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Evidence for aerobic ATP synthesis in isolated myelin vesicles

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ABSTRACT

Even though brain represents only 2–3% of the body weight, it consumes 20% of total body oxygen, and 25% of total body glucose. This sounds surprising, in that mitochondrial density in brain is low, while mitochondria are thought to be the sole site of aerobic energy supply. These data would suggest that structures other than mitochondria are involved in aerobic ATP production. Considering that a sustained aerobic metabolism needs a great surface extension and that the oxygen solubility is higher in neutral lipids, we have focused our attention on myelin sheath, the multilayered membrane produced by oligodendrocytes, hypothesizing it to be an ATP production site. Myelin has long been supposed to augment the speed of conduction, however, there is growing evidence that it exerts an as yet unexplained neurotrophic role. In this work, by biochemical assays, Western Blot analysis, confocal laser microscopy, we present evidence that isolated myelin vesicles (IMV) are able to consume O_2 and produce ATP through the operation of a proton gradient across their membranes. Living optic nerve sections were exposed to MitoTracker, a classical mitochondrial dye, by a technique that we have developed and it was found that structures closely resembling nerve axons were stained. By immunohystochemistry we show that ATP synthase and myelin basic protein colocalize on both IMV and optic nerves. The complex of data suggests that myelin sheath may be the site of oxygen absorption and aerobic metabolism for the axons.
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1. Introduction

The mammalian Central Nervous System (CNS) was estimated to consume more than 20% of the body chemical energy. Even though its weight is 2–3% of that of total body (Attwell and Laughlin, 2001), brain consumes 20% of total inspired oxygen (O_2) (Silver and Erecinska, 1998) and 25% of the body's total glucose with an immense metabolic demand (Kann and Kovacs, 2007; Silver and Erecinska, 1998). Neurons critically depend on O_2 supply, being most of neuronal ATP generated by oxidative metabolism (Ames, 2000).

Nevertheless mitochondrial density in brain is lower than in other tissues (Veltri et al., 1990). If we look at axons, mitochondria are few and localized only around in Ranvier's node (Edgar et al., 2008). This sounds paradoxical, considering the exclusivity of mitochondria in aerobic ATP production.

Taken together, these data would suggest that structures other than mitochondria are involved in aerobic ATP production. So we have focused our attention on myelin sheath, the multilayered membrane (Morell and Norton, 1980) produced by oligodendrocytes. Two characteristics of myelin are intriguing: its enormous surface extension, a prerequisite for O_2 absorption, and its high content of neutral lipids (cerebrosides and cholesterol), in which O_2 is known to be absorbed 3–5-fold more readily (Sidell, 1998).

Myelin has long been supposed to augment the speed of conduction (Kursula, 2001), however, there is growing evidence that it exerts an as yet unexplained neuro-trophic role. In fact, loss of myelin in demyelinating diseases does not simply cause a lowering of speed of conduction but axonal necrosis (Ferguson et al., 1997). Several authors have investigated the metabolic interaction between neurons and glia (Hertz et al., 2007). Hargittai and Lieberman (1991) observed that myelin contributes approximately 70% of the O_2 consumption in medial giant axon of the crayfish. Recently, lactate produced by astrocytes was hypothesized to be a

Abbreviations: GA, glutaraldehyde; MBP, myelin basic protein; WB, Western Blot; ANT, adenosine nucleotide translocase; AK3, adenylate kinase isoform 3; TIM, mitochondrial import inner membrane translocase; TOM, mitochondrial import outer membrane translocase; PBS, phosphate buffered saline; MW, molecular weight; R.O.D., Relative Optical Density; RH-123, Rhodamine 123; CLSM, Confocal Laser Scanning Microscopy; IMV, isolated myelin vesicles; MT, MitoTracker; MTP, mitochondrial transition pore.

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major neuronal energy substrate (Pellerin and Magistretti, 2004; Schurr, 2006) (astrocyte–neuron–lactate shuttle), but this hypothesis has been challenged (Korf, 2006).

Three proteomic studies are reported in literature, that identified the presence of many subunits of F1-Fo ATP synthase and redox chain, in isolated myelin preparation (Taylor et al., 2004; Vanrobaeys et al., 2005; Werner et al., 2007). Taylor pointed up the connection between energetics and myelin. In our recent proteomic study of the retinal rod outer segment disks (that are devoid of mitochondria), we reported the expression of most of F1-Fo ATP synthase (and redox chain) subunits which was active in ATP production (Panfoli et al., 2008).

2. Materials and methods

2.1. Myelin isolation

Myelin was isolated from five forebrain stems (5 g) of cattle of less than 1 year of age, collected in a slaughterhouse, by the 'floating up' sucrose gradient modification (Haley et al., 1981) of the method by Norton and Poduslo (1973) to reduce contaminants. Protease inhibitor cocktail (Sigma–Aldrich) 50 μg/ml 5-fluorouracil and 20 μg/ml ampicillin were present throughout isolation. Centrifugation was conducted in a Beckman FW-27 rotor (Beckman, Fullerton, CA, USA). *Step 1*: 20 g of sample were homogenized with a Potter-Elvehjem homogenizer in 20 vol. (w/v) of 0.32 M sucrose in 2 mM EGTA. For each tube, 30 ml of homogenate was layered over 28 ml of 0.85 M sucrose in 2 mM EGTA and the tube was centrifuged at 75 000 × *g* for 30 min. This step was repeat twice. *Step 2*: The layer of crude myelin at the interface of the two sucrose solutions was collected, and homogenized in water to a final volume of 60 ml. Suspension was centrifuged at 75 000 × *g* for 15 min. *Step 3*: Pellet was dispersed in a total volume of 60 ml of water and centrifuged 12 000 × *g* for 10 min. The cloudy supernatant was discarded. *Step 4*: The loosely packed pellet was dispersed in water and centrifuged at 12 000 × *g* for 10 min. The myelin pellet was suspended in 30 ml of 0.32 M sucrose in 2 mM EGTA, and suspension layered onto 0.85 M sucrose in 2 mM EGTA and centrifuged at 75 000 × *g* for 30 min.

2.2. TEM microscopy

IMV and optic nerves were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde (GA) included in gelatin and freezed in liquid nitrogen. Ultrathin sections (60 nm thick) were obtained with a microtome were put onto classical copper grids. These were labelled with the Ab anti-myelin basic protein (MBP), then anti-rat secondary Ab and protein-A bound to colloidal gold (15 nm). Labelling is fixed with 1% GA.

For colocalization analysis, the IMV and ultrathin optic nerve sections were labelled with the Ab anti-MBP and F1-Fo ATP synthase, then recognized by anti-rat secondary Ab bound to colloidal gold of 10 nm and by anti-rabbit secondary Ab bound to colloidal gold of 15 nm, respectively.

2.3. Electrophoresis, semiquantitive Western Blot (WB) and quantification

Denaturing electrophoresis (SDS–PAGE) was performed using a Laemmli (1970) protocol. Rabbit polyclonal Ab against recombinant human α/β F1-Fo ATP synthase subunits (Panfoli et al., 2008) was diluted 1:5000 in phosphate buffered saline (PBS); rat monoclonal anti-MBP, that identifies an 18–20 kDa band (a gift of Prof. Angelo Schenone, University of Genova); Anti-Na^+/K^+-ATPase, anti-adenosine nucleotide translocase (ANT) and adenylate kinase isoform 3 (AK3) (Santa Cruz, CA, USA) were diluted 1:400 in PBS. Anti-mitochondrial import inner membrane translocase (TIM), subunit 8A and mitochondrial import outer membrane translocase, subunit 20 (TOM) (Santa Cruz, CA, USA) were diluted 1:200 in PBS. Secondary Ab_s were from Sigma–Aldrich. Protein molecular weight (MW) markers were from Fermentas (Fermentas Life Sciences, Hanover, MD, USA). Quantitative densitometry was performed using the ImageJ 1.31v software (http://rsb.info.nih.gov/ij/). Each band was converted into a densitometric trace allowing calculations of intensity (Jarskog and Gilmore, 2000). Enhanced chemiluminescent (ECL) band signals were compared with the signal of whole protein pattern (Ravera et al., 2007a). Results were expressed as Relative Optical Density (R.O.D.). Quantification data come from five different bovine forebrains.

2.4. Biochemical assays

Na^+/K^+-ATPase was determined as the difference between the ATPase activities in the presence and in the absence of 0.1 mM ouabain (Balestrino et al., 1998). AK3 assay was performed according to Tomasselli et al. (1979).

2.5. Oxygraphic measurements

O_2 consumption was assayed in forebrain homogenate, crude myelin and isolated myelin, by a thermostatically controlled oxygraph apparatus equipped with an amperometric electrode (Unisense-Microrespiration, Unisense A/S, Denmark) and a rapid mixing device. In a typical experiment, sample was incubated, at 25 °C, in the following respiration solution: 120 mM KCl, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tris–HCl, pH 7.4 and 25 μg/ml ampicillin (final volume 1.7 ml) (Sgobbo et al., 2007). After 5 min, to observe the uncoupled respiration rate, 30 μM Nigericin was added before the addition of respirating substrates and inhibitors of O_2 consumption: 0.7 mM NADH, 20 mM succinate, 10 mM ascorbate, 40 μM rotenone, 50 μM antimycin A and 0.5 mM potassium cyanide. To observe the ADP-stimulated respiration rates 0.045 mM ADP was added after NADH and succinate addition. Mitochondria-enriched fraction was used as a positive control, in this case NADH was substituted by 5 mM pyruvate and 2.5 mM malate. The respiratory rates were expressed in μM O_2/min/mg.

2.6. Assay of redox complexes

All the four mitochondrial complexes were assayed according to Sottocasa et al. (1967), Janssen et al. (2007) and Baracca et al. (2003).

2.7. Spectrofluorimetric measurements of proton potential

Proton potential ($\Delta\mu_{H+}$) was assayed using Rhodamine 123 (RH-123) dissolved in ethanol (below 0.4%). Fluorescence intensity (excit.: 503; emiss. 527 nm) was monitored at 25 °C with a PerkinElmer LS 50B fluorimeter (Emaus et al., 1986) under continuous stirring. Sample (20 μg) was incubated for 5 min in: 0.32 M sucrose, 0.01 mM HEPES, 2 mM $MgCl_2$, 4 mM NaH_2PO_4, 5 mM KCl 10 mM glucose and 2.5 U/ml hexokinase (ADP regenerating system). Then 50 nM RH-123 was added to the mixture and $\Delta\mu_{H+}$ changes monitored. Additions were: 0.1 mM ADP, 0.01 mM rotenone (for four time), 20 mM succinate (for three time), 0.1 μM oligomycin (for four time) and 0.04 mM antimycin A (Baracca et al., 2003).

2.8. RH-123 fluorescence quenching rate

F1-Fo ATP synthase concentration was estimated by RH-123 fluorescence quenching rate using different concentration of oligomycin. Reaction mixture contained: 20 μg of protein, 0.32 M sucrose, 0.01 mM HEPES, 2 mM $MgCl_2$, 4 mM NaH_2PO_4, 5 mM KCl,

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