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The Energy Requirement for Protein Synthesis in Rat Brain Mitochondria Purified by Phase Partition

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Brain mitochondria purified by phase partition showed a higher rate of ^{14}C-leucine incorporation into proteins with an endogenous source of ATP than with an exogenous ATP-generating system. Under the former conditions the presence of atractyloside increased the ^{14}C-leucine incorporation into proteins. The effects of different valinomycin concentrations plus atractyloside on intramitochondrial ATP levels and ^{14}C-leucine incorporation into proteins have been studied. The results indicate that the protein synthesis in brain mitochondria is dependent on the intramitochondrial ATP concentration.

Key words: Brain mitochondria, Protein synthesis, Partition.

It is well established that the incorporation of amino acid into proteins of isolated mitochondria requires a constant ATP-generating system. Two methods may be used to provide the necessary source of ATP; an exogenous ATP-generating system consisting of ATP, phosphoenolpyruvate and pyruvate kinase, or an endogenous ATP-generating system by which the energy requirement is satisfied by a respiratory chain substrate plus ADP (1,16). Many mitochondrial preparations show a higher rate of amino acid incorporation into proteins when the endogenous system of generating ATP is used (7, 11, 12, 14). In this respect, MOCKEL and BEATTIE (11) suggested that the protein synthesis depends on the intramitochondrial ATP concentration, since the incorporation of amino acids into proteins is enhanced by the addition of atractyloside. MUTVEI and NELSON (12) described how a minimum rate of ATP production is needed in order to support the protein synthesis and suggested that a decreased efficiency of energy transduction could in part be compensated for by an increase in the respiratory rate. Furthermore, RABINOWITZ *et al.* (15) suggested that the initiation of the

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peptide chain in the mitochondrial proteins synthesis depends on the maintenance of the electrochemical gradient of protons ($\Delta\mu H^+$). However, the direct relationship between the intramitochondrial ATP concentration and the protein synthetic activity of isolated mitochondria has not been quantified.

In this work we have determined the ATP requirement of the protein synthesis in a highly purified preparation of brain mitochondria obtained by phase partition. This method yields a very pure preparation free of any synaptosomal or membrane contamination (10, 17) and avoids the use of high centrifugal forces that could disturb the metabolic integrity of the organelles (5, 19).

The results obtained here show the effect of the addition of valinomycin on the ATP concentration and on the amino acid incorporation into mitochondrial proteins, suggesting that protein synthesis in brain mitochondria is clearly related to the ATP concentration but is not directly dependent on the electrochemical potential.

Materials and Methods

Preparation of mitochondria. — Mitochondria were prepared from the brain of Wistar rats and purified under sterile conditions by the phase partition method (10, 17). Crude mitochondria were prepared as previously reported (10). One gramme of crude mitochondria was added to 7.0 g of potassium-containing two-phase system prepared by weighing up: 2.56 g 20 % (w/w) Dextran T-500, 1.28 g 40 % (w/w) Poly (ethylene glucol) 4000, 2.24 g 1.0 M sorbitol, 0.35 g 10 mM sodium EDTA, 0.70 g 1.0 % bovine serum albumin, 0.175 g 0.20 M potassium phosphate pH 7.8 and 0.01 g water. In some cases, an ammonium containing two-phase system was used by including 0.175 g of 0.20 M ammonium chloride, 0.20 ammonium hydroxide-phosphoric acid buffer pH 7.8, instead of the potassium phosphate buffer. The phase system was mixed by 20 inversions of the tube, and centrifuged for 2 minutes at 600 × *g* to shorten the time for phase setting. The lower phase, containing mitochondria, was diluted with 30 ml of medium containing: 0.32 M sorbitol, 0.1 mM EDTA, 0.1 % bovine serum albumin (fatty acid free) and 5 mM potassium phosphate pH 7.8 and centrifuged at 19,000 × *g* for 20 minutes.

Amino acid incorporation. — The incubation was carried out in the 100 mM KCl respiration medium described by LAI and CLARK (8) consisting of 100 mM KCl, 75 mM manitol, 25 mM sucrose, 10 mM phosphate/Tris, 10 mM Tris/HCl, 0.05 mM EDTA, pH = 7.4, 5 mM $MgCl_2$ and 0.1 % fatty acid free bovine serum albumin (BSA). The ATP requirement for ^{14}C-leucine incorporation was supported by an endogenous ATP-generating system consisting of 10 mM glutamate plus 2.5 mM malate and 2 mM ADP. Incorporation was started by the addition of 1 mg/ml of mitochondrial protein in a final volume of 0.6 ml and 1 μCi/ml of L-(U-^{14}C)-leucine diluted with cold leucine to give a final specific activity of 20 mCi/mmol. The incubation medium and $MgCl_2$, BSA, substrates and ADP solutions were previously sterilized by Millipore filtration (0.22 μm filters). The bacterial contamination of the incubation mixture was evaluated according to MOCKEL and BEATTIE (11) and never exceeded 100 colonies/ml. Precipitated proteins were treated and filtered as described by BEATTIE (1). The results were expressed as pmol of ^{14}C-leucine incorporated per mg of mitochondrial protein per hour.

Assay procedures. — For the determination of intramitochondrial ATP mitochondria were separated from the amino

acid incorporation medium according to the method of PALMIERI *et al.* (13). The ATP was measured by the luciferin-luciferase system in a LKB luminometer, model 1250, according to STREHLER (18).

Mitochondrial respiration was measured polarographically at 25° C according to LAI and CLARK (8). Mitochondria (0.75-1.0 mg/ml) was added to the respiration medium together with the substrates. Mitochondrial swelling was monitored by absorbance change at 600 nm with a Unicam SP-1800 spectrophotometer according to BERNARD and COCKRELL (2). Mitochondrial proteins were determined by the biuret method (4) using bovine serum albumin as standard.

Results and Discussion

Most of the results presented in this paper have been obtained using mitochondria purified by ammonium-containing two-phase systems since they showed a better metabolic performance (17) and a higher rate of ^{14}C-leucine incorporation into proteins (table I) than the mitochondria purified by potassium-containing two-phase systems. ^{14}C-leucine incorporation by brain mitochondria purified by phase partition was higher with the endogenous ATP-generating system than with the exogenous source of ATP. These results are similar to those reported for brain mitochondrial purified by Ficoll gradients (14) and skeletal muscle mitochondria (16).

Table I. *^{14}C-Leucine incorporation into brain mitochondria purified by phase partition.*
The endogenous ATP source consisted of 10 mM glutamate plus 2.5 mM malate and 2 mM ADP. The exogenous ATP source was generated by 2 mM, 5 mM phosphoenolpyruvate and 50 mg/ml of pyruvate kinase. Results are the average of at least four experiments ± Standard deviations.

Preparation and incubation conditions	Incorporation rate (pmol × mg^{-1} × h^{-1})
Purified by ammonium-containing two-phase system:	
Endogenous ATP source	37.6 ± 2.6
Exogenous ATP source	16.0 ± 2.4
Purified by potassium-containing two-phase system:	
Endogenous ATP source	20.2 ± 4.0
Exogenous ATP source	10.2 ± 2.2

Table II. *Effect of ADP concentration, atractyloside and valinomycin on the ^{14}C-Leucine incorporation into brain mitochondria purified by phase partition.*
The incubations were carried out using the endogenous ATP source in the presence of 10 mM glutamate + 2.5 mM malate with mitochondria purified from ammonium-containing two-phase systems. Atractyloside and valinomycin were added to the medium 5 minutes after starting the incubation by addition of the mitochondria. Results are the average of four experiments ± standard deviations.

Incubation conditions	Incorporate rate (pmol × mg^{-1} × h^{-1})	%
20 mM ADP	35.0 ± 1.4	93
10 mM ADP	38.4 ± 3.3	102
2 mM ADP	37.6 ± 2.6	100
2 mM ADP + 50 μM atractyloside	61.6 ± 4.8	164
2 mM ADP + 50 μM atractyloside + 10 μg/ml valinomycin	19.7 ± 2.5	53

In order to study the effect of the ATP/ADP ratio on the brain mitochondrial protein synthesis, the incorporation of ^{14}C-leucine into mitochondrial proteins at different ADP concentrations was assayed (table II). Rat brain mitochondria showed a respiratory activity under the experimental conditions in table II, of 184 ± 20 natons oxygen × min^{-1} × mg^{-1} with a P/O ratio of 2.6–2.9 under state 3 conditions (18). Since the incubation for the ^{14}C-leucine incorporation was carried out in 0.6 ml of medium with 1 mg/ml of mitochondrial protein, the

complete phosphorylation of 2 mM ADP during the incubation would take about 3 minutes. This fact means that in the presence of 2 mM ADP the mitochondria were respiring in a state 4 for 27 minutes of the incubation period. On the other hand, in the presence of 20 mM ADP, the incubation was entirely carried out in a state 3 of respiration.

The fact that ^{14}C-leucine incorporation is very similar in state 3 and state 4 of mitochondrial respiration suggests that the mitochondrial protein synthesis is not controlled by the intramitochondrial ATP/ADP ratio, which is higher in state 4 (3). Furthermore, since the conformation of mitochondria changes from state 3 respiration (shrunk) to state 4 (swollen) the results obtained show that the mitochondrial protein synthesis does not directly depend on the conformational state of the organelle. However, the addition of atractyloside, which blocks the ADP-ATP carrier, induced a clear stimulation of the ^{14}C-leucine incorporation in brain mitochondria maintained in state 4 during the period of incubation (table II), suggesting that the brain mitochondrial protein synthesis is sensitive to the intramitochondrial ATP concentration, as previously described by MOCKEL and BEATTIE (11) in skeletal muscle mitochondria.

The addition of atractyloside plus valinomycin produced an inhibition of the ^{14}C-leucine incorporation when compared to that observed in the presence of atractyloside alone (table II). Under these conditions, the two main processes using intramitochondrial ATP are the synthesis of mitochondrial proteins and the hidrolysis of ATP due to the uncoupling induced by valinomycin (6). And thus by changing the extent of uncoupling by valinomycin, the intramitochondrial ATP available for mitochondrial protein synthesis can be modified. Furthermore, BERNARD and COCKRELL (2), using brain mitochondria have shown that the valinomycin–dependent swelling and the uncoupling induced by potassium show a linear correlation, and therefore, the extent of uncoupling induced by different concentrations of the potassium ionophore can be correlated with the swelling rates.

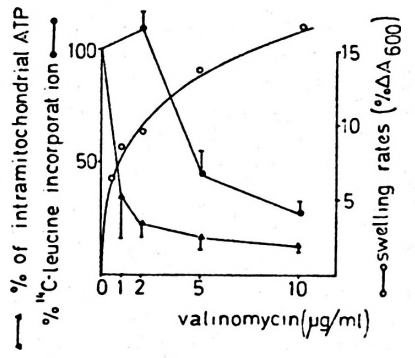

Fig. 1. *Effect of valinomycin on leucine incorporation, intramitochondrial ATP and mitochondrial swelling.*

The incubations were carried out using the endogenous ATP source in the presence of 10 mM glutamate + 2.5 mM malate and 2 mM ADP with mitochondria purified from ammonium-containing two-phase systems. Additions of atractyloside (50 M final concentration and valinomycin were as described in table II. (●), % of leucine incorporation, control value (100 %) was of 61.6 pmols of leucine × min^{-1} × mg^{-1} of mitochondrial protein. (▲) % of intramitochondrial ATP, control value (100 %) was of 2,638 pmols of ATP × mg^{-1} of mitochondrial protein. (O) Mitochondrial swelling. Swelling rates are expressed as the decrease in percentage of the initial absorbance at 600 nm × min^{-1}.

Fig. 1, shows the effect of different valinomycin concentrations plus atractyloside on mitochondria respiring in state 4 on ^{14}C-leucine incorporation, swelling rates and intramitochondrial ATP levels. The decrease of intramitochondrial ATP observed with 1 and 2 μg/ml of valinomycin did not affect the ^{14}C-leucine incorporation, which seems to indicate that at these ionophore concentrations intramitochondrial ATP is high enough to maintain the energy requirement for mitochondrial protein synthesis.

The later decrease in intramitochondrial ATP correlates well with a decrease in ^{14}C-leucine incorporation.

We have not observed under the experimental conditions used in this work a close direct dependence of mitochondrial protein synthesis on the transmembrane potential as suggested by RABINOWITZ *et al.* (15). The inhibition observed by these authors in puromycin treated mitochondria by the addition of uncounplers could be due to a decrease of the intramitochondrial ATP produced by the stimulation of the mitochondrial ATPase.

Although the amino acid incorporation appears to be independent of the ATP/ADP, as discussed above, with the results described here it is difficult to compare the rates of the amino acid incorporation obtained in state 3 and 4 of respiration in respect to the absolute intramitochondrial ATP concentration in both respiratory states. In fact, the main difference between both states consists in the different ATP/ADP but the very important changes in the volume of the mitochondrial matrix that takes place during the shrinking in state 3 may substantially increase the absolute ATP concentration.

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Resumen

Las mitocondrias de cerebro purificadas por partición en bifase muestran una mayor incorporación de C^{14}-leucina en sus proteínas en presencia de una fuente endógena de ATP que con un sistema exógeno de generación de ATP. En el primer caso la presencia de atractilósido aumenta la incorporación de C^{14}-leucina en sus proteínas. Se estudian los efectos de diferentes concentraciones de valinomicina más atractilósido sobre los niveles intramitocondriales de ATP y sobre la incorporación de C^{14}-leucina. Los resultados indican que la síntesis de proteína en mitocondria de cerebro es dependiente de la concentración mitocondrial de ATP.

Palabras clave: Mitocondrias, Cerebro, Síntesis proteica, Partición.

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