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Mitochondrial intermembrane inclusion bodies: The common denominator between human mitochondrial myopathies and creatine depletion, due to impairment of cellular energetics

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Abstract

Mitochondrial inclusion bodies are often described in skeletal muscle of patients suffering diseases termed mitochondrial myopathies. A major component of these structures was discovered as being mitochondrial creatine kinase. Similar creatine kinase enriched inclusion bodies in the mitochondria of creatine depleted adult rat cardiomyocytes have been demonstrated. Structurally similar inclusion bodies are observed in mitochondria of ischemic and creatine depleted rat skeletal muscle. This paper describes the various methods for inducing mitochondrial inclusion bodies in rodent skeletal muscle, and compares their effects on muscle metabolism to the metabolic defects of mitochondrial myopathy muscle. We fed rats with a creatine analogue guanidino propionic acid and checked their solei for mitochondrial inclusion bodies, with the electron microscope. The activity of creatine kinase was analysed by measuring creatine stimulated oxidative phosphorylation in soleus skinned fibres using an oxygen electrode. The guanidino propionic acid-rat soleus mitochondria displayed no creatine stimulation, whereas control soleus did, even though the GPA solei had a five fold increase in creatine kinase protein per mitochondrial protein. The significance of these results in light of their relevance to human mitochondrial myopathies and the importance of altered cell energetics and metabolism in the formation of these crystalline structures are discussed. (Mol Cell Biochem **174:** 283–289, 1997)

Key words: mitochondrial inclusion bodies, mitochondrial myopathies, electron microscopy, cryo-techniques, creatine kinase, guanidino propionic acid

Introduction

Mitochondrial intermembrane inclusion bodies (MIBs) have been described ever since Luft and others observed them in a patient suffering from a non-thyroidal hypermetabolism [1]. Electron microscopy of muscle biopsy from this patient revealed striking abnormalities in mitochondrial ultrastructure, and the remarkable electron dense, para-crystalline inclusion bodies found between the membranes of mitochondria situated mostly at the muscle periphery. These mitochondria are very often found in the area that gives rise to the 'Ragged Red Fibres' seen in the biopsies of patients suffering from the group of diseases called mitochondrial myopathies [2]. The fundamental genetic lesion, which gives rise to the many symptoms of this disease classification, has been shown to be mitochondrial in origin [2]. Similar, if not the same MIBs, have been reported in the mitochondria of sural nerves of humans suffering from hereditary/non-hereditary neuropathies (HMSN) [3].

Extensive research has been carried out on the ultrastructural characteristics of these MIBs, using conventional EM

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preparative techniques, ultimately showing that they are indeed crystalline and two main types of crystals are found in the different muscle types [4, 5]. Type 1 crystalline MIBs appear in type 1 fibres and type 2 crystals are only found in type 2 muscle fibres [4]. More outstanding differences between these two types are their size and position within the mitochondria. Type 1 crystals are on average 200 nm wide and 2 µm long, whereas the type 2 crystals are more cubic in their dimensions, usually 100–300 nm in all three dimensions. The unit cell dimensions of the former have been shown to be $38 \times 34 \times 8$ nm, and the latter having dimensions of $20 \times 17 \times 8$ nm. In addition, type 1 crystals are located in the intermembrane space of mitochondria but the type 2 crystals are found in the intercristal space [4].

Very similar MIBs have been shown to occur in the slow skeletal muscle of rats fed an analogue of creatine (Cr) called β-guanidino propionic acid (GPA) [6]. This analogue has the ability to competitively inhibit Cr uptake by the Cr-transporter positioned at the outer surface of cell membranes [7, 8]. This compound can be phosphorylated by creatine kinase, to produce phospho-GPA (P-GPA) and ADP, however P-GPA has a 2 fold higher Km and 1/1000 V_{max} than that of phospho-Cr (PCr) for CK [7, 9, 10]. Chronic GPA treatment eventually causes many metabolic alterations in several tissues of experimental animals, which are compensations for the missing CK/PCr circuit [7, 11, 12]. The chemical and structural composition of these MIBs has been hotly debated until recently when sarcomeric mitochondrial creatine kinase (Mi_b-CK) was localised within MIBs of Cr-depleted adult rat cardiomyocytes, at the ultrastructural and confocal microscopical level [13]. The inclusion bodies seen in skeletal muscle of creatine depleted rats also label intensely for Mib-CK [14, 15]. Stadhouders and others found extensive labeling for Mi_b-CK over the MIBs in biopsies from patients suffering mitochondrial cytopathies, e.g. Chronic Progressive External Opthalmoplegia (CPEO), Kearns Sayre Syndrome and MELAS [4].

Therefore, it would seem that different alterations to normal muscle metabolism (inherent mitochondrial genetic mutations in the human case, leading to defects in the respiratory chain of the effected mitochondria, and decreased Cr levels in the rat model) lead to similar MIBs enriched with Mi_b-CK.

This short report wishes to delve into the functionality of Mi_b-CK within these inclusion bodies and speculate the reason for the occurrence of these phenomena.

Materials and methods

GPA production and animal care and feeding

GPA was synthesized in bulk according to the method described by [16]. Female Sprague Dawley rats were fed at 3 weeks of age on control diet or a diet containing 2.5% GPA, and also 1% GPA in their water supply for 3–4 months. Rats were killed using CO_2 or cervical dislocation and then exsanguinated.

Electron microscopy

Human biopsy material was taken from a patient suffering from hereditary motor and sensory neuropathy [3] and processed as described therein, using conventional EM-techniques.

Diaphragm samples of control and creatine depleted rats were taken immediately after cervical dislocation with the aid of an ophtalmologic puncher (Ø 2 mm; Grieshaber and Co AG, Winkelriedstrasse 52, CH-8203 Schaffhausen, Switzerland).

Subsequently, high pressure freezing was applied to the diaphragm samples. The samples were freeze-substituted in acetone containig 2% osmium tetroxide at –90°C for 16 h, –60°C for 12 h, –30°C for 8 h and 0°C for 1 h and then they were stepwise embedded in Epon (30, 70 and finally 100%) [17]. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined in a Phillips EM 301 and CM-12.

Preparation of skinned soleus fibres

Solei, from both control and GPA-fed animals, were treated in the same way. Soleus muscle was removed after killing the rat and exsanguination, and then placed into the isolation buffer (EGTA 10 mM, $MgCl_2$ 9.5 mM, $KHPO_4$ 3 mM, taurine 20 mM, K-Mes 49 mM, imidazole 20 mM), pH 7.1, Ca^{2+} 2.27 mM and at 4°C. They were then teased apart to produce fibre bundles of equal size i.e. 15 mm by 3 mm. Fibres were then introduced to the isolation buffer containing 50 µg/ml digitonin and mixed gently for 20 min at 4°C. Subsequently fibres were removed from this medium and washed with the respiration buffer (see below) and maintained at 4°C on ice [18].

Respiration analyses

The respiration analyses were carried out at 25°C, with a Space Cyclobios oxygraph (Anton Paar, Innsbruck, Austria) in the respiration buffer (mannitol 75 mM, sucrose 25 mM, 2 mM K-MES, KH_2PO_4 10 mM, EDTA 0.5 mM, BSA 1 mg/ml, Tris HCl 20 mM), pH 7.4 and using fibre bundles with a wet weight of approximately 15 mgs.

Stimulation of Oxidative Phosphorylation (OXPHOS) was carried out in the presence of 5 mM succinate and 5 mM $MgCl_2$, with 0.1 and 2.0 mM ADP, with and without 10 mM Cr present. Maximum stimulation was that which was measured after stimulation with 2.0 mM ADP and all other stimulations were expressed as a percentage of this maximal stimulation [19].

Mitochondrial isolation from soleus muscle

Solei were removed from control and GPA rats and placed into a medium containing 220 mM D-mannitol, 70 mM sucrose, 10 mM Hepes, 5 mM BME and 0.2 mM EDTA (MSH buffer), and minced with a surgical scissors. The mince was washed with excess buffer to remove the maximum amount of blood from the tissue. The minced tissue was then ground manually with a glass tissue grinder, and then homogenised at 4°C. The homogenate was centrifuged at 1000 g for 10 min, and the supernatant removed. The pellet was resuspended in 20 ml MSH buffer and centrifuged again for 10 min. Both supernatants were pooled and centrifuged at 8000 g for 20 min, the supernatant discarded and the pellet resuspended in fresh MSH buffer. This was again centrifuged at 8000 g for 20 min and the mitochondrial pellet suspended in 200 µl of buffer and kept for biochemical analysis at 4°C or frozen with liquid nitrogen.

Gel electrophoresis and Western blots

Protein was determined using the BioRad method and a 10% polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of 0.1% sodium dodecyl sulphate (SDS) and 20 µg of mitochondrial protein were loaded in each lane (see ref. [12]). After semi-dry blotting, protein transfer efficiency was observed by Ponceau S staining of the nitrocellulose membrane and then the membranes were blocked by a buffer containing 1% BSA in phosphate buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2PO_4, 1.4 mM KH_2PO_4, pH 7.4). Antibody labelling was carried out for Mi_b-CK at concentrations of 1:1000 and 1:400 respectively. After 1 h the membranes were washed 3 times with the above blocking buffer. The secondary antibody, peroxidase-conjugated goat anti-rabbit IgG, was then applied at 1:3000 concentration, and detected using H_2O_2 and luminol (2.5 mM luminol, 0.5 mM p-jodophenol, 50 mM Tris-HCl pH 7.5, and 0.15% H_2O_2) exposure to X-ray film (Fugi Medical RX) for densitometric analyses, using the Image Quant 3.3, Molecular Dynamics California.

Results

GPA feeding and rat weights

We found that rats fed the analogue GPA maintained a consistent 20% lighter total body weight than control fed rats, after 4 weeks of feeding. Body weights were measured up to week 12 of feeding (data not shown).

Electron microscopy

Ultrastructural examination of the human muscle biopsy material, revealed clear aggregations of abnormal subsarcolemmal mitochondria at the muscle periphery. Many of these giant mitochondria contained crystalline inclusion bodies, the same as those described in [3, 5]. Figure 1a is a micrograph showing such Type 2 inclusion bodies in muscle from a patient suffering from HMSN.

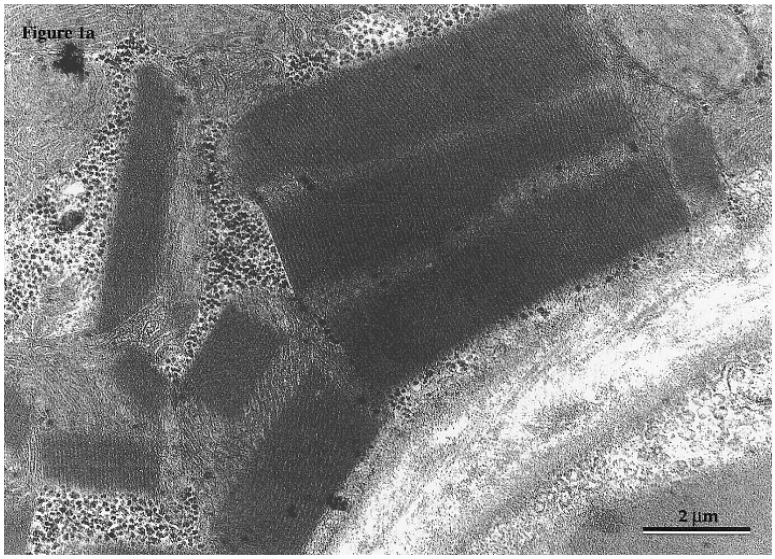
High pressure frozen diaphragm from control rats (Fig. 1b) showed no inclusion bodies but diaphragm from GPA fed rats revealed extensive accumulation of giant mitochondria at the muscle periphery, with inclusion bodies that were rod-like and cristae membrane-enveloped, but show no sign of membrane attachment, Fig. 1c.

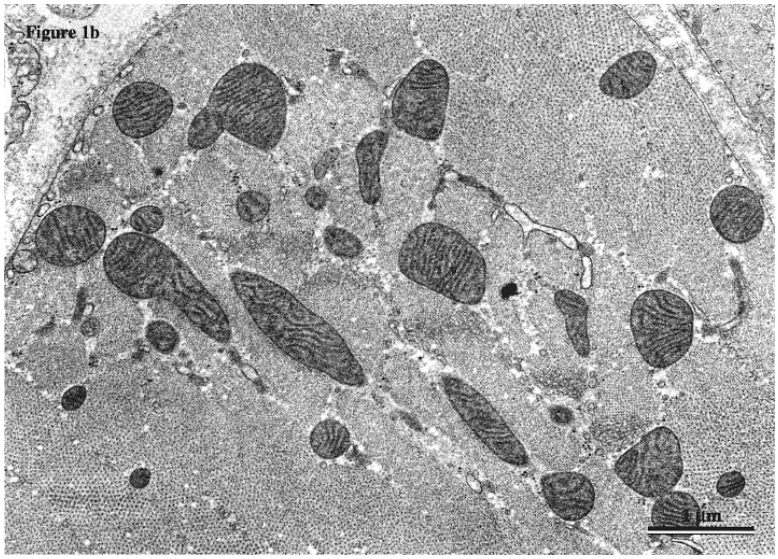
Function of mitochondrial creatine kinase in skinned soleus muscle fibres after chronic GPA feeding

We studied this role of Mi_b-CK in the regulation of oxidative phosphorylation after chronic treatment of rats with GPA. Respiration of skinned soleus fibres, that are permeable to external substrates, was followed by oxygen electrode respirometry, as a means to monitor activity of OXPHOS (Table 1). Under conditions saturating OXPHOS with phosphate, substrate and oxygen, addition of 0.1 mM ADP was not sufficient to fully activate the system. In both groups of GPA treated and control rats only 76% of the maximal oxygen consumption rate (State 3 max.), compared to that seen with 2 mM ADP, was achieved. Considering that inhibition of OXPHOS by atractyloside decreased the total respiration only by 50%, addition of 100 µM ADP produced half of the possible oxygen consumption by mitochondria. However, complete activation of oxidative phosphorylation was achieved by the controls at this low ADP concentration through Mi_b-CK activity (in the presence of 10 mM creatine). In contrast, this was no longer possible in solei from GPA treated rats. Thus a clear difference in creatine stimulated oxidative phosphorylation between soleus muscle of normal and GPA-fed animals was observed. This indicates that in the latter case the enzyme is somehow inactivated and incapable to mediate creatine stimulation of OXPHOS.

Densitometric analysis of Mi_b-CK immunoblots

Our results indicate an increase of approximately 5 fold in the amount of Mi_b-CK measured per mitochondrial protein, in GPA soleus mitochondria when compared to control samples (Table 2). This is supportive of other observations that the major component of the inclusion bodies seen in GPA fed rats are composed of Mi_b-CK [12–15]. Such a large accumu-





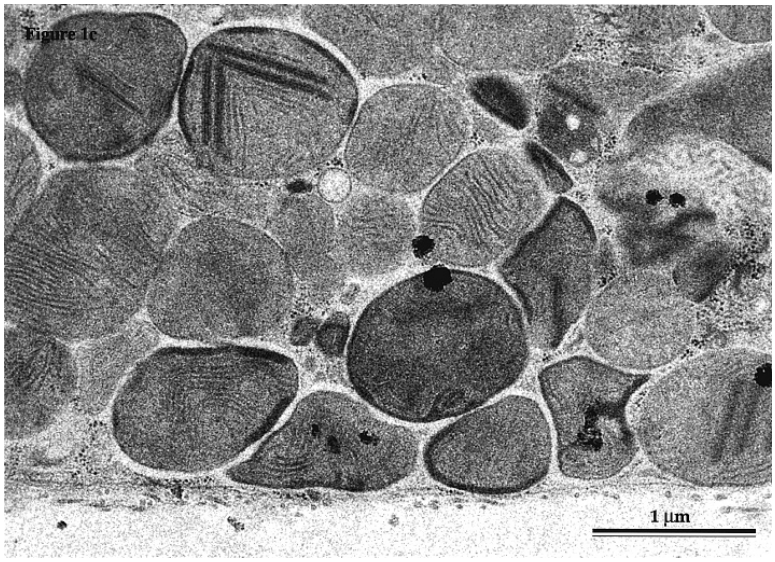


Fig. 1. Electron micrographs. Ultrastructural examination of the human muscle biopsy material, revealed clear aggregations of abnormal subsarcolemmal mitochondria at the muscle periphery. Many of these giant mitochondria contained crystalline inclusion bodies, the same as those described by [5]. Figure 1a is a micrograph showing such Type 2 inclusion bodies in muscle from a patient suffering from HMSN [3]. High pressure frozen diaphragm from control rats (Fig. 1b) showed no inclusion bodies but diaphragm from GPA fed rats revealed extensive accumulation of giant mitochondria in the muscle periphery, with inclusion bodies that were rod-like and cristae membrane-enveloped, Fig. 1c.

lation of Mi_b-CK between the membranes of GPA soleus mitochondria, probably plays a major role in the induction of these crystalline MIBs. As is seen in the creatine stimulated respiration experiments, the activity of Mi_b-CK in the GPA mitochondria is compromised to such an extent that it is unable to stimulate oxygen consumption as Mi_b-CK in control mitochondria does in the presence of 10 mM Cr and 10 mM ADP [12]. It is unclear why such an increase in Mi_bCK occurs other than it being a compensatory response to low Cr and ATP levels in the cell, concomitant with an increased mitochondrial volume [6, 12, 29]. Intriguingly the cytosolic form of CK in the soleus, M-CK, actually decreases in activity, leading to an overall decrease in the overall activity of CK in GPA solei [6, 12]. However it has been shown that inclusion body formation is not necessary to negate Cr stimulatable respiration, as GPA fed rat hearts, whose mitochondria do not contain inclusion bodies or even excessive amounts of Mi_b-CK, are also unable to be stimulated by 10–15 mM Cr and low ADP concentrations [12, 20]. Therefore these MIBs must represent an end stage of inactivation of Mi_b-CK.

Discussion

The results presented here indicate clearly that mitochondrial myopathies and creatine depleted muscles of rats share a striking phenomenon, the formation and accumulation of MIBs in extremely large mitochondria, predominantly positioned at the periphery of the muscle fibre. In human muscle biopsy samples (Fig. 1a), such abnormal mitochondria are quite often found in the area which gives rise to the so called 'Ragged Red Fibres', a diagnostic factor for some mitochondrial myopathies [2]. These humans have already shown to be highly enriched with Mi_b-CK but it is still not known whether or not these aggregates in human muscle are enzymatically functional [4].

MIBs of Cr depleted muscles have also been shown to be enriched with Mi_b-CK, so we used this as a model to test it's functionality within these structures. The skinned muscle fibre experiments we carried out with control soleus, show that Mi_b-CK can stimulate OXPHOS to maximum State 3 respiration at 0.1 mM ADP, however this is not the case in creatine depleted soleus (Table 1). As can be seen from Fig. 1c, MIBs were present in muscle of the GPA fed rats. Therefore it can be stated that the five fold accumulation state of Mi_b-CK in the GPA solei mitochondria (Table 2), is not functionally advantageous for CK mediated OXPHOS stimulation [12]. To fully qualify this conclusion, the same experiments must be carried out with human biopsy material from mitochondrial myopathy patients, who have mitochondria with similar MIBs.

Therefore, the question of why and how these MIBs occur arises, seeing as they are not beneficial for CK mediated mitochondrial respiration, remains to be answered. Furthermore, one can ask whether the reasons for MIB formation are the same in creatine depleted muscle and human mitochondrially defective muscle.

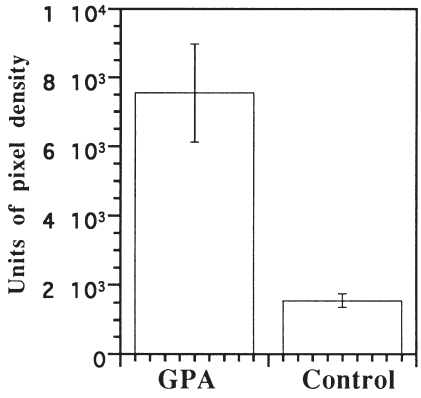
To get a deeper insight into this problem we must look at the literature that describes MIBs in rodent skeletal muscle, treated in ways other than creatine depletion, and compare the metabolic effects of such treatments. For example, ischemia

Table 1. Succinate dependent respiration analysis data of skinned soleus fibres from control and GPA fed rats. Respiration analysis was carried out for Cr and non Cr stimulated OXPHOS

Additions	GPA % of State 3 maximum	Control % of State 3 maximum
0.1 mM ADP	76 ± 7	76 ± 9
2.0 mM ADP	100	100
0.1 mM Atract	54 ± 9	55 ± 9.4
10 mM Creatine		
0.1 mM ADP	71 ± 17	100 ± 4
2.0 mM ADP	92 ± 2	101 ± 1
0.1 mM Atract	46 ± 11	45 ± 16

Stimulation of OXPHOS was carried out in the presence of, firstly, 0.1 mM ADP followed by 2.0 mM ADP, in the presence or absence of 10 mM Cr (see Materials and methods). 100% state 3 was taken as the oxygen consumption in the presence of 2.0 mM ADP for both GPA and control soled fibres. 0.1 mM atractyloside was added to inhibit ADP dependent OXPHOS (n = 6). The other values are the relative percentages of this maximum State 3 rate of oxygen consumption. Note that Cr stimulated respiration is 100% even at relatively low (0,1 mM) ADP in control mitochondria, but is significantly reduced (only 71%) in mitochondria of EPA fed rats. (Reprinted with permission from [12]).

Table 2. Histogram showing the relative amounts of Mi_b-CK protein per mitochondrial protein of GPA and control solei mitochondria



Western blots followed by immuno peroxidase staining for Mi_b-CK in isolated soleus mitochondria from control and GPA fed rats. The respective bands on the X-Ray film given after 10 sec exposure to luminol luminesence, representing the Mi_b-CK protein, were analysed via densitometry, (see Materials and methods). Error bars represent standard deviation (n = S). (Data taken from [12] with permission).

is known to produce these lesions [21, 22]. Both of these reports show MIBs as seen in our creatine depleted rat soleus and diaphragm. Hanzlikova and Schiaffino (1977) found that after 12–24 h of ischemia, (induced by surgical separation of the aorta from the vena cava), MIBs were most commonly observed. Interestingly, they found granular inter-cristal inclusions at an earlier stage of ischemia, but never after 12 h. These granular inclusions could represent an early stage in the development of higher structurally ordered MIBs.

Very similar results were found in degenerating and regenerating ischemic rabbit tibialis anterius and locally cold-injured adult mouse triceps surae [23]. All the referred-to studies, and our own model, involve the proliferation of mitochondria prior to the appearance of MIBs.

What are the common consequential lesions between these experiments? Serious impairment of normal muscle energy metabolism certainly seems to be an important factor. Creatine depletion has the effect of diminishing PCr levels and impairing the CK/PCr shuttle, which upsets the ability of Cr depleted cells to maintain high levels of ATP in areas which depend upon this [23], e.g. at the sarcoplasmic reticulum calcium pump [25] and the M-band Mg^{2+}-ATPase [9]. Ischemia starves the muscle of a normal oxygen supply, which leads to a decreased mitochondrial ATP production. Cold exposure has to have a more drastic effect upon global muscle metabolism, so a specific link is difficult to point out. All the above experimental models have a common effect of disrupting ATP production and supply, most likely affecting the phosphorylation potential of the cell [11]. These induced defects in normal energy metabolism can be compared with the genetic defects in mitochondria of patients suffering from mitochondrial myopathies. Such mutations ultimately lower the muscle cells capacity to provide ATP via OXPHOS [2]. Therefore, it is clear that when normal muscle energy metabolism is disrupted, which is often followed by mitochondrial proliferation and enlargement, the MIBs appear.

One can only hypothesise about why these inclusion bodies occur and the only possibility that we can propose is that oligomeric forms of Mi_b-CK accumulate in abnormally excessive quantities within the intercristal spaces under stress. Ultrastructurally, after negative staining, these can be studied at an early stage, due to the large size of the enzyme i.e. the octamer is $9.3 \times 9.3 \times 8.3$ nm [26, 27]. As an ultrathin section can be 30–40 nm in thickness, this could represent 3–4 layers of the enzyme, accounting for the electron density between the membranes. Admittedly, one should keep in mind that the fine structure of MIBs depends heavily upon the EM preparation method used, as was clearly demonstrated [13].

However, why the enzyme crystallises could be due to the environment within the intermembrane spaces of mitochondria. First of all Mi_b-CK has been shown to interact with the inner and outer membranes of mitochondria [28, 29]. Cardiolipin is the only negatively charged phospholipid of the innermembrane and Mi_b-CK has been shown to interact with cardiolipin vesicles [29] and crystallise 2 dimensionally on cardiolipin layers [26]. Therefore, cardiolipin in the inner membrane may be a foundation for Mi_b-CK oligomerisation and along with spatial and orientational restrictions within the membrane spaces, may induce Mi_b-CK crystallisation. Furthermore accumulation of the enzyme in the intermembrane and/or intercristal space, as we show here, i.e. a five fold increase of Mi_b-CK has been seen in rat solei after 3 months of GPA feeding [12], must be another important factor in MIB development. Therefore, we believe the conditions which we have mentioned above, together induce crystallisation of Mi_b-CK octamers in the intermembrane and intercristal spaces of mitochondria. This is reversible upon a return to normal cellular metabolism and energy state as has been shown [13, 30]. It would be interesting to analyse muscle tissue from mitochondrial myopathy patients after long term creatine administration, to observe whether a change occurs in MIB structure or quantity.

Finally, it is important to mention the fact that Cr supplementation has been revealed as beneficial to mitochondrial myopathy patients (MELAS), in so far as alleviating the severity of muscle and brain symptoms [31].

Very recent structural and biochemical characterization of isolated intra-mitochondrial inclusion bodies by high-resolution electron microscopy and immuno-blotting, respectively, indeed revealed Mi-CK octamers as the main component of MIBs in mitochondria of GPA-fed rats [32].

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