms and tissues, especially in rat liver (Latruffe and Gaudemer, 1974), beef heart (Nielsen et al., 1973), or in Jerboa

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(Mountassif et al., 2008). BDH catalyses the reversible oxidation of BOH to AcAc in presence of NAD(H) as cofactor, according to an ordered bi bi mechanism where the coenzyme binds first to the enzyme catalytic site (Latruffe and Gaudemer, 1974; Nielsen et al., 1973). While the enzyme is soluble in bacteria (Bergmeyer et al., 1967), in contrast, in eucaryotes it is anchored in inner mitochondrial membrane (Latruffe and Gaudemer, 1974; Nielsen et al., 1973). The mitochondrial BDH requires lecithin for its activity where phosphatidylcholine have been demonstrated to induce a discrete but significant conformational change of the active site (El Kebbaj et al., 1986). On the other hand, in bacteria, the role of BDH in the energetic metabolism is to oxidize D-3-hydroxybutyrate to acetoacetate leading to the availability of NADH, the main electron donor molecule, to the membrane bound oxidative phosphorylation machinery producing ATP. In such procaryotic organism, D-3-hydroxyburate is produced from the hydrolysis of poly-β-hydroxybutyrate (PHB), an energy storage polymer.

In the recent past researchers paid attention to *Tetrahymena*, a unicellular ciliated lower eukaryotic of microorganism either in the field of phylogeny (Mukai and Endoh, 2004), mitochondrial dependent-apoptosis (Kobayashi and Endoh, 2003; Kobayashi and Endoh, 2005; Endoh and Kobayashi, 2006), heavy metal pollution (Rico et al., 2009) or mitochondrial linked-phospholipids metabolism (Tellis et al., 2003). Interestingly, BDH is also present in *Tetrahymena pyriformis* and considered as a good indicator of water pollution (Mountassif et al., 2006a). In this protozoan, the enzyme is located in the mitochondria (Conger and Eichel, 1965). However, its metabolic role is unknown. We postulate that acetyl-CoA produced from fatty acid oxidation cycle would be converted into acetoacetyl-CoA. Following this, the carbon chain of acetoacetyl-CoA would be incorporated into poly--hydroxybutyrate (PHB). Then when needed, the D-3-hydroxybutyrate produced from PHB hydrolysis would be oxidized by BDH as enzyme coupled to the mitochondrial electron transport chain.

Our group has shown that *T. pyriformis* BDH was inhibited by peroxisome proliferators as it is the case for rat liver BDH and that inhibition process occurs in the active site of the enzyme (El Kebbaj et al., 1995). However its energy supply is poorly understood. This work was focused on the metabolism of hydroxybutyrate through the study of the membrane bound mitochondrial NAD+ -dependent D-3-hydroxybutyrate dehydrogenase (EC. 1.1.1.30) (BDH), a ketone body catalyzing enzyme involved in the interconverting D-3-hydroxybutyrate to acetoacetate. Due to the lack of information concerning the use of BOH as mitochondrial energy substrate and the biochemical properties of the converting enzyme in protozoan, we isolated mitochondria of *T. pyriformis* in order to study its respiratory properties during the BDH oxidation process. Then, we determined BDH catalytic parameters, that is, reaction mechanism, themodynamic characteristics and sensitivity towards cations and cofactors.

The aim of this study was to provide a better understanding in the biology and the metabolism of *T. pyriformis*, a water living unicellular organism which can be exposed to environmental changes, that is, substrate availability, salts, toxic, temperature and pH changes.

MATERIALS AND METHODS

Biological preparations

T. pyriformis, wild strain, was grown aerobically without shaking, in a broth medium containing 1.5% proteose-peptone, 0.25% yeast extract at 27°C during one week (Latruffe et al., 1982). Preparations of coupled mitochondria were performed as described in (Weinbach, 1961) after washing the cells with the dry medium (0.58 g/l Na- citrate, 0.15 g/l NaH_2PO_4, 0.14 g/l Na_2HPO_4 and 0.2 g/l $CaCl_2$).

Submitochondrial vesicles (SMV) which are inside out inner mitochondrial membrane particles are prepared from mitochondria (Kielley and Bronk, 1958) by swelling mitochondria in 20 mM KH_2PO_4 hypotonic buffer and sonication in order to break mitochondrial membrane and release the matrix. Protein estimation was done using the Bio-Rad assay according to the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Oxygen consumption

Freshly isolated and intact mitochondria (10 mg per essay) were incubated and oxygen consumption was measured using a Clarke Electrode (Oxygraph Gilson). Respiratory control ratio and respiratory chain oxidative activities, in state 4 or in state 3, were measured at 37°C in the Ernster medium containing 62 mM sucrose, 8 mM $MgCl_2$, 50 mM KCl, 20 mM Hepes, pH 7.3 as described in (Estabrook, 1967) using the following substrates either, glutamate + malate, D-3-hydroxybutyrate, succinate, α-ketoglutarate, citrate or pyruvate at 5 mM (state 3 was obtained with the presence of 0.2 mM ADP and 4 mM inorganic phosphate…).

Enzymatic activities measurements

BDH activity was measured as usual (Mountassif et al., 2006b) at 37°C by following NADH production at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}.cm^{-1}$) using either SMV or frozen- thawed mitochondria (as indicated in the legends of figures) 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+ and 2.5 μg rotenone (final addition) to prevent NADH reoxidation by the respiratory chain. The activity was started by the addition of DL- 3-hydroxybutyrate to 10 mM final concentration. Kinetic parameters of BDH were determined by measuring the initial rate at 37°C (excepted if indicated conditions cf Figure 2) in a standard medium as above described for the oxidation of D-3-hydroxybutyrate using the following coenzyme and substrate concentrations:

$[NAD^+]$ = 0.2, 0.4, 0.6 or 0.8 mM ; [D-3-hydroxybutyrate] = 1.25, 2.5, 4.5 or 10 mM; or in the same medium at pH 7 without NAD^+ and rotenone and in presence of varying acetoacetate concentrations (0.2, 0.4, 0.6 or 0.8 mM) and NADH concentrations (0.2, 0.4, 0.6 or 0.8 mM) (using 0.8 mM NADH, the absorbance was mesued at 366 nm ($\varepsilon = 3.0 .10^3\ M^{-1}.cm^{-1}$) in order to measure optical density). In all cases, different NaCl concentrations were ajusted to the medium in order to keep constant salt concentration. Graphical determination of parameters was made from mathematical analysis according to

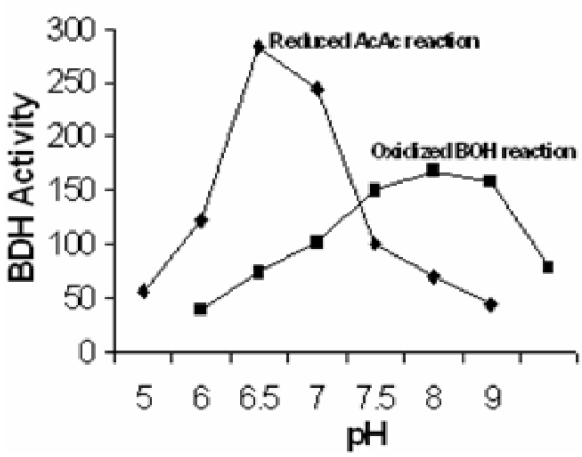

Figure 1. pH dependency of BDH activity on forward –black squares - or reverse –black diamonds - reaction. Aliquots of submitochondrial vesicles (1.7 mg protein/ml) were used to measure BDH activity in function of pH with a buffer coktail containing 50 mM of Tris-HCl, Mes, Hepes, and potas-sium phosphate ; pH was adjusted at 37°C either with KOH or HCl. BDH activity was expressed in nmol NAD reduced or NADH oxidized. mn $^{-1}$. mg protein $^{-1}$. Data correspond to one of three representative experiments.

was measured as reported (Bruni et al., 1965) by estimation of inorganic phosphate using the ''Fiske and Subbarow'' method.

Chemicals

DL-3-hydroxybutyrate (sodium salt) was purchased from Fluka (Buchs Switzerland); NAD+ (free acid) and NADH were from Boehringer (Mannheim, Germany); acetoacetate, methylmalonate and succinate were from Sigma (St Louis, USA) and all other chemicals were of analytical grade.

Statistical data analysis

In each assay, the experimental data represent the mean of three independant assays. Student test was used as value lower than 0.05 was considered significant.

Remark

Despite that *T. pyriformis* grows experimentally at 27°C or possibly ambiante varying temperatures in its natural biotope, we measured enzymatic activities at the usual temperature of 37°C since Figure 2 shows that BDH activity inceases almost regularly from 15 to 37°C (optimum temperature activity).

RESULTS

Mitochondrial oxidative characteristics of *T. pyriformis*

Coupled mitochondria were purified from *T. pyriformis* wild strain. Mitochondrial oxidative activities were measured using several substrates, D-3-hydroxybutyrate, succinate, 2-ketoglutarate, citrate, pyruvate and a mixture of glutamate + malate.

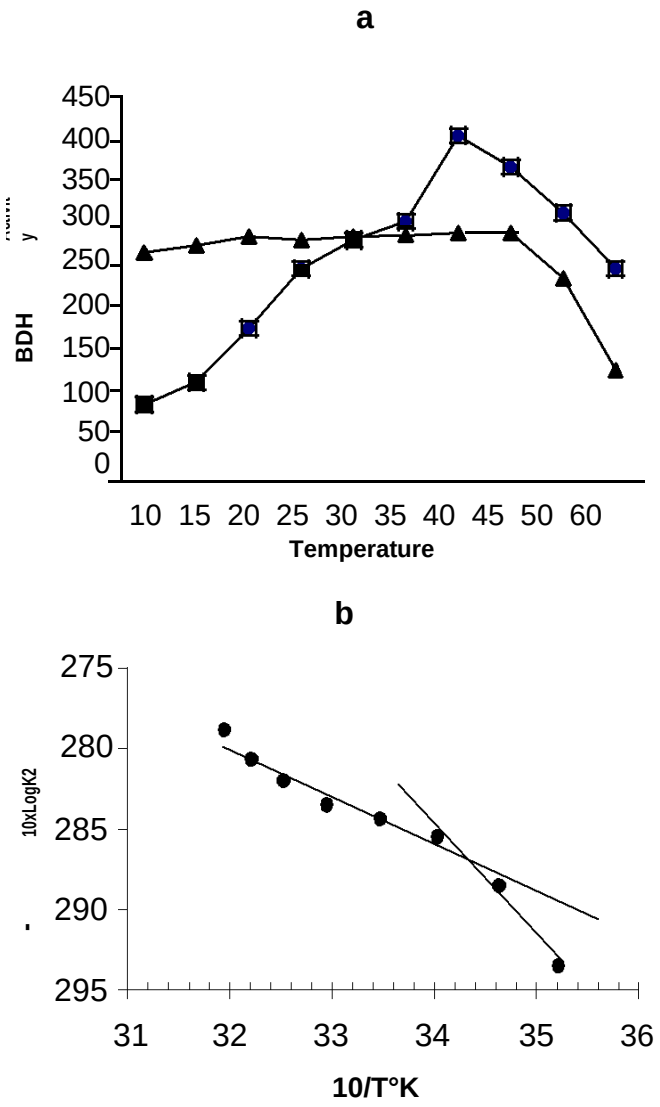

Figure 2. Effect of temperature on BDH activity. For thermal activation –black squares of the enzyme, aliquots of submitchondrial vesicles (1.9 mg protein/ml) were used to measure BDH activity at different temperatures (from 10 to 60°C). For thermal inactivation –black diamonds of the enzyme, aliquots of submitochondrial vesicles (1.9 mg protein/ml) were incubated for three minutes at each fixed temperature (from 10°C to 60°C) before performing BDH activity assay at 37°C. BDH activity was expressed in nmol NAD reduced. min^{-1}. mg protein^{-1}. (Figure 2a). Data are from one of five representative experiments. For Arrhenius plot of BDH activity (Figure 2b), the logarithm of the catalytic activity (Ln k 2) is plotted as a function of the reciprocal of temperature (in Kelvin degrees, T°K) . The lines represent the best linear regression of data points. The slopes of the line correspond to two activation energy values (-Ea/R). The arrow indicates the break in the activity. The adjacent numbers correspond to apparent Ea values (in Kcal/mol). Data are from two representative experiments.

As shown in Table 1, the oxygen consumption is the highest with D-3-hydroxybutyrate as substrate. The stimulation of oxidative activities in presence of ADP is si-

Table 1. Mitochondrial oxygen consumption and respiratory control ratio of *T. pyriformis.*

Mean of oxygen consumption (n. atoms O_2 / min / mg prot) at 37°C	Respiratory control ratio		
Substrates	**State 4**	**State 3**	**State 3 / State 4**
Glutamate + Malate	1.22	2.34	1.91
3-hydroxybutyrate	1.70	3.34	1.96
Succinate	1.29	2.20	1.70
2-ketoglutarate	1.27	3.22	2.53
Citrate	1.26	2.16	1.71
Pyruvate	0.95	3.02	3.16

For experimental conditions, see materials and methods. Freshly isolated and intact mitochondria were used in this study. Assays have been done in duplicate and experiments have been repeated twice. No significant variations were observed in these conditions.

Table 2. Specific activities of mitochondrial BDH, SDH and ATPase from *T. pyriformis*.

Enzyme	Specific activity at 37°C (n.mol. product / min / mg prot.)	
	Control	**+CAP**
BDH	110.15 ± 16.41	107.81 ± 3.12 (x 0.98)
SDH	39.84 ± 3.91	64.84 ± 5.86 (x 1.63)
ATPase	208.59 ± 23.43	100 ± 33.2 (x 0.48)

For experimental conditions, see materials and methods. The specific activities correspond to NADH oxidation, DCIPH2 oxidation or inorganic phosphate production for BDH, SDH and ATPase respectively. The measurements using frozen-thawed mitochondria (5 mg protein) have been done in triplicate for BDH and SDH, and in duplicate for ATPase. Chloramphenicol (CAP), a mitochondrial protein synthesis inhibitor was added in the *T. pyriformis* medium during growth at 50 µg/ml final concentration. Experiments were repeated twice.

milar for BOH and all other substrates. The specific activities of inner mitochondrial bound enzymes were reported in Table 2. As shown, BDH activity is higher than SDH activity but lower than ATPase activity. The presence of chloramphenicol (CAP), a mitochondrial protein synthesis inhibitor (Turner and Lloyd, 1971) in the culture medium, stimulates SDH activity and decreases ATPase activity but does not significantly modify BDH activity.

Catalytic mechanism of BDH

Following the Lineweaver and Burk analysis (Lineweaver and Burk, 1934), the obtained kinetic parameters are summarized in Table 3. It appears, in results not reported, using Cleland analysis (Cleland, 1963), that BDH from *T. pyriformis* follows an ordered bi bi catalytic mechanism where NADH is a competitive inhibitor towards NAD+ (Ki = 0.047 mM) and non competitive towards D - 3-hydroxybutyrate (Ki = 0.11 mM), while acetoacetate is non competitive towards both BOH (Ki = 2.65 mM) and NAD+ (Ki = 1.8 mM).

Effect of pH on BDH activity

The variation of BDH activity versus medium pH is shown in Figure 1. The highest activity was obtained at pH 8 for the conversion of D- 3-hydroxybutyrate to acetoacetate, while an optimum pH of 6.5 was observed for the conversion of acetoacetate to D-3-hydroxybutyrate.

Effect of temperature on BDH activity

The study of enzyme stability towards temperature is reported in Figure 2. It can be seen that BDH denaturetion process occurs at 40°C. On the other hand, the Arrhenius plot reveals a break at 19°C with two activation energy values, that is, Ea = 6.77 Kcal.mol^{-1} at temperatures higher than the break and Ea = 13.53 Kcal .mol^{-1} at temperatures lower than the break.

Effect of ligands and cations on BDH thermal stability and activity

The effect of temperature on enzyme stability was stu-died in the presence of cations, coenzyme or substrate. It appeared (Table 4) that Zn^{2+} strongly protects BDH activity against denaturation (93% after 20 min of incubi-tion) . A low but significant protection was also observed with Mg^{2+}, Mn^{2+}, Ca^{2+} and with NAD(H). Thermodynamic parameters of the NAD^{+} binding to BDH were determined at 37°C. The calculated values were the following: G = - 3.91 Kcal.mol^{-1}, H = 5.97 Kcal.mol^{-1}, S = 32.18 cal

Table 3. Apparent kinetic parameters of mitochondrial BDH from *T. pyriformis* in forward (oxidation) and in reverse (reduction) reactions.

Forward reaction (Vmax = 130 nmol NADH produced / mg of protein / min)			Reverse reaction (Vmax = 280 nmol NADH oxidized / mg of protein / min)		
K_M NAD^+ (mM)	K_D NAD^+ (mM)	K_M BOH (mM)	K_MNADH (mM)	K_D NADH (mM)	K_MAcAc (mM)
0.33 ± 0.04	2.5 ± 0.5	2.22 ± 0.13	0.11 ± 0.03	1.11± 0.05	1.15 ± 0.07

Aliquots of submitochondrial vesicles (SMV) (3 - 5 mg/ml) were used to measure BDH activity at 37°C at various substrates concentrations as indicated in materials and methods section. The mean values are given ± SEM as the result of three independent experiments.

Table 4. Effect of coenzymes, substrates and cations on BDH activity and against thermal inactivation.

	Remaining BDH activity (%) after preincubation at 37°C			
	0 min (instant measurement)	5 min	10 min	20 min
Control	100	60 ±10	40 ±10	20 ±3.0
NAD^+ (1.5 mM)	-----	73.7 ± 10	52.6 ± 15	25 ±7.0
NADH (1.5 mM)	-----	100 ± 00	75 ±3.0	30 ±12
$NADP^+$ (2 mM)	-----	57 ±25	35.7 ± 11	14.3 ± 10
BOH (1.3 mM)	-----	60 ±7.0	50 ±14	20 ±7.0
AcAc (1 mM)	-----	66 ±8.0	41 ±8.0	20 ±10
$MgCl_2$ (3 mM)	180 ± 10 (+ 80%)	63 ±7.0	63 ±9.0	47.3 ± 6.0
Control + EDTA 1.66 mM	50	----------	----------	----------
Control + EDTA 1.66 mM + $MgCl_2$ (1 mM)	90	----------	----------	----------
$CaCl_2$ (3 mM)	150 ± 10 (+ 50%)	84.2± 4.0	63 ±17	26 ±4.0
$MnCl_2$ (3 mM)	100 (0)	89 ±8.0	58 ±8.0	52.6 ± 3.0
$ZnCl_2$ (3 mM)	98.5 (- 1.5%)	93 ±8.0	93 ±4.0	93 ±10
NaCl (3 mM)	100 ± 6 (0%)	62 ±16	45 ±6.0	22 ±9.0

Submitochondrial vesicles (SMV) (3 mg protein / ml) were preincubated in 50 mM Hepes, pH 7.4 at 37°C in presence or in absence of effectors. Aliquots were removed at different times to measure BDH activity, as described in materials and methods. Results are the means ± SEM of three separate experiments.

Table 5. Effect of divalent cations (Mg^{2+} and Ca^{2+}) on BDH kinetic parameters and on BDH competitive inhibitor-inhibition constant.

Kinetic constants	K_M NAD^+ (mM)	K_D NAD^+ (mM)	K_M BOH (mM)	Ki methyl malonate (mM)
Control	0.33 ± 0.04	2.22 ± 0.02	2.5 ± 0.1	0.12 ± 0.02
+ Mg^{2+} (2 .5 mM)	0.22 ± 0.03$^{(NS)}$	1.25 ± 0.36 $^{(NS)}$	1.29 ± 0.38$_{(NS)}$	3.33 ± 0.15$^{(*)}$
+ Ca^{2+}(1.5 mM)	0.34 ± 0.10 $^{(NS)}$	1.33 ± 0.4 $^{(NS)}$	1.16 ± 0.08$_{(NS)}$	1.50 ± 0.2$^{(*)}$

Aliquots of submitochondrial vesicles (3 mg protein/ ml) were used to determine kinetic parameters in the presence or in the absence of cations, at 37°C and at pH 8 (see Materials and Methods, and legend of table I). The mean values ± SEM represent three independent experiments. (NS): non significant differences (*): Statistically significant differences

cal.mol^{-1} and Ea = 6.77 Kcal.mol^{-1}.

Two D -3-hydroxybutyrate structural analogs (methylmalonate and succinate) were tested as competitive inhibitors towards BOH; methylmalonate is the strongest inhibitor as shown by its inhibition constant value (Ki = 0.12 mM) as compared to the one for succinate (Ki 1.8 mM). This difference could be due to the presence of the branched methyl group beared by methylmalonate.

The effects of some monovalent and divalent cations on BDH activity were also studied. One can see in Table 4 that Ca^{2+} and Mg^{2+} are the only activators. These two cations increase the affinity binding of NAD^+ (decrease of KD) and decrease the affinity binding of BDH (strong increase of Ki methylmalonate binding) (Table 5). In the order to confirm the activating role of Mg^{2+}, the effect of EDTA, a chelating compound, was tested. It was found that EDTA inhibits BDH activity (Table 4) and this inhibition was reversed by the addition of Mg^{2+}. The effect

of Mg^{2+} or EDTA is a concentration dependent process where high concentrations of Mg^{2+} lead to a decrease of BDH activity (not shown). Moreover, the presence of EDTA leads to a lack of catalytic process inhibition in excess of D-3-hydroxybutyrate or NAD+ (not shown). The maximum velocity is reduced at an average of 20%. In this case, two molecules of each substrate bind to the enzyme and their dissociation constants were estimated following the Kuhn's method (Kuhn, 1974) and corresponded to KD = 3.1 mM and KD'= 1.07 mM for NAD^{+}, and KD = 2.08 mM and KD'= 19.95 mM for BOH.

DISCUSSION

Mitochondrial oxidative characteristics of *T. pyriformis* were investigated following the oxidation of several substrates and by measuring the activities of BDH, SDH and ATPase in absence or in presence of chloramphenicol (CAP) since it is known that the presence of CAP in the growth medium modifies the phopholipid metabolism (Turner and Lloyd, 1971) but also inhibits mitochondrial protein synthesis. BDH and SDH are encoded by nuclear genes while several ATPase (F1) subunits are encoded by mitochondrial DNA. Thus, as expected the presence of CAP gives only to a decrease of ATPase activity. It must be pointed out that frozen-thawed mitochondrial leads to the formation of mixed particles when most of them are inside-out mitochondrial inner membrane. In this case, swelling interference on substrate permeability and accessibility can be considered negligible since BDH, SDH and ATPase activities are all located on the inner face of the inner mitochondrial membrane.

Concerning BDH, we found (not shown) that the activity was linked to submitochondrial vesicles (SMV) and that the freezing and thawing of SMV leads to the progressive loss of the activity. The activity could be recovered by the addition of phospholipid vesicles in accordance with the need of lipid requirement of this enzyme (not shown). Related to this, the lack of CAP effect on BDH activity was due to the fact that the possible phospholipid changes surrounding BDH in mitochondria remain unchanged after CAP treatment while it does for SDH since an activation is observed. Alternatively, the stimulation of SDH activity after CAP treatment may reveal a compensation of the decrease of oxidative phosphorrylation by an increase of succinate dehydrogenase units. The kinetic study of BDH revealed that the enzyme catalytic mechanism follows an ordered bi bi. The same mechanism was previously shown for BDH from other species, that is, rat liver (Latruffe and Gaudemer, 1974), beef heart (Nielsen et al., 1973) and bacterial *Rhodopseudomonas spheroides* (Preuveneers et al., 1973).

The highest activity obtained at pH 8 for the D-3-hydroxybutyrate oxidation and at pH 6.5 for the acetoacetate reduction was similar with BDH of other species like bacterial *Pseudomonas lemoignei* (Delafield et al., 1965) and rat liver (Latruffe and Gaudemer, 1974). In results not shown, we found that the NAD+ binding increased at optimum pH while the affinity for D -3-hydroxybutyrate is not significantly affected. This was as a result of a pH induced - change in the coenzyme binding site. Such change has been previously reported by our group in rat liver BDH active site concomitant with a charge transfer (El Kebbaj and Latruffe, 1997).

The BDH denaturation process occurs at 40°C and the break in Arrhenius plot at 19°C with two activation energy values. These values are lower than those obtained for rat liver BDH (Latruffe and Gaudemer, 1974). This break suggests either a change of tertiary structure of the enzyme, as proposed for rat liver BDH (El Kebbaj and Latruffe, 1986) or a phase transition dependency of phospholipid surrounding the enzyme, as reported in other systems (Lenaz et al., 1972). The thermodynamic parameters values (Ross et al., 1981) are in agreement with the existence of hydrophobic interactions between coenzyme and BDH. The protection by NAD+ against thermal denaturation is in accordance with results for rat liver BDH (Wise and Lehninger, 1962). In contrast NADP+ (an inactive coenzyme for BDH) and substrate alone (BOH or AcAc) do not exhibit thermal protection. This is in agreement with the lack of substrate binding to the active site without the present of the specific coenzyme.

The activating and protecting effects of divalent cations, especially Ca^{2+} and Mg^{++} can be compared with the effect of such cations on protein conformation previously repor-ted for several mitochondrial enzymes : L-glycerol-3-phosphate dehydrogenase from different mam-malian tis-sues (Beleznai et al., 1988) and pig heart 2-ketoglutarate dehydrogenase complex (Panov and Scarpa, 1996). The inhibition of BDH activity by EDTA and the removal of this inhibition restored by the addition of Mg^{2+} show that the EDTA exhibits its effect through chelating action. Similar results were reported for BDH from bacterial Rhodopseudomonas spheroides (Bergmeyer et al., 1967). All these results are in favor of the implication of divalent cations on the structure-function relationships of BDH. This is supported by the fact that the NAD^{+} dissociation constant and the methylmalonate inhibition constant were modified in the presence of the two studied cations (Ca^{2+} and Mg^{2+}) where the highest change occurs with Ca^{2+} (Table 5). These two cations may exhibit their effect on BDH binding site either directly or indirectly by modifying the membrane structure as previously reported for beef heart BDH (Mc Intyre et al., 1988) where calcium or mag-nesium cations induces enzyme conformational changes. Interestingly, the positive and protecting effects of non heavy metal cations contrast with the inhibitory effect of the water pollutant heavy metal cations (Mountassif et al., 2006a; Rico et al., 2009).

In conclusion, the present work provides new insights on the energy metabolism of *T. pyriformis* wild strain where D-3-hydroxybutyrate is a choice substrate. More-

over, the properties of BDH reveal the following features: 1. the BDH kinetic study shows that the enzyme follows a bi bi order catalytic mechanism; 2. the enzyme is sensitive to several ligands. Indeed, Ca^{2+} and Mg^{2+} are activators, while Zn^{2+} strongly stabilizes the BDH against thermal inactivation; 3. EDTA inhibits the enzyme while leads to the lack of inhibition process by excess of substrate or of coenzyme; 4. succinate and methylmalonate are competitive inhibitors where methylmalonate gives the strongest effect; 5. BDH is sensitive to pH and temperature, where pH affects the binding of the coenzyme while the temperature dependency shows a break near 19°C. Such break suggests a conformational change of BDH in relationship with change of phospholipid physical state surrounding BDH.

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ABBREVIATIONS

AcAc, Acetoacetate; **BDH**, D-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30); **BOH**, DL-3-hydroxybutyrate; **CAP**, Chloramphenicol; **DCIP (H2)**, Dichloroindolphenol oxidized and reduced form; **EDTA**, Ethylenediamine tetraacetic acid; **Hepes**, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; **Mes**, 4-(N-morpholino) ethanesulfonic acid; **NAD + (H)**, Nicotinamide adenine dinucleotide oxidized/reduced forms; **SDH**, Succinate dehydrogenase; **SMV**, Submitochondrial vesicles; *T. pyriformis*, *Tetrahymena pyriformis*; **Tris**, Trihydro-xymethyl-aminomethane.

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