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Dependence of flux size and efficiency of oxidative phosphorylation on external osmolarity in isolated rat liver mitochondria: role of adenine nucleotide carrier

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Abstract

The aim of this work was a thermodynamic and kinetic study of the influence of varying external osmolarity on overall oxidative phosphorylations in isolated rat liver mitochondria. When external osmolarity is increased from 100 to 400 mosM by using a non-penetrant sugar: (i) matrix volume diminishes, (ii) state 3 respiratory rate decreases when state 4 slightly varies, (iii) states 3 and 4 protonmotive force and NAD(P)H level increase, whereas oxidative phosphorylation efficiency (ATP/O) decreases. Indeed, respiratory flux versus protonmotive force relationships depend on the osmolarity considered: the lower the external osmolarity, the higher the span of overall driving force necessary for the same respiratory rate.

To further investigate the mechanism of the decrease in respiratory and ATP synthesis flux leading to a lowering in oxidative phosphorylation efficiency, we determined the adenine nucleotide carrier control coefficient on respiratory and ATP synthesis rates respectively. The main result is that the adenine nucleotide carrier control coefficient on respiratory rate decreases, and conversely that adenine nucleotide carrier control on ATP synthesis rate increases, from iso- to hyperosmolarity. Furthermore, whatever the osmolarity, when state 3 respiratory rate is titrated with carboxyatractyloside, the same relationship is observed between ATP/O ratio and respiratory flux. From many previous studies, it has been shown that an increase in external osmolarity and a consequent decrease in matrix volume inhibits almost all mitochondrial proton pumps (coupling site 1 and 2 of respiratory chain, ATPase) in different ways. In this work, we show that in phosphorylating mitochondria, the adenine nucleotide carrier plays a key role: its inhibition as the external osmolarity increases lowers the state 3 respiration close to state 4 level and consequently leads to a decrease in oxidative phosphorylation efficiency.

Keywords: Oxidative phosphorylation efficiency; Mitochondrion; External osmolarity; Matrix volume; Adenine nucleotide carrier; Swelling; (Rat liver)

1. Introduction

It has long been recognized that in order to perform their functions, cells have to accumulate or to lose a number of osmotically active substances which tend to swell or to shrink the cells ([1] for review). For instance, to keep a constant volume, cells have to develop many regulatory volume mechanisms; yet despite the latter, volume variations may remain [2–4]. In the liver, cell volume alterations can be seen in conditions of osmotic portal blood alterations (meddling of large quantities of water, increase in amino-acid concentrations). Moreover, it has been shown that the effect of either hormones or amino-acids transported with sodium on cellular metabolism primarily induces modifications of cell volume. Numerous metabolic pathways, which induce the formation or disappearance of osmotically active substances, i.e., protein and carbohydrate metabolism, have been shown to be particularly sensitive to modifications in cell volume. So it is now admitted that cell volume alterations are a new principle of metabolic control [5–7]: an increase in cell volume acts as an anabolic signal [8–10], whereas a decrease in cell volume acts as a catabolic signal [8,9]. Moreover, changes in cell volume are associated with modifications in cytosolic osmolarity which could induce modifications of mitochondrial volume in situ.

The regulation of mitochondrial functions through changes in mitochondrial volume induced in different ways

Abbreviations: ΔpH, proton chemical potential difference across mitochondrial inner membrane; $\Delta\Psi$, electrical potential difference across mitochondrial inner membrane; Δp, protonmotive force; ANT, adenine nucleotide carrier.

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(on isolated rat liver mitochondria) has long been studied by several groups (see [11] for review, [12]). Firstly, in conditions of increasing matrix volume (α-adrenergic agonist pretreatment of rat, hypoosmotic incubation conditions, intramitochondrial increase in $[Ca^{2+}]$) the mitochondrial metabolism has been shown to be strongly affected. For instance, in the presence of glucagon, mitochondrial respiration, pyruvate metabolism and citrulline synthesis were stimulated [13] and in conditions of decreasing external osmolarity, fatty acid oxidation was increased and cytochromes were more reduced [14]. Furthermore, it has been shown that an increase in matrix $[Ca^{2+}]$ stimulates an electrogenic flux of potassium into the mitochondrial matrix which induces mitochondrial swelling [15]. Moreover, increasing the matrix volume by decreasing external osmolarity and measurements of the flavoprotein reduction state in these conditions showed that flavoproteins were less reduced in conditions of increasing matrix volume, which in turn suggests that intramitochondrial regulation of fatty acid β-oxidation through the effect of matrix volume occurs between flavoprotein and ubiquinone [16]. Similar studies have been done on isolated rat heart mitochondria, giving similar results [17]. In conditions of matrix volume condensation, an inhibition of mitochondrial substrate oxidation has been observed and interpreted as an osmotically sensitive diffusion of quinones through the mitochondrial membrane [18]. It has also been shown that, under conditions of matrix condensation, state 3 respiration was inhibited as ATPase activity [19] and ADP/O ratio decreased [20]. These studies show that whatever the way by which matrix volume varies in vitro, an increase in matrix volume increases state 3 respiratory rate while a decrease in matrix volume decreases this respiratory rate.

All these data come mainly from metabolic studies of the effect of varying matrix volume on mitochondrial metabolism. Moreover, the hypothesis implying an activation of the respiratory chain between electron-transfering flavoprotein and ubiquinone in hypoosmolarity appears to us improbable, because of the slight control coefficient of this part of the respiratory chain on oxidative phosphorylations in isoosmotic incubation conditions [21]. The influence of varying matrix volume on essential thermodynamic parameters in cellular energetics, i.e., redox status of coenzymes, protonmotive force, has still not been studied. So our aim has been to undertake a thermodynamic and kinetic study of the effect of external osmolarity on overall oxidative phosphorylations. We show, as previously established, that increasing external osmolarity decreases both the state 3 oxygen consumption rate and the matrix volume but has little or no effect on state 4 respiration under the same conditions. Moreover, we measured an increase in protonmotive force and the NAD(P)H level associated with a decrease in ATP/O ratio. Thus, the effects of osmolarity on overall oxidative phosphorylations are essentially an inhibition of one or some Δp-consuming processes, inducing state 3 moving nearer to state 4 oxygen consumption rate at high external osmolarity. Furthermore, a control analysis shows that the adenine nucleotide carrier plays a key role in such a process leading to a lowering in oxidative phosphorylation efficiency.

2. Materials and methods

2.1. Mitochondrion preparation

Liver mitochondria were prepared from male Wistar rats (250–300 g body weight) starved overnight. Rats were killed by cervical dislocation and each liver was rapidly removed and put into an ice-cold isolation medium containing 225 mM sucrose, 20 mM Tris-HCl (pH 7.2) and 1 mM EGTA. Mitochondria were isolated according to [22] in the same medium. The mitochondrial pellet was finally resuspended in the isolation medium. Protein concentration was estimated by the biuret method using bovin serum albumin as standard [23].

2.2. Mitochondrial respiration and ATP / O measurements

The rate of oxygen uptake at various osmolarities was measured polarographically at 26°C using a Clark-type oxygen electrode connected to a microcomputer giving an online display of rate values. Respiration buffer contained 5 μM $TPMP^+$ (tetraphenylmethylphosphonium), 5 μM DMO (5,5-dimethyl oxazolidine-2,4-dione), 5 μM mannitol, 20 mM Tris-HCl (pH 7.2), 1 mM EGTA, 6 mM respiratory substrates, 5 mM Tris-P_i and an amount of sucrose to make up the osmolarity to the required value. Before use, sucrose, purchased from Merck, was passed through a Dowex 50W column in order to remove contaminating cations. From 1 to 4 mg of mitochondrial protein were used as a function of experimental conditions after checking that the respiratory rate was always directly proportional to the milligramme of protein, whatever the osmolarity.

Oxygen concentrations in the different osmolarity media were determined with NADH quantitated spectrophotometrically and yeast mitochondria.

Phosphorylation rate was measured by $[^{32}P]P_i$ incorporation in adenine nucleotides as previously described [24]. Phosphorylation rate measurements were done in respiratory buffer supplemented with 1 mM ADP, at various sucrose osmolarities. The ATP/O ratio stoichiometries were determined from the yield of oligomycin-sensitive phosphorylation rates versus respiratory rates.

2.3. Measurement of matrix space, ΔpH and $\Delta\Psi$ using radiolabeled elements

Routinely, the protonmotive force under different steady states was determined as follows. Matrix space was determined using [^{3}H]water and [^{14}C]mannitol, an inner mem-

brane impermeable sugar, in the respiratory buffer. After equilibration (3 min) 0.5 mM ADP was added in order to reach state 3 respiration. After 15 s, mitochondria were rapidly centrifuged (20 s) and then treated as described previously [24]. For state 4 measurements, the rapid centrifugation was done when the net ATP synthesis flux was nul (state 4). The delay after ADP addition necessary to obtain state 4 depended on the medium osmolarity and was checked by oxygen consumption determinations. ΔpH was measured by distribution of [^{14}C]DMO according to [25] and $\Delta\Psi$ by [^{3}H]TPMP^{+} distribution.

TPMP^{+} is a lipophilic cation which is partially linked to membranes; we applied to our measurements a correction coefficient of 0.38, determined in our laboratory [26], for this binding. Since our experimental conditions implied changes in membrane structures, we also measured $\Delta\Psi$ in parallel experiments using ^{86}Rb (known as a marker which does not bind to membranes) in the presence of valinomycin for two different osmolarities. We observed the same linear relationship between [^{3}H]TPMP^{+} and ^{86}Rb accumulation whatever the osmolarity. So we considered that the correction factor used for TPMP^{+} binding was available whatever the osmolarity. We also determined the matrix volume using [^{32}P]P_i in the presence of mersalyl, in order to inhibit the P_i/H^{+} carrier [27]. The evolution of matrix volume as a function of external osmolarity was comparable to its evolution measured with [^{14}C]mannitol and [^{3}H]H_2O. Moreover, the matrix volume did not significantly vary during a period of 15 min (not shown). Taken together, these facts indicate that mannitol is a suitable marker of the matrix volume.

2.4. Mitochondrial NAD(P)H fluorescence measurements

NAD(P)H measurements make it possible to qualitatively evaluate the reducing equivalent supply to the respiratory chain. NADH + NADPH fluorescence was monitored at 26°C with a Kontron fluorimeter as previously described [28]. Excitation wavelength was 340 nm and fluorescence emission wavelength was 465 nm. Mitochondria (0.1 mg protein) were suspended in the respiratory buffer at various sucrose osmolarities to a final volume of 3 ml. Results are expressed as a percentage of NAD(P)H fluorescence: 0% was an endogenous signal corresponding to a steady state of mitochondria placed in respiratory buffer without added substrate, state 3 fluorescence corresponds to mitochondria in the presence of 6 mM respiratory substrates and 50 μM ADP. State 4 fluorescence was measured when the net flux of ATP synthesis was nul. Maximal NAD(P)H fluorescence (100%) was measured in the presence of 1 μg/ml antimycin. This technique does not allow us to determine quantitatively the NADH concentration because we simultaneously measure free and bound NAD(P)H and we cannot evaluate the part taken by the former and the latter; furthermore, maximal NAD(P)H fluorescence depends on the enzyme involved and we could not quantitate the NAD^{+} content.

2.5. Mitochondrial swelling

The initial rate of swelling of non-respiring mitochondria in a potassium acetate medium of various osmolarities in the presence of a non-limiting concentration of valinomycin depends on mitochondrial permeability to protons [29]. Decrease in 546 nm absorbance was monitored with an Eppendorf spectrophotometer at 26°C. The swelling buffer contained 20 mM Tris-HCl (pH 7.2), 25 μg/ml oligomycin, 1 mM KCN and various concentrations in potassium acetate. Swelling was initiated by the addition of 0.25 μg valinomycin/ml and the maximal rate of swelling was determined in the presence of 1 μM CCCP, which is the concentration inducing maximal rate of swelling whatever the osmolarity.

3. Results

3.1. Respiratory rates and matrix volume

Respiratory fluxes, expressed as a function of external osmolarity, were measured under different steady states with various respiratory substrates. External osmolarity was varied using sucrose as osmotic support. Fig. 1 shows the evolution of state 3 and 4 oxygen consumption rates as a function of external osmolarities using glutamate and malate as respiratory substrates. As previously shown by several groups [13,20], increasing external osmolarity from 100 to 500 mosM inhibits drastically state 3 respiration. Under state 4, the respiratory rate decreased slowly but significantly when external osmolarity increases from 100 to 225 mosM and remained constant for higher osmolarities until 500 mosM. The uncoupled state, measured by using 2,4-DNP was inhibited by increasing external osmolarity in the same range as state 3 but it remained greater

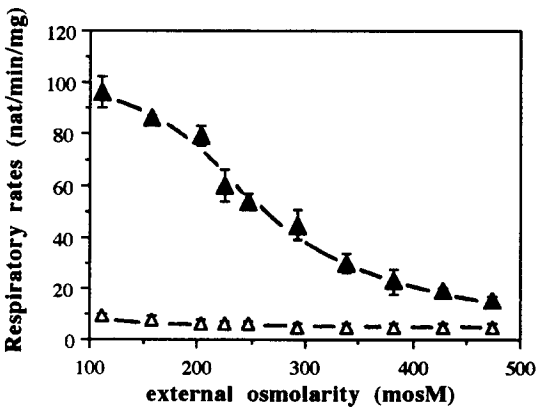

Fig. 1. State 3 (▲) and 4 (Δ) respiratory rates as a function of external osmolarity. Mitochondria (1 mg/ml) were suspended in respiratory buffer as described in Materials and methods section. The values presented are from at least three different experiments carried out with three different mitochondrial preparations.

than state 3 respiration (not shown). Similar results were obtained using succinate as respiratory substrate (not shown). TMPD/ascorbate oxidation was far less sensitive to such changes in osmolarity as described previously (not shown but see [18]). Indeed, when physiological substrates were used in phosphorylation conditions, increasing external osmolarity decreased the respiratory rate, while in the non-phosphorylating state, i.e., state 4, the respiratory rate seemed almost insensitive to osmolarity. Further measurements were done with glutamate and malate as substrates.

In buffer commonly used for mitochondrial preparation and respiratory flux measurements, the osmolarity of the medium is almost the same as the internal osmolarity of the mitochondria. In our experimental conditions, as the osmolarity of the medium was modified with a non-permeant sugar, the matrix volume changed to equilibrate the osmotic pressure across the inner mitochondrial membrane. So the matrix volume needed to be defined under each incubation condition. Fig. 2a shows the evolution of matrix volume as a function of external osmolarity. Indeed, an increase in external osmolarity induced a decrease in matrix volume, whatever the respiratory state. For a given osmolarity, state 3 and 4 matrix volumes were not significantly different. As previously shown [30] decrease in matrix volume when external osmolarity is increased correlates well with decrease in JO_2 in phosphorylating conditions (Fig. 2b).

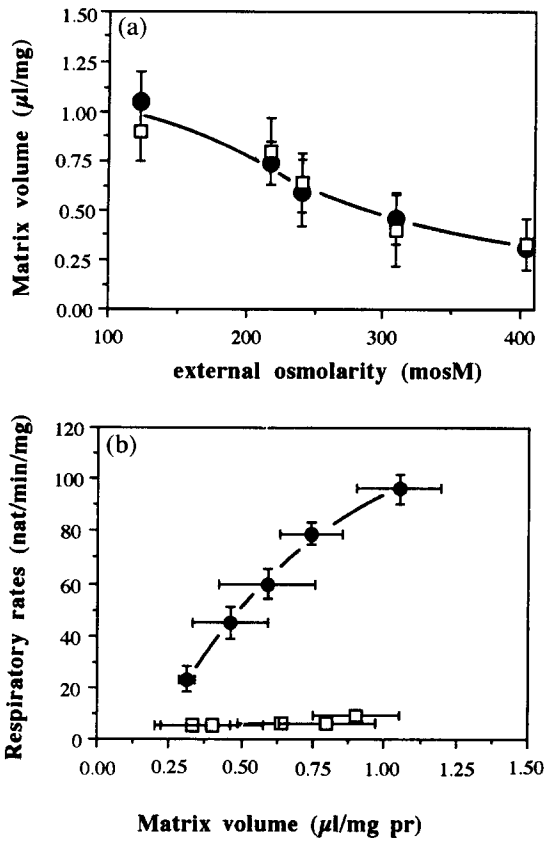

Fig. 2. (a) Dependence of state 3 (●) and 4 (□) matrix volume on external osmolarity. Mitochondria (4 mg/ml) were incubated in respiratory buffer supplemented with [^{14}C]mannitol and ^3H_2O as described under Materials and methods. The values presented are from at least six different experiments carried out with six different mitochondrial preparations. (b) Respiratory rates as a function of matrix volume under state 3 (●) and 4 (□). The values used for this representation are those presented under Figs. 1 and 2a. For each osmolarity studied, it was checked that the relationship between JO_2 and the mitochondrial amount was not modified whatever the mitochondrial concentration used in this study.

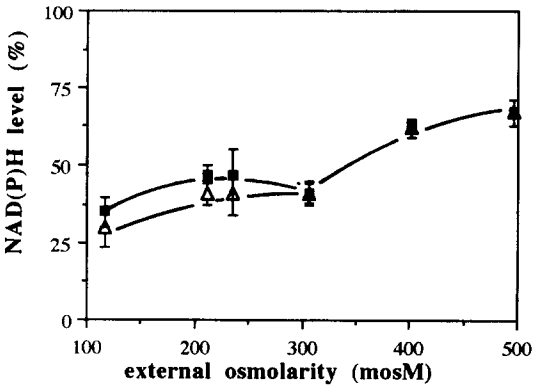

Fig. 3. State 3 (■) and 4 (Δ) NAD(P)H level as a function of external osmolarity. NAD(P)H level was measured fluorimetrically as described in Materials and methods. The values presented are from at least three different experiments carried out with three different mitochondrial preparations.

3.2. NAD(P)H level and protonmotive force

NAD(P)H level was fluorimetrically measured under different steady states, using glutamate and malate as substrates. This level is qualitatively a function of the reduced equivalent supply to the respiratory chain. Fig. 3 shows that increasing external osmolarity slightly increases the NAD(P)H level in isolated rat liver mitochondria; as the osmolarity approaches 300 mosM, no difference between state 3 and 4 NAD(P)H level can be measured.

According to the chemioosmotic theory, the protonmotive force is the energetic intermediary between respiratory and ADP phosphorylation flux. So, to furthermore investigate the effect of osmolarity on oxidative phophorylations, we measured the evolution of protonmotive force relative to an increase in external osmolarity. Fig. 4 (a and b) shows that when increasing the osmolarity, ΔpH and $\Delta\Psi$ increase, whatever the respiratory state. For each osmolarity, it is worth noting that, as expected, the protonmotive force under state 3 is less than under state 4, essentially owing to a decrease in $\Delta\Psi$. Fig. 5 shows that for each steady state, either 3 or 4, we obtained different relationships between JO_2 and Δp. Under phosphorylation conditions, an increase in Δp was directly related to a decrease in JO_2, whereas, under state 4, a large increase in Δp was linked to either a slight change in respiratory rate or none at all. Moreover, in hyperosmolarity (400

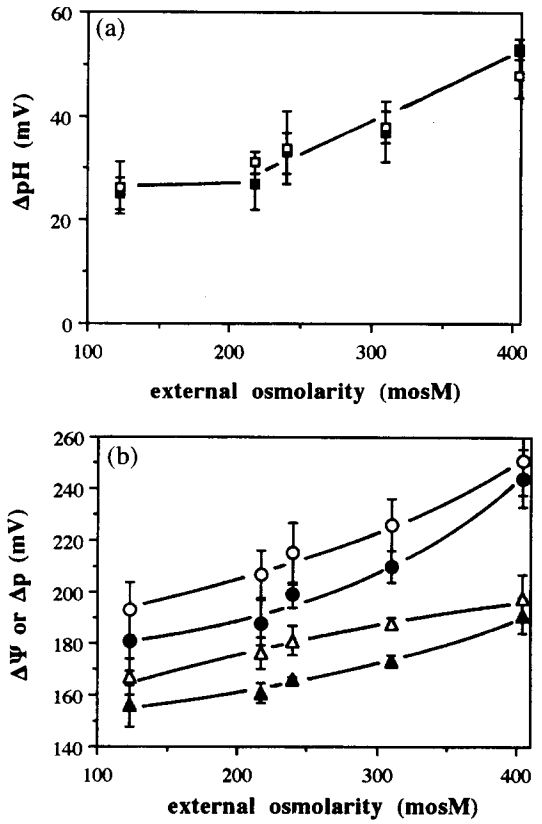

Fig. 4. (a) ΔpH under state 3 (■) and 4 (□) respiration as a function of external osmolarity. ΔpH was determined as described in Materials and Methods. The values presented are from at least three different experiments carried out with three different mitochondrial preparations. (b) $\Delta\Psi$ and Δp under state 3 (▲ and ●, respectively) and 4 (Δ and ○, respectively) respiration as a function of external osmolarity. $\Delta\Psi$ was determined as described in Materials and methods. The values presented are from at least three different experiments carried out with three different mitochondrial preparations.

mosM sucrose), the state 3 protonmotive force was greater than the state 4 protonmotive force in hypoosmolarity (100 mosM sucrose), while for the oxygen consumption rate, state 3 respiration in hyperosmolarity is 3-times greater than state 4 respiration in hypoosmolarity (see Figs. 1 and 4b). In state 4, the flux of protons coupled to the oxygen consumption rate is strictly equal to the passive reentry of protons, $J^{+}_{Hout} = L_H \cdot \Delta p$ (where L_H is the membrane conductance to protons). If JO_2 does not vary when Δp increases, it can be supposed that the membrane conductance to protons decreases. Indeed, for a constant state 4 respiration rate, protonmotive force depends on the osmolarity considered.

3.3. Passive permeability to proton as a function of external osmolarity

There is no unquestionnable method for measuring membrane conductance to protons for high protonmotive force [31]. However, it is now accepted that the initial rate of passive swelling in potassium acetate medium in the presence of a non-limiting concentration of valinomycin is proportional to L_H [32]. So, mitochondrial permeability to protons was determined at near null Δp by the initial rate of swelling of non-respiring mitochondria in potassium acetate medium of various osmolarities. Fig. 6 shows that increasing the external osmolarity in potassium acetate decreased the initial rate of mitochondrial swelling. So, mitochondrial permeability to protons seemed to decrease when external osmolarity increased. This correlates well with state 4 oxygen consumption rates which did not significantly vary when Δp increased. However, if the inner mitochondrial membrane conductance seems to decrease when increasing external osmolarity, we have to keep in mind that in our experimental conditions, the matrix volume varied and even if the membrane was still the same (lipid and protein composition) its elasticity could vary so, these measurements are not completely reliable.

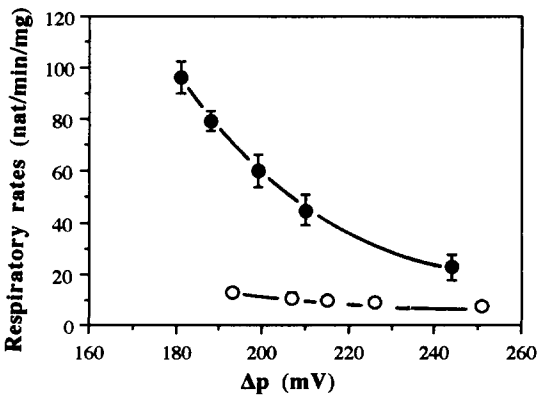

Fig. 5. State 3 (●) and 4 (○) respiratory rates as a function of protonmotive force. The values used for this representation are from Figs. 1 and 3.

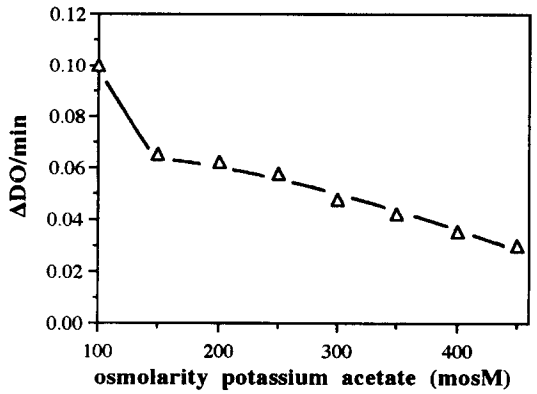

Fig. 6. Dependence of the initial rate of passive swelling (Δ) in potassium acetate medium on external osmolarity in potassium acetate. Incubation conditions were as described in Materials and methods. This experiment is representative of three such experiments carried out with two different mitochondrial preparations.

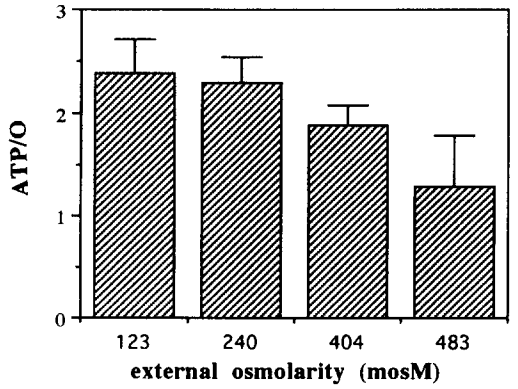

Fig. 7. Efficiency of oxidative phosphorylations as a function of external osmolarity with glutamate and malate as substrates. ATP synthesis and respiratory flux were measured as described in Materials and methods. The values presented are from at least three different experiments carried out with three different mitochondrial preparations.

3.4. Efficiency and control of oxidative phosphorylations

It has previously been shown that when increasing external osmolarity, the ADP/O ratio tends to decrease together with the respiratory flux [20]. Fig. 7 shows an inhibitory effect of osmolarity on overall oxidative phosphorylation efficiency: the ATP/O ratio decreases almost half of the control value (isoosmolarity conditions) at 480 mosM with glutamate and malate as substrates. Such an inhibition was also observed with succinate as substrate (not shown). So, J_{ATP} is more markedly affected by hyperosmolarity than JO_2 even if both fluxes are decreased. When external osmolarity increases, protonmotive force and the NAD(P)H level also increase, and oxidative phosphorylation efficiency and state 3 respiratory rate decrease. Taken together, these facts indicate that in state 3 and in hyperosmolarity sucrose, phosphorylation processes become more controlling. In isolated phosphorylating rat liver mitochondria, one of the most important enzymatic steps involved in oxidative phosphorylation control is the adenine nucleotide carrier [21]. Thus, we measured adenine nucleotide carrier control on both JO_2 and J_{ATP} by the inhibitor titration method [21,33,34], using carboxyatractylate, a quasi-irreversible inhibitor of adenine nucleotide carrier [35]. Indeed, when an irreversible inhibitor is used, the control coefficient can be calculated directly from an inhibition curve:

$$C_i = \frac{dJ/J}{dI/I_m}$$

where I_m is the amount of inhibitor required for total inhibition of the enzyme.

Table 1 shows that the control exerted by the adenine nucleotide carrier on either JO_2 or J_{ATP} was different for each osmolarity considered. In isoosmolarity (225 mosM sucrose) as previously shown, adenine nucleotide carrier control is nearly the same on JO_2 and J_{ATP} [36]. In

Table 1
Control coefficient of adenine nucleotide carrier on either ATP synthesis or state 3 oxygen consumption rate

Osmolarity sucrose (mosM):	100	225	400
$C_{ANT}^{JO_2}$	0.7 ±0.14	0.51±0.07	0.34±0.09
$C_{ANT}^{J_{ATP}}$	0.85±0.045	0.54±0	0.97±0.14

Experimental procedures were as described in Materials and methods. The values presented are from at least three different experiments carried out with three different mitochondrial preparations.

hypoosmotic conditions, the control exerted by the adenine nucleotide carrier on both JO_2 and JATP increased. From iso- to hyperosmolarity, adenine nucleotide carrier control on JO_2 decreased, although on J_{ATP} it increased when JO_2 decreased. The main interpretation is that when external osmolarity increases, the phosphorylating respiration moves nearer to state 4 respiration as described previously when external temperature decreases [37]. Indeed, three experimental results sustain this interpretation: (i) kinetic constraints which appear on J_{ATP} when the osmolarity increases from 225 to 400 mosM; (ii) a decrease in ATP/O ratio and JO_2 when external osmolarity increases; (iii) state 3 respiration and ATP/O titrations with carboxyatractylate at three distinct osmolarities show that a unique relationship between JO_2 and ATP/O exists whatever the osmolarity (Fig. 8).

Furthermore, it is well known that the cytochrome-*c* oxidase segment of the respiratory chain is only slightly affected by changes in external osmolarity [18]. If our interpretation is correct, when external osmolarity rises, the kinetic limitation of ATP synthesis due to the adenine nucleotide carrier must induce a large decrease in ATP/O ratio with TMPD and ascorbate as electron donors. Fig. 9 shows that from iso- to hyperosmolarity (500 mosM sucrose), the ATP/O ratio drastically decreased. The fact that the respiratory rate under state 3 was only slightly

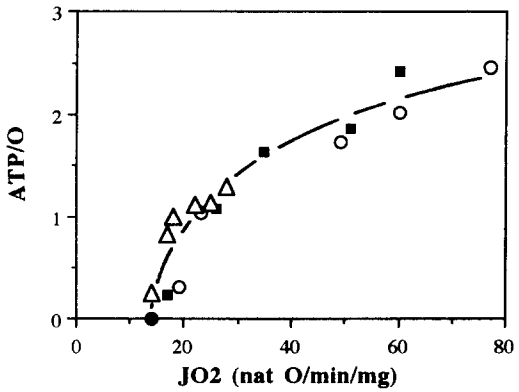

Fig. 8. Carboxyatractylate titration of oxidative phosphorylation efficiency for three different osmolarities (400 mosM sucrose Δ, 225 mosM sucrose ■, 100 mosM sucrose ○). ATP synthesis and respiratory flux were measured as described in Materials and methods. The values presented are from a single representative experiment.

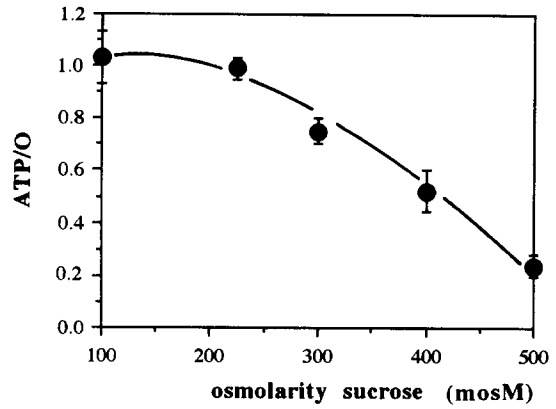

Fig. 9. Oxidative phosphorylation efficiency as a function of external osmolarity with TMPD + ascorbate as electron donors. ATP synthesis and respiratory flux were measured as described in Materials and Methods. The values presented are from at least three different experiments carried out with a single mitochondrial preparation.

affected by hyperosmolarity is due to a weak dependence of oxygen consumption rate on ATP synthesis when TMPD + ascorbate are used as substrates.

4. Discussion

Under our experimental conditions, i.e., energized mitochondria under either state 3 or 4 respiration with glutamate and malate as substrates, there is a linear relationship between matrix volume and 1/osmolarity from iso to hyperosmolarity incubation conditions (Fig. 10). This has previously been described on isolated rat liver mitochondria under deenergized conditions [30]. These elements, i.e., linear relationship between matrix volume and 1/osmolarity, same matrix volume whatever the respiratory rate and the energetic status at a given osmolarity, point to the fact that in sucrose medium (impenetrant sugar), isolated rat liver mitochondria behave like an osmometer. Meanwhile, in hypoosmolarity conditions, the matrix volume is less than expected by the linear relationship obtained between matrix volume and 1/osmolarity in iso- and hyperosmolarity incubation conditions. This may indicate that in a hypoosmotic medium, volume regulatory mechanisms occur: as previously proposed, K^+/H^+ carrier in such conditions (i.e., increase in matrix volume inducing a decrease in intramatricial $[Mg^{2+}]$) can induce an extrusion of potassium associated with an intrusion of protons [38].

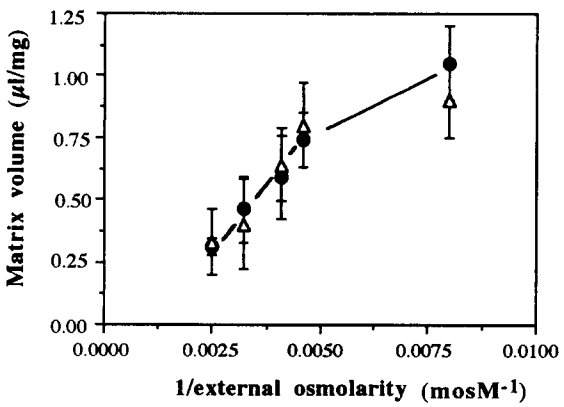

Fig. 10. Evolution of state 3 (●) and 4 (Δ) matrix volume as a function of 1/osmolarity. The values used for this representation are from Fig. 1.

In our laboratory, studies on isolated rat hepatocytes have shown that in hypoosmolarity incubation conditions, mitochondrial NADH/NAD^+ ratio and Δp diminish while JO_2 does not vary, in such a manner that the absolute value of the term $2\Delta E'h - 10\Delta p$ (where $2\Delta E'h$ is the redox span between NADH/NAD^+ and O_2/H_2O and assuming 10 protons excreted by atom oxygen reduced) increases [26]. Clearly, these changes are such that under hypoosmotic incubation conditions, a greater overall driving force is necessary for the same respiratory rate compared to isoosmolarity. This indicates that the respiratory chain is not activated, as previously described [16], but conversely that there are kinetic constraints involving a modification of respiratory chain response to thermodynamic forces.

On isolated mitochondria and under a resting state, JO_2 is only linked to passive reentry of protons ($J_H^+ = L_H \cdot \Delta p$). When external osmolarity is increased from 225 to 500 mosM, JO_2 does not significantly vary when Δp increases. The main interpretation is that L_H decreases when external osmolarity increases. This hypothesis implies that the H^+/O stoichiometry of the respiratory chain does not vary, a supposition which is sustained by the unique relationship obtained between ATP/O and JO_2 whatever the osmolarity with carboxyatractyloside titration (see Fig. 8). Furthermore, the decrease in the initial rate of passive swelling in potassium acetate medium when external osmolarity increases is another argument in favor of this interpretation (see Fig. 6).

Preliminary studies on mitochondrial swelling in hypoosmotic medium or after rat hormonal pretreatment (see [11] for review, [12]) have shown that an increase in matrix volume induces an increase in the state 3 oxygen consumption rate of isolated rat liver mitochondria. Moreover, measurement of the redox state of flavoproteins and ubiquinones shows that flavoprotein reduction state diminishes when the matrix volume increases [16]. The interpretation of these experimental results is that of a respiratory chain activation between flavoproteins and ubiquinones linked to the increase in matrix volume [16]. In our study, decreasing the osmolarity increased state 3 respiration and decreased NAD(P)H level. This correlates well with the hypothesis of an activation of the respiratory chain. But the fact that JO_2 stimulation is linked to an activation of the respiratory chain is not confirmed by the fact that in hypoosmotic medium, state 3 protonmotive force decreases. Moreover, under a resting state, the NAD(P)H level and protonmotive force decrease when external os-

molarity decreases, while JO_2 varies less than in state 3. Furthermore, when external osmolarity increases, adenine nucleotide carrier control on JO_2 decreases, and phosphorylation and respiratory flux titration with carboxyatractyloside give an unique relationship between ATP/O ratio and JO_2 whatever the osmolarity. These results show that the main phenomenon observed when external osmolarity increases is an inhibition of ATP synthesis, and consequently an increase in both protonmotive force and NAD(P)H level linked to a decrease in state 3 oxygen consumption rate. But these results do not exclude the sensitivity of the respiratory chain to osmolarity, which has previously been shown [18]. Moreover, our results in the presence of an uncoupler (not shown) indicate that when external osmolarity increases, the uncoupled respiratory rate decreases. On isolated rat liver mitochondria, it has previously been shown that when external osmolarity increases, there are kinetic constraints applied to respiratory chain proton pumps [18]. Indeed, after segmental analysis of the electron transport chain, the authors concluded in an osmotically sensitive diffusion of quinones through the bilayer, considering that phosphorylation, per se, is not sufficient to account for the osmotic sensitivity of respiration, since osmotic sensitivity is present in uncoupled respiration. However, these kinetic constraints on respiratory chain do not play a great part in phosphorylation conditions, in which a decrease in ATP/O ratio and an increase in adenine nucleotide carrier control on J_{ATP} are related to an increase in Δp and NAD(P)H level when external osmolarity increases. Thus, our thermodynamic and kinetic study of the effect of external osmolarity on both ATP synthesis and respiratory flux points to the main role of kinetic constraints on Δp-consuming systems, particularly the adenine nucleotide carrier, when external osmolarity increases; as a matter of fact, state 3 moves nearer to state 4 respiratory rate and oxidative phosphorylation efficiency decreases.

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