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Creatine transporters: A reappraisal

Oliver Speer,[1]* Lukas J. Neukomm,[1]* Robyn M. Murphy,[2] Elsa Zanolla,[1] Uwe Schlattner,[1] Hugues Henry,[3] Rodney J. Snow[2] and Theo Wallimann[1]

[1]*ETH-Zürich, Institute of Cell Biology, ETH-Hönggerberg, Zurich, Switzerland;* [2]*School of Health Sciences, Deakin University, Burwood, Australia;* [3]*University Hospital, Clinical Chemistry Laboratories, Lausanne, Switzerland*

Abstract

Creatine (Cr) plays a key role in cellular energy metabolism and is found at high concentrations in metabolically active cells such as skeletal muscle and neurons. These, and a variety of other cells, take up Cr from the extra cellular fluid by a high affinity Na^+/Cl^--dependent creatine transporter (CrT). Mutations in the *crt* gene, found in several patients, lead to severe retardation of speech and mental development, accompanied by the absence of Cr in the brain.

In order to characterize CrT protein(s) on a biochemical level, antibodies were raised against synthetic peptides derived from the N- and C-terminal cDNA sequences of the putative CrT-1 protein. In total homogenates of various tissues, both antibodies, directed against these different epitopes, recognize the same two major polypetides on Western blots with apparent Mr of 70 and 55 kDa. The C-terminal CrT antibody (α-CrT_{COOH}) immunologically reacts with proteins located at the inner membrane of mitochondria as determined by immuno-electron microscopy, as well as by subfractionation of mitochondria. Cr-uptake experiments with isolated mitochondria showed these organelles were able to transport Cr via a sulfhydryl-reagent-sensitive transporter that could be blocked by anti-CrT antibodies when the outer mitochondrial membrane was permeabilized. We concluded that mitochondria are able to specifically take-up Cr from the cytosol, via a low-affinity CrT, and that the above polypeptides would likely represent mitochondrial CrT(s). However, by mass spectrometry techniques, the immunologically reactive proteins, detected by our anti-CrT antibodies, were identified as E2 components of the α-keto acid dehydrogenase multi enzyme complexes, namely pyruvate dehydrogenase (PDH), branched chain keto acid dehydrogenase (BC-KADH) and α-ketoglutarate dehydrogenase (α-KGDH). The E2 components of PDH are membrane associated, whilst it would be expected that a mitochondrial CrT would be a transmembrane protein. Results of phase partitioning by Triton X-114, as well as washing of mitochondrial membranes at basic pH, support that these immunologically cross-reactive proteins are, as expected for E2 components, membrane associated rather than transmembrane. On the other hand, the fact that mitochondrial Cr uptake into intact mitoplast could be blocked by our α-CrT_{COOH} antibodies, indicate that our antisera contain antibodies reactive to proteins involved in mitochondrial transport of Cr. The presence of specific antibodies against CrT is supported by results from plasma membrane vesicles isolated from human and rat skeletal muscle, where both 55 and 70 kDa polypeptides disappeared and a single polypeptide with an apparent electrophoretic mobility of ~ 60 kDa was enriched. This latter is most likely representing the genuine plasma membrane CrT.

Due to the fact that all anti-CrT antibodies that were independently prepared by several laboratories seem to cross-react with non-CrT polypeptides, specifically with E2 components of mitochondrial dehydrogenases, further research is required to characterise on a biochemical/biophysical level the CrT polypeptides, e.g. to determine whether the ~ 60 kDa polypeptide is indeed a bona-fide CrT and to identify the mitochondrial transporter that is able to facilitate Cr-uptake into these organelles. Therefore, the anti-CrT antibodies available so far should only be used with these precautions in mind. This holds especially true for quantitation of CrT polypeptides by Western blots, e.g. when trying to answer whether CrT's are up- or down-regulated by certain experimental interventions or under pathological conditions.

*These authors have contributed equally to this work.

Address for offprints: T. Wallimann, Institute of Cell Biology, ETH-Hönggerberg HPM F39, CH-8093 Zürich, Switzerland
(E-mail: theo.wallimann@cell.biol.ethz.ch)

In conclusion, we still hold to the scheme that besides the high-affinity and high-efficiency plasmalemma CrT there exists an additional low affinity high Km Cr uptake mechanism in mitochondria. However, the exact biochemical nature of this mitochondrial creatine transport, still remains elusive. Finally, similar to the creatine kinase (CK) isoenzymes, which are specifically located at different cellular compartments, also the substrates of CK are compartmentalized in cytosolic and mitochondrial pools. This is in line with ^{14}C-Cr-isotope tracer studies and a number of [^{31}P]-NMR magnetization transfer studies, as well as with recent [^{1}H]-NMR spectroscopy data. (Mol Cell Biochem **256/257**: 407–424, 2004)

Key words: creatine transporter isoforms, mitochondrial membrane transporters, 12-transmembrane helix transporter family, creatine pools, bioenergetics, phosphocreatine shuttle, creatine kinase and mitochondria

Abbreviations: ANT – adenine nucleotide translocator; AGAT – arginine glycine amino-transferase; BC-KADH – branched chain keto acid dehydrogenase; Cr – creatine; CK – creatine kinase; CrT – creatine transporter; CsA – cyclosporin A; GAA – guanidine acetic acid; GAMT – guanidine-acetate methyltransferase; β-GPA – β-guanidino propionic acid; α-HCA – α-cyano-4-hydroxycinnamic acid; α-KGDH – α-ketoglutarate dehydrogenase; LC-ESI-MS/MS – liquid-chomatograph-electro-(nano)spray-ionization tandem mass spectroscopy; MALDI TOF – matrix-assisted laser desorption-ionisation and time-of-flight; MCK – muscle type creatine kinase; PDH – pyruvate dehydrogenase; PCr – phosphocreatine; PKC – protein kinase C; PTP – permeability transition pore; TCA – trichloric acid; TFA – trifluoroacetic acid; VDAC – outer mitochondrial membrane voltage dependant anion channel

Introduction

Recent publications have shed new light on the importance of creatine (Cr). In particular, mutations in the two genes involved in Cr synthesis all lead to an absence of creatine in the brain, which seems to evoke severe disturbances of brain function [1–9]. In patients with these inborn errors of creatine metabolism [4, 10–12] several research teams have found treatment with Cr was able to increase the Cr content of the brain. However in patients with mutations of the Cr transporter (CrT) protein no such increase following Cr treatment was observed [2, 7].

This work will briefly discuss Cr metabolism, as well as the identification of the CrT. For more detailed reviews, the reader is directed to [13, 14]. The predominant part of this work will present new challenging data on the molecular identification of the CrT isoforms. Cr supplementation in health and disease will not be mentioned here, but can be found in other parts of this issue or also see [9, 13].

Creatine metabolism

Cr and phosphocreatine (PCr) are guanidino compounds, which together with creatine kinase (CK) isoforms constitute part of the cellular energy network in cells that typically display large fluctuations in energy demand such as skeletal muscle, brain, heart and many other tissues [15–20]. In these tissues, Cr is the substrate of creatine kinase, which transfers a phosphate group from ATP to Cr to produce PCr at sites of energy production (mitochondria) and recycles ATP by consuming the PCr at sites of high energy turn over (for review see [16, 21]).

In mammals, the final step of Cr synthesis takes place mainly in the liver and pancreas by the enzyme guanidine-acetate methyltransferase (GAMT) using the Cr precursor guanidine acetic acid (GAA). GAA itself is synthesised in the kidney by arginine glycine armino-transferase (AGAT) [22–25]. Interestingly, tissues, which contain the highest concentrations of PCr and total Cr, do not synthesize their own Cr or do so only to a limited extent [1]. This shortcoming is compensated by absorption of Cr into the respective tissues by a specific CrT from the circulating blood [26–29], (for review see [14, 17]). Blood Cr levels are maintained by endogenous Cr synthesis or by ingestion of Cr-containing food (fish and meat).

The creatine transporter

Various cell systems and *in vivo* human studies have shown that the regulation of Cr uptake may be governed by a number of different mechanisms (see [14]). Putative phosphorylation and glycosylation consensus sequences (Fig. 1) have been identified on the CrT cDNA that suggest the protein may be regulated in either one or both of these ways. Recently, it was shown that serine phosphorylation of CrT decreased after starvation in rats with a concomitant increase in Cr uptake into skeletal muscle vesicles [30], whereas tyrosine phosphorylation of CrT was decreased after Cr supplementation [31]. These data suggest that changes in the extra- and/or intracellular Cr content alters the phosphorylation state of the CrT, and thereby its activity. In parallel to the elevated phosphorylation levels, c-Src kinase activity also increases such that it might be speculated that c-Src kinase could possibly tyrosine phosphorylate CrT since this kinase was enriched by immunoprecipitation together with the CrT [30–32].

Recent data obtained with anti-CrT antibodies involving immuno-localization, cell fractionation and Cr uptake studies, suggest the existence of CrT isoforms with localisation in the plasma membrane and with in mitochondria [29, 33]. This would support the existence of cytosolic and mitochondrial pools [34, 35].

Inborn errors in CrT

Several mutations of the CrT have been described in humans [2, 3, 7, 36]. These patients present with hypotonia, epilepsy and delay in development, speech and expressive language function, and have an absent Cr peak in their brain proton-MR spectra, elevated Cr in blood and in urine [2, 3, 7, 36]. Fibroblasts from these patients contained hemizygous nonsense mutations in the CrT gene and were defective in Cr uptake [2, 3]. Interestingly, the Xq28 locus – where the *crt1* gene is localized – has been linked to the genes for several neuromuscular disorders, such as Barth syndrome [37–40], causing several authors [17, 41, 42] to speculate that dysfunctional *crt1* gene may be responsible for some of these diseases.

A growing number of families with genetic defects in CrT have been identified. Consequently, it is of interest to study the CrT protein(s) in terms of structure, function and localization, with the aim to study the Cr system and to develop possible diagnostic tests for clinical use. One such approach would be the generation of highly specific antibodies against CrT protein(s) or domains thereof, e.g. for clinical screening by Western blot analysis of patients white blood cells or fibroblasts in the future.

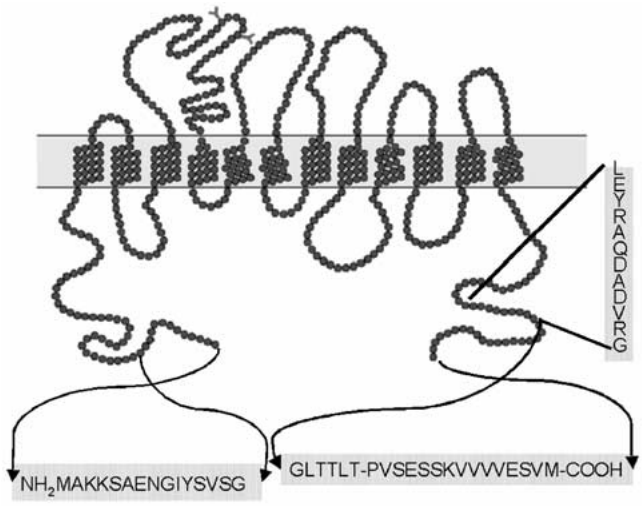

Fig. 1. Proposed topology of the hypothetic human creatine transporter 1 protein (hCrT1), adapted from [87]. Depicted are the three peptides against which antibodies have been produced [17, 44, 45].

Generation of antibodies against CrT

Antibodies against CrT have been generated independently in several laboratories [17, 43–45]. Dodd *et al*. produced a peptide antibody against an over expressed C-terminal polypeptide stretch of 56 amino acids of CrT protein [45] and immuno purified this serum with a heterologously expressed 21 C-terminal polypeptide stretch. This antibody shows 5 signals between 50 and 100 kDa on Western blots. However, only a 90 kDa signal is obtained from HEK293 cells after the enrichment of cell surface exposed proteins via biotinylation and pulled down with Streptavidin coated beads [45]. This immunoband represents a glycosylated protein (personal communication). With the same method of antibody production Kekelidze *et al*. obtained three distinct signals at 55, 70 and > 100 kDa in a C6 glioma cell line whereas, in a L6 muscle cell line only the 55 and 70 kDa signals were seen [43]. Our research group produced sera against N-terminal (NH_2-M-A-K-K-S-A-E-N-G-I-Y-S-V-S-G) or C-terminal peptides (P-V-S-E-S-S-K-V-V-V-V-E-S-V-M-$_{COOH}$) (Fig. 1) [17]. Using these antibodies on Western blot, two main signals at 55 and 70 kDa were obtained in rat muscle, heart, skeletal muscle, brain, liver, kidney, lung, testis and intestine total homogenates. In rat heart, however, a 150 kDa signal has been seen, whereas in enriched sarcolemma fractions from rat skeletal muscle, a single ~ 60 kDa signal has been described [29]. In mitochondria isolated from rat gastrocnemius and soleus muscle, heart, brain, and kidney mitochondria both anti-N- and anti-C-terminal peptide antibodies revealed two strong signals at 55 and 70 kDa [29, 33], whereas in mitochondria from rat liver and kidney an additional 112 kDa protein was also seen [29].

Tran *et al*. produced another antibody directed against a more internal C-terminal cDNA-derived protein sequences (L-E-Y-R-A-Q-D-A-D-V-R-G) (Fig. 1) [44]. These authors report signals at apparent molecular weight of 55, 60, 75 and > 100 kDa in extracts from C_2C_{12} cells. After treatment with tunicamycin (an N-linked glycosylation inhibitor) the 75 kDa signal disappeared, whereas the signal at 60 kDa was significantly increased, suggesting that this could be the core CrT protein. Only the 55 kDa signal, however, was captured by streptavidin after cellular surface biotinylation. This 55 kDa polypeptide is not present at the cell surface after Cyclosporin A treatment [44]. These authors discuss the possibility that Cyclosporin A inhibits the chaperone-like cyclophilin, which might be necessary for a correct folding process of CrT in the endoplasmic reticulum.

A commercially available antibody (a 20 amino acid peptide near the cytoplasmic N-terminal of CrT sequence) was used to immune precipitate CrT [30–32]. These precipitations were afterwards analysed with phosphotyrosine and phosphoserine antibodies. So far these authors reported one single representative Western blot signal with apparent Mr of

55 kDa after probing with both phospho-amino acid antibodies [30–32]. The actual Western blot signal after probing with α-CrT antibody was reported between 50 and 80 kDa [30].

The common denominator in terms of immune reactivity of all these different antibodies is their consistent detection of strong Western blot signals of two main polypeptides in the range of 55 and 70 kDa, with slight variations in the apparent molecular weight, depending on the gel system, the animal species or cell types used. Since the predicted molecular weight of CrT derived from the CrT cDNA is approximately 70 kDa, the detection of a strong signal with all of these anti-CrT antibodies at 70 kDa seemed appropriate. Interestingly, with other transporters of the 12-helix membrane transporter family (e.g. the serotonin transporter), these highly hydrophobic membrane proteins seem to generally show a rather anomalous electrophoretic behaviour in SDS-PAGE with apparent Mr of over 100 kDa, as opposed to the expected molecular weight of 70 kDa calculated from the cDNA [46, 47].

Present state of knowledge

Encouraged by these data, and in an attempt to characterize the tissue distribution and subcellular localization of CrT, e.g. on the plasmamembrane, the ER or Golgi etc., we started to use our antibodies for Western blotting, as well as for immuno- and EM localization studies [29, 33]. Western blot quantification of the two major polypeptides of 55 and 70 kDa in different muscle types from rats treated either with β-guanidinopropionic acid (β-GPA), a known competitive inhibitor of Cr transport [48], or Cr to deplete or increase intracellular Cr pools, respectively, showed that chronic long-term supplementation by high-dose Cr led to a down-regulation in the accumulation of both of these protein species, whereas the opposite was true after βGPA treatment [17, 49]. These facts indicated that both of these bands might be CrTs since they responded to interventions affecting cellular Cr levels in the expected manner.

Tissue specific expression of CrT proteins

The first surprise with these antibodies came from Western blot analysis of the tissue distribution as the highest expression of both CrT-species was found in heart. Even though these data corresponded to those from Northern blot analysis [42, 50–52, 58] it was somewhat unexpected, since heart neither expresses the highest levels of CK nor accumulates the highest amount of Cr compared to other tissues [53]. One would, perhaps expect fast-twitch glycolytic, white muscle with high concentrations of PCr and total Cr and very high expression levels of cytosolic CK [16] to express the highest levels of CrT. The opposite to this, however, was found in rat skeletal muscle where slow-twitch oxidative muscle showed a greater expression of the CrT compared with the fast-twitch glycolytic muscle [54, 55]. On the other hand, cardiac muscle contains the highest proportion of mitochondrial volume fraction of all muscles, and slow-twitch oxidative muscle has a greater mitochondrial number than fast-twitch glycolytic muscle. Strikingly, the liver, despite being the tissue of Cr synthesis, does not express creatine kinase [56]. Nevertheless liver was found to express high concentrations of both immunoreactive anti CrT bands. By contrast, Northern blot analysis provided no evidence of significant levels of CrT mRNA in liver since either no CrT mRNA, or only very low amounts thereof have been reported [42, 50–52, 57, 58]. However, this might contradict the physiological role of liver in terms of creatine metabolism, since this organ, as the Cr synthesising organ, must be able to export Cr, but the Na^+ gradient would not support the CrT being responsible for the export of Cr.

Localization of the 55 and 70 kDa polypeptide species by cellular fractionation and immune histochemistry

Using the anti-CrT antibodies, two major peptides at 55 and 70 kDa were also detected in brain, spleen, testis, heart, kidney and liver (Fig. 2). On tissue sections of heart and skeletal muscle analysed by immuno confocal microscopy it was evident that both antibodies recognized proteins within mitochondria [29, 33]. By isolation of mitochondria via gradient centrifugation these polypeptide doublets were enriched in the mitochondrial fractions (Fig. 2). In addition, by immunoelectron microscopy, the mitochondrial localisation could also be confirmed [33]. These findings somehow matched parallel findings by Dodd [45] who employed an independently produced antibody, which recognises the transiently expressed CrT as intracellular spots possibly resembling mitochondria. Finally, in our hands, isolated mitochondrial membranes revealed the presence of the 55, 70 and 112 kDa proteins co-purified with the inner mitochondrial membrane (Fig. 3) and [33].

Mitochondrial creatine uptake

Thus, in the inner mitochondria membrane, there are at least 3 polypeptides, which are recognized by our anti COOH CrT sera (Fig. 3). The presence of a CrT protein within mitochondria seems reasonable, as PCr was found within mitochondria [34, 35]. Consequently, it had to be addressed whether mitochondria are indeed able to take up Cr. In fact, our subsequent experiments showed that mitochondria isolated from different tissues were able to take up Cr (Fig. 4A). According to our recent measurements, this mitochondrial Cr uptake

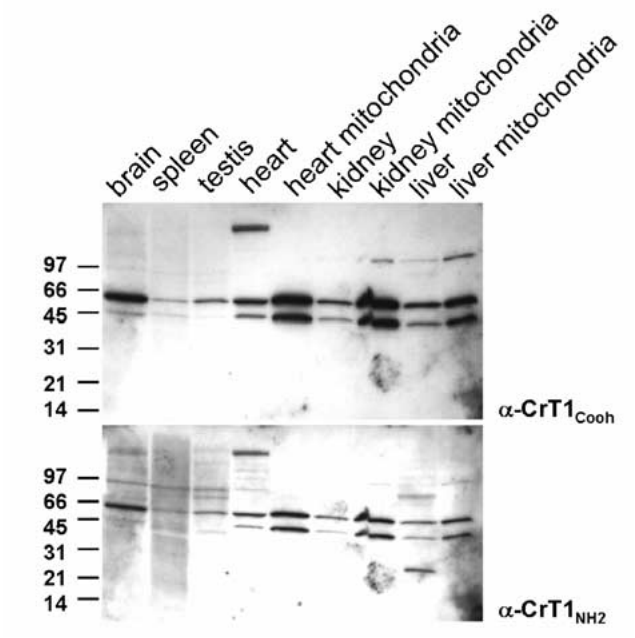

Fig. 2. Immunoblot of total tissue extracts and highly purified mitochondria obtained by fractionated and density gradient centrifugation (20 μg of protein loaded per lane). Proteins were separated by 12% SDS PAGE, transferred to Nitrocellulose membrane. Proteins were detected with anti-CrT C-terminal peptide antibody, and reprobed with anti-CrT N-terminal peptide antibody.

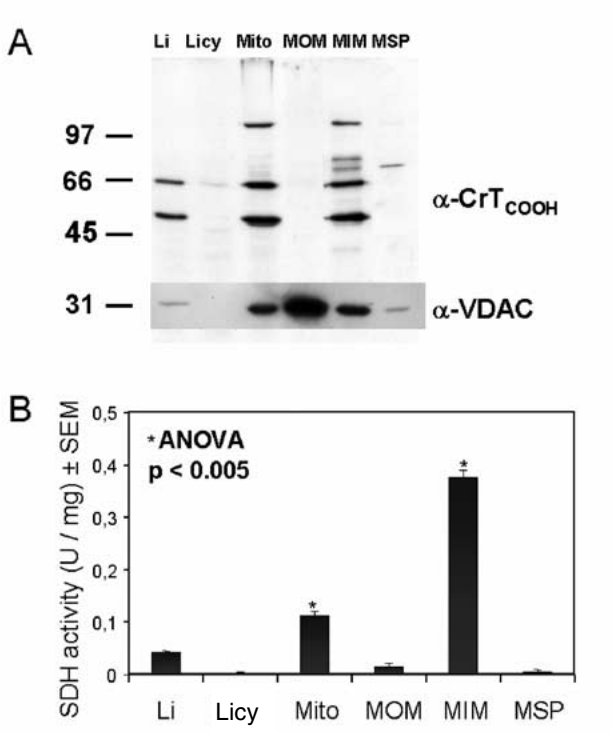

Fig. 3. Submitochondrial localization of CrT by fractionation of mitochondrial membranes. After isolation of mitochondria (Mito) and cytoplasm (Licy) from adult rat liver homogenate (Li), mitochondrial outer (MOM) and mitochondrial inner membrane (MIM), as well as soluble mitochondrial proteins (MSP), were enriched by a swelling-shrinkage procedure and a non-linear sucrose ultra-centrifugation [63]. Twenty μg protein extract each were separated with SDS-PAGE and blotted onto a nitrocellulose membrane. CrT was detected by using polyclonal rabbit α-CrT_{COOH} antibodies and secondary goat α-rabbit coupled HRPO antibodies in liver homogenate, enriched in mitochondria and highly enriched in MIM, but absent in the cytosol and MSP. The same blot was reprobed with α-VDAC monoclonal mouse antibody, a marker for the mitochondrial outer membrane. VDAC was detected highly enriched in the MOM. (B) Succinate dehydrogenase (SDH) activity assay of subcellular fractions. SDH as an enzymatic marker for the mitochondrial inner membrane was used to test the enrichment of MIM by measuring the specific SDH activity (U/mg) of the various fractions. Note the almost complete absence of SDH in Licy, MOM and MSP.

displays a V_{max} of 12 nmoles min^{-1} mg^{-1} protein and a Km of 16 mM representing an efficient but low affinity CrT [33]. The rather high K_m of the mitochondrial Cr uptake seems reasonable, as the CrT in the plasma membrane representing a high-affinity transporter (Km for Cr 20–50 μM) is able to efficiently accumulate Cr into the cell leading to an intracellular Cr concentration in muscle in the range of 20–40 mM [13, 16, 29].

Mitochondrial Cr uptake was sensitive to the SH-modifying reagent *N*-ethylmaleimide (NEM) [33]. This compound has been tested to inhibit enzymatic activities at low concentrations [59, 60]. Most importantly the anti C-terminal CrT sera were also able to inhibit the mitochondrial Cr accumulation, whilst a control antibody had no such effect (Fig. 4B) [33]. This however was only possible if the outer mitochondrial membrane was disrupted either by digitonin (Fig. 4 B) or by osmotic shock [33]. All these data seemed convincing to predict the existence of a mitochondrial Cr uptake protein. Even more, we were convinced that we had identified a new mitochondrial CrT protein, as the physiological characteristics of the mitochondrial Cr uptake were distinct from the Cr transport at the plasma membrane. Raising question to the existence of a specific mitochondrial CrT was that, in contrast to the plasmalemmal Cr uptake, mitochondrial Cr uptake activity showed a high Km but was not very effectively inhibited by β-GPA [33]. Another finding, which favoured a protein different from plasmalemma CrT, was the sensitivity of mitochondrial Cr transport to the protonophoric uncoupler carbonyl cyanide p-trifluoromethoxyphenyhydrazone (FCCP), which disrupts the mitochondrial membrane potential [33]. Furthermore, the question of whether this mitochondrial Cr uptake was facilitated via a non-specific amino acid transporter must also be considered, given the competitive inhibition of Cr uptake by arginine and lysine [33]. Finally, the existence of two CrT mRNAs reported on Northern blots by many groups [28, 42, 50, 52, 57, 58, 61, 62] seemed also to support the existence of at least two different CrT species.

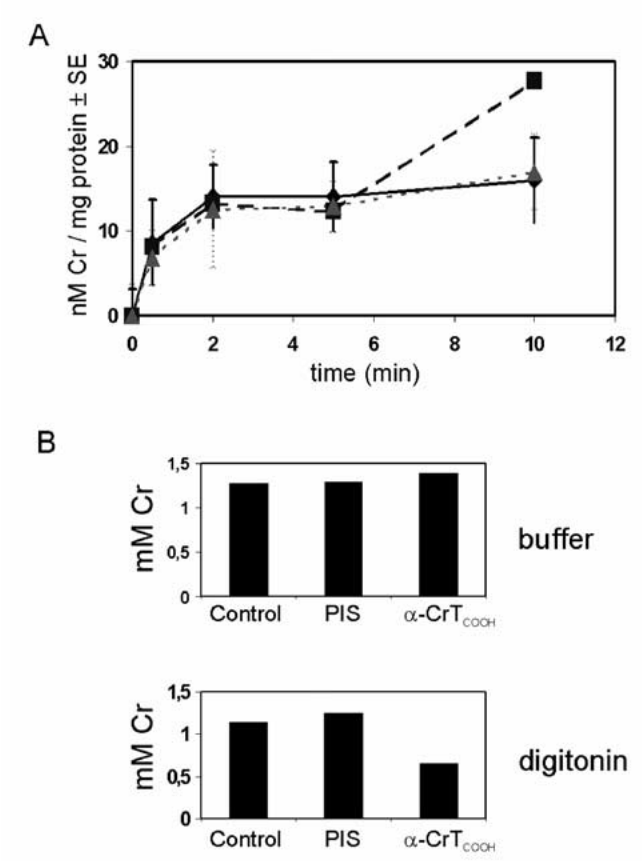

Fig. 4. (A) Mitochondrial Cr uptake. Time course experiments of Cr uptake (panel A) into isolated mitochondria from rat heart (■), liver (♦), and kidney (▲), measured at 20 mM of external Cr concentration in the presence of substrates for oxidative phosphorylation. The amount of Cr uptake is expressed as nM/mg total mitochondrial protein. (B) Inhibition of mitochondrial Cr uptake. Mitochondria were pretreated for 1 h at 22°C in 250 mM sucrose buffer, or in 250 mM Sucrose buffer containing 100 μg/mg^{-1} protein digitonin, together with rabbit anti-CrT C-terminal peptide serum, pre-immune-serum (PIS) or with the same volume of the corresponding sucrose buffer only (control). Subsequently, mitochondria were washed 3 times with 250 mM sucrose, 10 mM Hepes KOH, pH 7.4, 0.1 mM EGTA. The Cr transport assays were performed subsequently as in Fig. 4A and described in Experimental procedures.

Recently obtained insights and new developments

Having identified a new mitochondrial Cr uptake activity that is clearly distinct from the plasma membrane Cr uptake, we tried to identify the molecular identity of this mitochondrial Cr uptake protein. In an attempt to further characterize the two major mitochondrial CrT-related protein species, we started a proteomics approach to identify and partially sequence the two major proteins of 50 and 70 kDa, recognized by our anti-CrT antibodies. Since initial work showed similar results between our COOH- and NH_2-terminal antibodies, most subsequent data has been collected using the α-CrT_{COOH} antibody, unless otherwise stated.

Identification of α-CrT reactive proteins

Two dimensional gel electrophoresis, Western blot and advanced liquid chromatography nano-spray ionization tandem mass spectrometry (LC-ESI-MS/MS) were used for the identification of the different α-CrT_{COOH} immuno reactive proteins that were detected in enriched mitochondrial inner membrane preparations [70].

Proteins of the mitochondrial inner membrane were separated by 2D SDS-PAGE transferred to nitrocellulose membrane, stained reversibly with Ponceau and probed by Western blotting with our anti-CrT antibodies. Ponceau stain and Western blot signals were merged after digitalisation (Fig. 5A, red spots represent proteins visualized with Ponceau, blue spots represent the α-CrT_{COOH} Western blot signals). Spots that were recognized by α-CrT_{COOH} antibodies (Fig. 5A, spots 5–8), as well as other spots (Fig. 5A, spots 1–4), were cut out from paralleled SYPRO Ruby stained 2D gels (Fig. 5B) and then digested with trypsin. The eluted peptides were subsequently analysed by LC-ESI-MS/MS. The MS/MS spectrum of a representative protein, which was identified, is shown in Fig. 6. Eight protein spots in the region of interest could be identified as shown in Fig. 5B and listed in Table 1.

Using LC-ESI-MS/MS, the α-CrT_{COOH} immuno reactive proteins at 70 and 55 kDa in the mitochondrial inner membrane, which entered the 2D PAGE, were identified as three dihydrolipoamide acyltransferases (spots 5, 6 and 8 in Fig. 5 and Table 1), which are subunits of different α-keto acid dehydrogenase multi-enzyme complexes. The 70 kDa protein recognized by α-CrT_{COOH} is the dihydrolipoamide S-acetyltransferase (EC 2.3.1.12), a 70 kDa protein of the pyruvate dehydrogenase complex (PDH), known as the E2 component. Its peptide MS/MS spectrum is shown in Fig. 6. One of the 55 kDa α-CrT immuno reactive proteins was identified as the dihydrolipoamide S-acetyltransferase (EC 2.3.1.61), a 47 kDa protein of the α-ketoglutarate dehydrogenase complex (α-KGDH), also known as the E2 component. The other 55 kDa protein was identified as the dihydrolipoamide branched chain transacylase, a 53 kDa protein of the branched chain keto acid dehydrogenase complex (BC-KADH), also known as the E2 component. Each of these multi-enzyme complexes is a constituent of the α-keto acid dehydrogenase multi-enzyme complex. The fourth identified protein was aldehyde dehydrogenase (spot 7, Fig. 5 and Table 1). Four mitochondrial proteins (ATP synthase, HSP60, NADH dehydrogenase Fe-S protein, cytochrome c reductase core protein 1) listed in Table 1, which did not react with the α-CrT antibodies were also identified and used as a control for our methods.

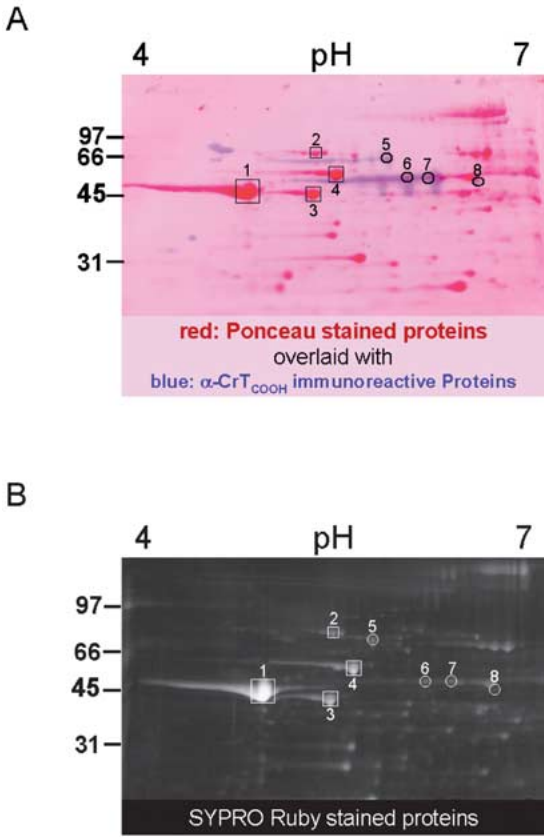

Fig. 5.

Fig. 5. Identification of α-CrT immuno reactive proteins. (A) 2D Western blot against CrT. Two mg of mitochondrial inner membrane proteins were separated by 2D SDS-PAGE and blotted on a nitrocellulose membrane. The proteins on the membrane were stained with Ponceau (red) and digitized. After destaining, the membrane was incubated with polyclonal rabbit α-CrT$_{COOH}$ antibody and CrT isoforms (blue) were visualized by goat α-rabbit coupled HRPO secondary antibodies. (B) SYPRO Ruby stained 2D Gel. Stained proteins marked with circles were excised and used for digestion, and peptides were then analyzed by LC-ESI-MS/MS.

Fig. 6. (A) Amino acid sequence of spot no. 5. Identified amino acid stretches are shown in red. (B) An overall score of the data corresponding to the protein no. 5. Below, different representation of locations within the protein sequences of the identified peptides (yellow) are shown. (C) Peptide MS/MS spectrum. A spectrum of one of the identified peptides is shown including the y- and b-ions.

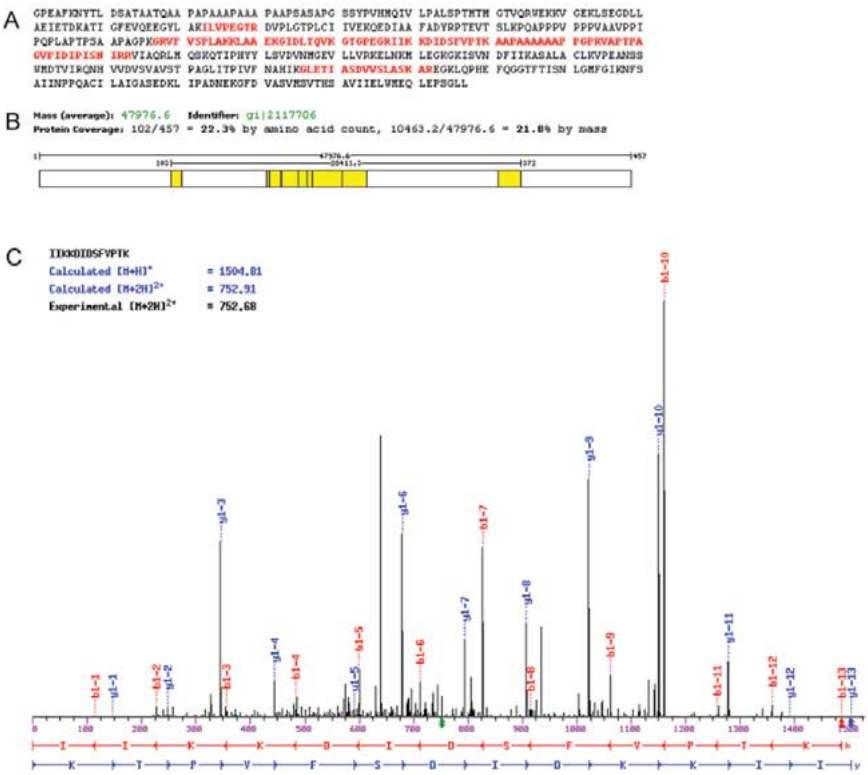

Fig. 6.

Table 1. Proteins identified by LC-ESI-MS/MS and MALDI-TOF

Spot No.	Protein (origin, if recognized)	Mass (Dalton)
1	ATP synthase β-chain (EC 3.6.3.14) mitochondrial, fragment (*R. norvegicus*)	56353
2	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (*Mus musculus*)	79417
3	60 kDa heat shock protein, mitochondrial precursor (*R. norvegicus*)	60955
4	Ubiquinol-cytochrome c reductase core protein 1	53420
5	Dihydrolipoamide S-acetyltransferase (EC 2.3.1.12) liver (*R. norvegicus*)	68764
6	Dihydrolipoamide S-succinyltransferase (EC 2.3.1.61) mitochondrial precursor (*R. norvegicus*)	47413
7	Aldehyde dehydrogenase (EC 1.2.1.3) mitochondrial precursos (*R. norvegicus*)	56488
8	Dihydrolipoamide branched chain transacylase, mitochondrial precursos (*Mus musculus*)	53160

Two mg of mitochondrial inner membrane proteins were separated by 2D SDS-PAGE, and stained with SYPRO Ruby (Fig. 5B). Spots were cut out of the gel, proteins were digested and resulting peptides prepared for LC-ESI-MS/MS or MALDI-TOF. Spots 5–8 were identified as being both α-CrT_{COOH} and α-CRT_{NH2} immuno reactive (Fig. 5A).

Examining the complete CrT sequence or the peptide sequences used for antibody generation (Fig. 1), no homology with other proteins was found using a BLAST search. Aligning N- and C-terminal CrT peptide sequences (NH_2-M-A-K-K-S-A-E-N-G-I-Y-S-V-S-G and P-V-S-E-S-S-K-V-V-V-V-E-S-V-M-COOH, respectively) with the amino acid sequences of the identified α-keto acid dehydrogenases, however, revealed some sequence homologies, which might also be recognised by the peptide antibodies, thus providing a rational for the observed cross-reactivity of our anti-CrT antibodies. Interestingly, aligning those peptides with HSP60 or ATP synthase gave the same amount of sequence homology, although neither HSP60 nor ATP synthase were recognised by our antibodies (Fig. 5A). Consequently, sequence homologies derived from two-dimensional alignments may not entirely explain the cross reactivity of our antibodies with the α-keto acid dehydrogenases.

The major α-CrT immunoreactive polypeptides are not genuine membrane proteins

Three spots that corresponded to α-CrT immuno reactive spots within the range of the upper 70 and the lower 55 kDa bands were identified by LC-ESI-MS/MS as dihydrolipoamide acyltransferases. These three enzymes are not integral membrane-spanning proteins, but all belong to PDH, BCKADH and α-KGDH multi subunit complexes that are bound to the inner matrix faced leaflet of the inner mitochondrial membrane [71].

Carbonate washing of mitochondrial membranes

The results derived from LC-ESI-MS/MS suggested that those proteins identified after 2D PAGE would be membrane-associated proteins rather than integral membrane-spanning proteins. After conventional one-dimensional SDS PAGE and Western blotting at least three different α-CrT signals were observed, whereas after 2D PAGE and Western blotting, we could observe only α-CrT reactive proteins with the size of 70 and 55 kDa. It is well known that highly hydrophobic proteins such as 12 membrane spanning domain-containing proteins, do not enter commercial pre-cast gels for iso-electric focusing (unpublished observation). We therefore concluded that the higher molecular weight signal identified in 1D Western blot at ~ 112 kDa, but not seen in the 2D PAGE, using the α-CrT antibodies, represents a typical membrane protein, for which the CrT is a good candidate. To test this hypothesis, mitochondrial membranes were washed with 100 mM sodium carbonate at pH 11. In that buffer system, integral membrane proteins cannot be washed off the membranes, whereas membrane-associated proteins should be removed [68, 69].

Rat liver and kidney mitochondria were sonicated and washed in sodium carbonate buffered at pH 11 and membranes were sedimented. Pellets and corresponding supernatant were separated by SDS-PAGE, Western blotted and tested by α-CrT_{COOH} (Fig. 7A). Control Western blots were also tested with α-VDAC antibodies. VDAC, a well-known membrane spanning protein of the outer mitochondrial membrane, remained with the mitochondrial membrane pellet. In contrast, the 55 and 70 kDa polypeptides partially separated from the membranes and were detected in both the supernatant and the pellet. Surprisingly the 112 kDa α-CrT reacting proteins did not detach from the mitochondrial membranes. We therefore concluded that this high Mr protein could be involved in the mitochondrial Cr uptake rather than those of dehydrogenase complexes (55 and 70 kDa).

Our carbonate washing experiments were fully supported by results derived from phase partitioning by Triton X-114 [67], whereby membrane proteins stay in the detergent phase, while soluble proteins are found in the aqueous phase (Fig. 7B). In these experiments both α-CrT_{COOH} immuno reactive 70 and 55 kDa protein band signals remained in the aqueous phase with a much weaker signal seen in the detergent phase. This indicates that both 70 and 55 kDa α-CrT_{COOH} immuno reactive proteins in mitochondria are not bona fide integral membrane proteins, although the membrane preparation [63] indicated a definite association with the mitochondrial inner membrane.

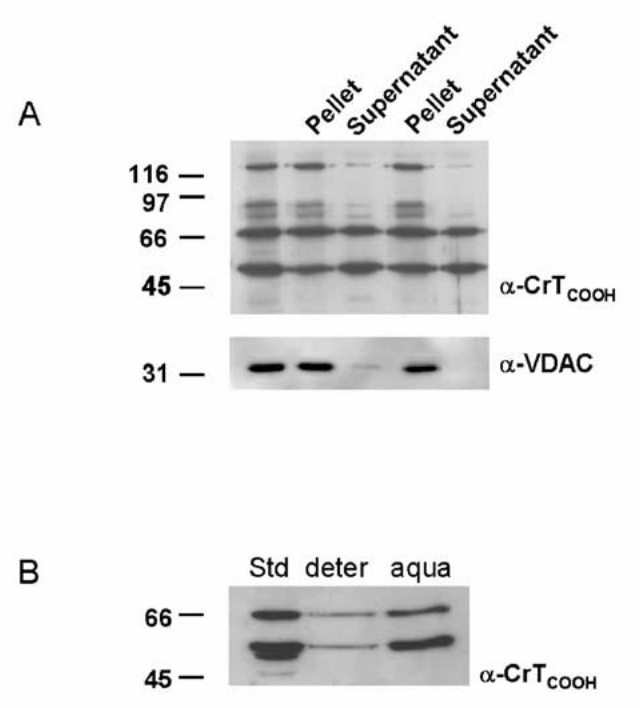

Fig. 7. (A). Western blot of rat liver mitochondria washed with 100 mM sodium carbonate at pH 11. After incubation and sonication mitochondria in carbonate, membranes were pelleted and compared to the aqueous supernatant. After separation by SDS-PAGE and Western blotting, membranes were probed with α-CrT$_{COOH}$ and reprobed with α-VDAC antibodies. (B) Phase Partitioning of α-CrT$_{COOH}$ immuno reactive protein bands. Seventy-two µg mitochondrial proteins were incubated in 2% Triton X-114. After centrifugation, the pellet containing integral membrane proteins and detergent (deter), and the supernatant containing aqueous proteins (aqua) were precipitated and compared to 20 µg mitochondrial proteins (Std). After separation and blotting onto a nitrocellulose membrane, the fractions were probed with α-CrT$_{COOH}$ antibodies.

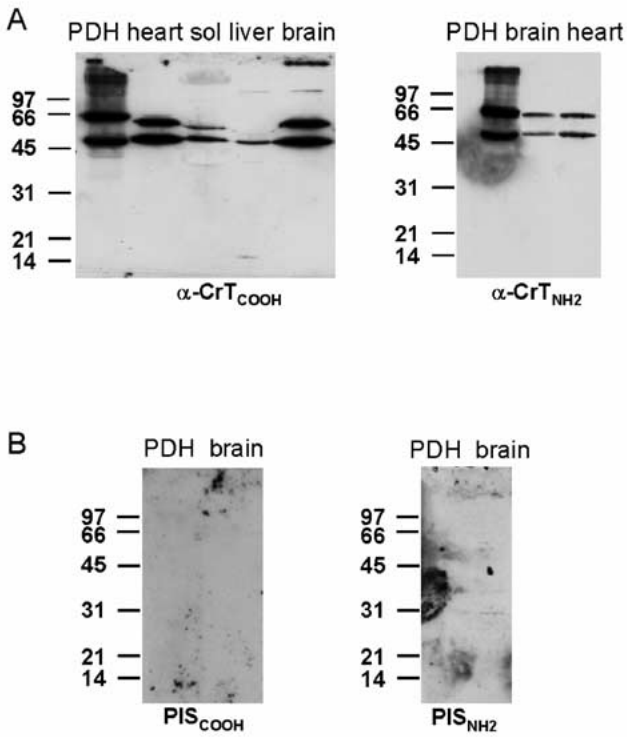

Fig. 8. (A) Comparison of purified PDH to rat heart and soleus muscle by Western blot analysis. Three µg PDH, rat heart and soleus muscle, each 20 µg, were separated by SDS PAGE and Western blotting. Membranes were probed with polyclonal rabbit α-CrT$_{COOH}$ and α-CrT$_{NH2}$ antibodies both 55 and 70 kDa signal were visualized. (B) Purified PDH and brain extracts run on Western blots as above and probed with preimmune serum (PIS) from rabbits injected with either the COOH- or NH$_2$-terminal peptides. Note the cross reactivity of both α-CRT antibodies with PDH (A) and absence of reaction with pre-immune serum (B).

Comparison with adenine nucleotide translocase (ANT) as a genuine transmembrane protein of the inner mitochondrial membrane, and miCK as a more soluble protein in the intermembrane space, being weakly associated with both mitochondrial membranes, indicated also in isolated kidney mitochondria that the immuno reactive 55 and 70 kDa 'CrT' protein bands do not have the behaviour of membrane-attached proteins rather than of integral transmembrane proteins (not shown).

α-CrT$_{COOH}$ reactivity with pyruvate dehydrogenase enzyme complexes

A major component of the 70 kDa immuno reactive protein, thought to be the CrT$_{70}$ isoform, was identified here as the E2 component of the PDH. To verify the result derived from LC-ES-MS/MS, 3 µg of PDH purified from porcine liver (Sigma) was compared by Western blot analysis to rat liver homogenate, brain, heart and soleus muscle (Fig. 8A). In this purified PDH fraction, our α-CRT antibodies detected two major polypeptide signals, one at about 70 kDa, and the other at about 55 kDa. Both signals in the PDH fraction, when compared to the signals obtained with brain, liver, heart and soleus muscle, support our result derived from LC-ESI-MS/MS. Obviously, our α-CrT$_{COOH}$ and α-CrT$_{NH2}$ antibodies bind to the E2 component of PDH, and probably additionally to one of the other E1 or E3 components of PDH, as indicated by the 55 kDa immuno reactive protein band. As a standard control Western blots of purified PDH and brain extract, show no signals with the preimmune sera of either the COOH- or NH$_2$ terminal antibodies (Fig. 8B).

Protein identified in plasma membrane and red blood cells using α-CrT$_{COOH}$ antibody

To date, a number of studies using our CrT antibodies have reported the presence of CrT in whole cell extracts of human [17, 54, 72, 73] skeletal muscle. It is now evident that the

proteins described in these studies are predominantly of mitochondrial localisation and hence detecting proteins associated with the E2 component of PDH, as described in the beginning of this section. To examine whether a protein localised exclusively to the plasma membrane in both rat and human skeletal muscle was also detectable using our α-CrT_{COOH} antibody, enriched plasma membranes were isolated using the giant vesicles method. A single immunoreactive band was detected on a Western blot in both rat (Fig. 9B lanes 1–3) and human (data not shown) skeletal muscle, which differed to the more abundant proteins that have been found to be of mitochondrial origin, and seen in whole muscle extracts (Fig. 9A, lanes 1 and 2 and Fig. 9B, lane 4). No mitochondrial contamination of the plasma membrane fractions was seen in rat skeletal muscle as assessed by immunoprobing for cytochrome oxidase (Fig. 9B, bottom). Therefore, this band may represent the high affinity, low Km CrT expected to be localised to the plasma membrane. In giant vesicles prepared from rat and human skeletal muscle, an immunoreactive band was identified migrating to the same point on the gel using either our α-CrT_{COOH} or the commercially available CrT antibody (Alpha Diagnostics – AD, 10 μg.ml^{-1} in blocking buffer, Fig. 9C). Examination of the protein isolated from RBC membranes, reveals a single band of ~ 68 kDa using our α-CrT_{COOH} antibody (Fig. 9A, lanes 3–5). A single band has also been described previously in rat red blood cells using a similar method of extraction [29]. We have preliminary evidence that the immunoreactive band seen in human RBC using the α-CrT_{COOH} Ab is slightly different in apparent molecular weight to the skeletal muscle plasma membrane CrT protein, however further work is required to determine this. Nevertheless, in both rat and human skeletal muscle and RBC, it is apparent that a single membrane associated protein is detected using the α-CrT_{COOH} antibody, which does not correspond to either of the predominant bands at ~ 55 and 70 kDa that have now been discovered to be proteins associated with the E2 component of PDH. It is possible that this protein is the CrT protein, responsible for over 90% of Cr uptake into muscle. Sequencing data, however, is vital to clarify this.

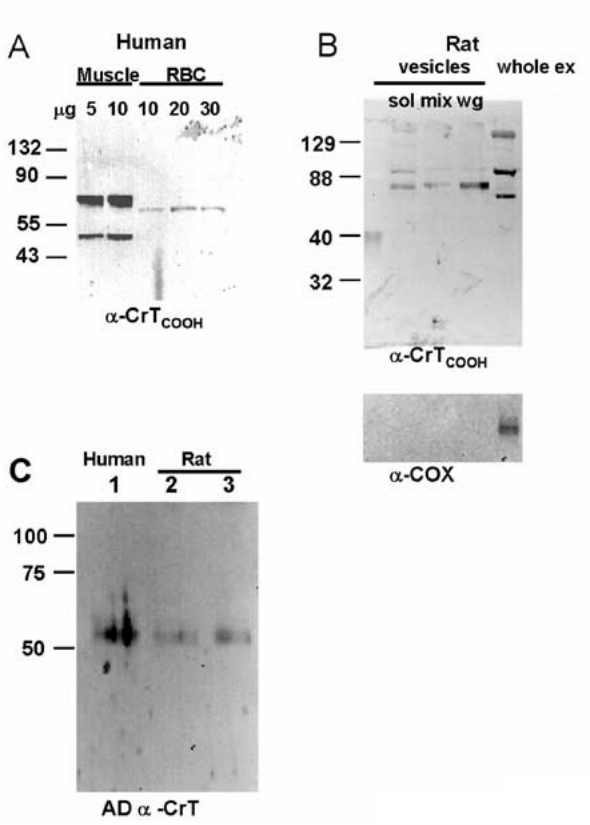

Fig. 9. (A) Human skeletal muscle extracts (lanes 1 and 2) and proteins from red blood cell membranes (lanes 3–5) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were probed with polyclonal rabbit α-CrT_{COOH} antibodies. The amount of protein loaded into each lane is indicated. (B). Rat skeletal muscle giant vesicle plasma membrane (lanes 1–3, soleus, mixed muscle and white gastrocnemius, respectively) and whole muscle extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were probed with polyclonal rabbit α-CrT_{COOH} antibodies (top) and cytochrome C oxidase (α-COX, bottom). (C) Giant vesicle plasma membrane preparations from human (lane 1) and rat (lanes 2 and 3) skeletal muscle were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were probed with affinity purified rabbit α-CrT (Alpha Diagnostics, AD). In all figures a molecular weight marker is indicated on the left side of the figure.

Discussion

A word of caution concerning the major immunoreactivity of α-CrT antibodies

Several research groups have independently produced antibodies against the $COOH^-$ and/or NH_2-terminus of the CrT [17, 43, 44, 74], as well as the commercially available antibody. All antibodies recognise at least one protein with a molecular mass of approximately 55, 70 and over 100 kDa in whole cell extracts. At least our antibodies [17, 29, 33, 54] recognise mitochondrial proteins also at 55 and 70 kDa. We have shown that the main part of these mitochondrial proteins is clearly water-soluble and not membrane spanning. This is supported by carbonate washing and phase partitioning experiments using the inner mitochondrial membrane preparations. With modern protein detection methods these were identified as different E2 components of the α-keto acid dehydrogenase multi enzyme complexes, namely PDH, BCKADH and α-KGDH. This goes nicely in hand with data seen with confocal microscopy, electron microscopy and biochemical fractionation which all provide evidence that these proteins are localised within mitochondria. In addition we have shown, that these antibodies recognize a protein

within muscle plasma membrane, which is distinct from those recognized in mitochondria, shown in Fig. 9 and [28, 29]. However, so far we have not succeeded in identifying this plasma membrane CrT protein by mass spectrometry or other molecular biology techniques.

Upon close inspection of the PDH subunit sequences, very short stretches of sequence homology are seen with our N-terminal CrT peptides. Homology search with M-A-K-K-S-A-E-N-G-I-Y-S-V-S-G reveals only CrT. However, an alignment of M-A-K-K-S-A-E-N-G-I-Y-S-V-S-G with the Dihydrolipoamide acetyltransferase (EC 2.3.1.12) sequence shows homology of M-A-K-K-S-A-E-N-G-I. But only a homology search with the fused consensus sequence M-A-K-K-S-A-A-E-N-G-I reveals CrT and Dihydrolipoamide acetyltransferase (EC 2.3.1.12) with the same probability. However, the homology searches under the same conditions with the C-terminal CrT sequence P-V-S-E-S-S-K-V-V reveals only CrT and GABA transporter. Aligning both termini with the amino acid sequences of the identified proteins might explain the cross-reactivity. However, after aligning both termini with HSP60 one would expect also a cross reactivity with this protein, which is experimentally not observed (Fig. 5A). These data may explain the very unexpected cross-reactivity, the chances of which, however, would appear to be marginal at first glance. However, the fact that our antibodies clearly cross-react with purified PDH is a clear argument that there is indeed cross-reactivity of our anti-CrT antibodies with this enzyme.

Interestingly, in humans suffering from primary biliary cirrhosis, auto-antibodies against PDH have been detected. Primary biliary cirrhosis is a chronic idiopathic liver disease characterized by the specific destruction of intrahepatic bile ducts [75]. This disease is characterized by the presence of anti-mitochondrial antibodies in patient sera, and many lines of evidence suggest the involvement of an autoimmune response in the pathogenesis of primary biliary cirrhosis. The major mitochondrial antigens recognized by anti-mitochondrial antibodies include the constituents of α-keto acid dehydrogenase complexes, namely the PDH, BC-KADH and the α-KGDH. It has been demonstrated that the E2 components of PDH, α-KGDC and BC-KADH are the major determinants of the anti-mitochondrial antibodies in the sera of patients with primary biliary cirrhosis [75–80]. These considerations may explain the presence of auto-PDH antibodies in our rabbits, although they were clearly negative before immunisation as seen in Fig. 8 B, that is, immunisation of the rabbits with these peptides could have led to elicitation of auto-antibodies against PDH.

On the other hand it seems very unlikely that this should happen in several laboratories to the same extent unless the injection of CrT related sequences to generate antibodies against this protein, or the appearance of these antibodies in the animals bloodstream, by some mechanism of cell damage, would lead to the formation of auto-anti-PDH antibodies. This would indicate that anti-CrT antibodies in the blood stream would do some cell damage in the injected animals.

Thus, although our anti-sera and probably also those of other laboratories clearly contain some antibodies against genuine CrT, the most prominent signals on whole cell extracts with these antibodies are always at 55 and 70 kDa. These latter two proteins are more likely to represent subunits of PDH and other keto acid dehydrogenase's and are unlikely to have anything to do with genuine CrT unless unknown CrT species would share the same Mr of 55 and 70 kDa, as discussed above. However as shown with purified plasma membrane, which is free of mitochondria protein, a third protein is detected. This protein is different in its size from those detected in mitochondria and may represent plasma membrane CrT (Fig. 9) [28, 29, 54].

Thus, all quantification results of CrT obtained so far with the different anti-CrT antibodies, either directed against synthetic peptides or fusion proteins, should be viewed with caution, due to the fact that the prominent signals at 55 and 70 kDa are due to cross-reactivity of the anti-CrT antibodies with PDH. The latter question remains elusive until a clear-cut identification of CrT polypeptides is achieved either from immunoprecipitated material or by 1D SDS-PAGE/mass-spectrometer analysis.

Explanation of the unexpected results by the new data

Assuming now that the two major polypeptides recognized by the anti-CrT antibodies are not related to CrT, but represent two subunits of mitochondrial keto acid dehydrogenase, peripherally attached to the inner mitochondrial membrane (matrix side), this would explain the preponderance of the 55 and 70 kDa polypeptides seen in heart and liver which are both tissues with high mitochondrial volume content. Additionally, ours and the work of others showing the fibre type dependence of the CrT in both rat [54, 55] and human [73] whole skeletal muscle needs to be re-examined.

The prominent localization signals both by immunofluorescence staining [29, 33, 54, 73], as well as be immunoelectron microscopy [33] may now be explained by cross-reactivity of the anti-CrT antibodies with mitochondrial keto acid dehydrogenase's. However, on the other hand, mitochondria have been shown recently to possess a Cr uptake system, by simple Cr transport assays, independently of the use of antibodies [33, 35]. This Cr uptake, although showing low affinity for Cr, was inhibited by compounds reacting with sulfhydryls, as well as by our α-C-terminal CrT antibody. However, additional competition experiments have to be performed concerning the specificity of mitochondrial Cr uptake, for it could be, as indicated by its high Km for Cr of 15 mM, which corresponds approximately to the internal free Cr concentra-

tions in muscle, that this transporter may not be exclusively for Cr. As indicated by the competitive effect of arginine and lysine on mitochondrial Cr transport [33] it may also transport other related compounds into mitochondria. Again, interestingly, our α-CrT peptide antibodies have been shown to interfere with mitochondrial Cr uptake so that it has to be assumed that these sera indeed contain α-CrT activity, the question remaining is whether this activity is related to the two prominent signals of 55 and 70 kDa or to some of the low-abundance signals, specifically to a faint immunoreactive high-Mr polypeptide in the range of 112 kDa that is often seen on Western blots of isolated mitochondria. It is to be expected that the amount of plasma membrane CrT expressed in cells may be very low and thus the signals of genuine CrT seen when enriched plasma membrane is examined may be very weak on Western blots when whole tissue extraction methods are used. Unfortunately though, it seems, that the 55 and 70 kDa signals were so clear-cut and strong that the entire community was mislead to believe that these were the important signals. To sort this multifaceted enigma, which is holding up the entire CrT research community, a new series of experiments is necessary to identify the genuine CrT isoforms in tissues and in sub cellular fractions.

Conclusions and outlook

Based on our results and those of other groups in the field, we still hold to the general scheme (Fig. 10) that besides the high-efficiency plasmalemma CrT [26–28, 42, 44, 51, 58, 81–83] there exists an additional low affinity high K_m Cr uptake mechanism in mitochondria [29, 33].

In line with the above observation, *in vivo* isotope tracing studies with labeled Cr have shown that CK does not have access to the entire cellular Cr and PCr pool(s) [84], which indicates that intracellular Cr and PCr pools may exist that are not in immediate equilibrium with one another. Such interpretations are in agreement with a number of ^{31}P NMR magnetization transfer studies [85], as well as with recent ^{1}H NMR spectroscopy data [86], where monitoring the Cr and PCr levels in human muscle pointed to the existence of a pool of Cr that is not NMR 'visible' in resting muscle, but appears in NMR spectra of muscle in ischemic fatigue or post mortem [86].

As far as the identification of CrT species is concerned, the exact protein biochemical nature of these polypeptides, – this holds true also for the plasmalemma, as well as for the mitochondrial Cr uptake, remains elusive until clear-cut identification and sequence data are available. Until such data are available, we suggest, in light of the facts demonstrated above, that α-CrT antibodies, if at all, should only be used with these precautions in mind. CrT-quantification as a function of certain interventions, e.g. Cr supplementation or Cr depletion, obtained by α-CrT Western blot quantification have to be considered with caution and need to be re-evaluated in the future, when the molecular identity of CrT isoforms are known.

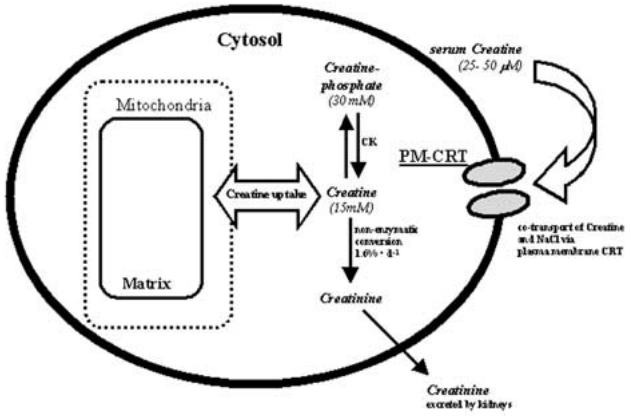

Fig. 10. General scheme of cellular Cr transport. A compartmentation of three Cr pools, that is, in blood serum, cytosol, and mitochondria, are shown. These pools are interconnected via two different Cr uptake mechanisms, the high affinity (low *Km*) plasma membrane Cr transporter (*PM-CrT*) [28, 29] and the low affinity (high *Km*) mitochondrial Cr transport [33]. The high Cr concentration gradient (300–600 fold) between serum and cytosol was maintained using an outside-in-directed NaCl gradient, which was used to co-transport Cr across the plasma membrane against a huge Cr concentration gradient. Two-thirds of the Cr that has entered the cytosol becomes trans-phosphorylated by the creatine kinase reaction to PCr, which is not a substrate of the plasma membrane CrT . The strict discrimination of the plasma membrane CrT between Cr and PCr leads to entrapment of PCr inside the cell, since PCr escapes equilibration. This thermodynamically facilitates further Cr uptake by the plasma membrane CrT and helps maintaining the enormous total Cr concentration gradient (600–1,000 fold) across the plasma membrane. The mitochondrial CrTs present in the inner mitochondrial membrane mediate Cr transport into mitochondria. Biological membranes, impermeable for Cr and PCr, not being in equilibrium with each other via diffusion, separate all three Cr compartments. These Cr transporters are likely to be regulated to mediate the exchange and channeling of Cr between these independent compartments, which may differ in their total Cr content, as well as in their PCr/Cr ratios according to their specific metabolic needs. The concentrations of PCr (30 mM) and Cr (15 mM) given here are those of a glycolytic fast twitch skeletal muscle [13, 16], with a typically very high Cr content. Adopted from [33].

Note added in proof

Identification of the a-CrT reactive, high molecular weight protein

As seen in Figs 2, 3 and 7, there was another > 100 kDa polypeptide reacting with our α-CrT_{COOH} serum. Carbonate washing experiments, similar to those shown here in Fig. 7,

showed that this high Mr polypeptide was resistant to high-pH extraction and remained with the inner mitochondrial membrane fraction. Therefore, it was tempting to speculate that this high Mr protein must be a genuine transmembrane protein, in contrast to both the 55 and 70kDa proteins (Fig. 7). While this work was in press, the high molecular weight region containing the > 100 kDa polypeptide in question was subsequently cut out from a Coomassie Blue stained gel, followed by trypsin digestion and analyzed by MALDI-TOF mass spectrometry. Based on the molecular mass determinations of the resulting peptides, this polypeptide was unambiguously identified as carbamoyl-phosphate synthase (CPS). The amino acid sequence of CPS was covered to 24% by the peptides, and the identification score reached 140. CPS was identified from two different mitochondrial membrane preparations and two samples each were analyzed, giving the same results. CPS is a highly hydrophobic mitochondrial enzyme that is associated with the MIM [88]. A hydrophobicity plot for CPS revealed two transmembrane domains, which would indicate that some portion of CPS is exposed on the outer side of the MIM, a region possibly recognized by the α-CrT antibody [33]. Thus, after having eliminated the 55, 70 and > 100 kDa polypeptides as candidates for CrT, there only remains one α-CrT-reactive polypeptide of approximately 60–65 kDa, depending on the tissue and the species, that is very likely to constitute the genuine CrT protein. This protein, however, is not present in mitochondria, but can be detected in plasma membrane preparations, e.g. in isolated sarcolemma vesicles from muscle (see Fig. 4 in [29] and Fig. 9B in this work) and from erythrocytes (Fig. 9A in this work). We thus have to conclude, that mitochondria, do not contain a protein related to genuine CrT. However, mitochondria can take up significant amounts of Cr in a highly reproducible fashion [33] by an unidentified transport mechanism [29]. This Cr uptake was slightly inhibited by FCCP, NEM and α-CrT antibodies, as well as by arginine [33], but surprisingly not affected by β-GPA, a well-known specific inhibitor of CrT [29]. The absence of inhibition by β-GPA, but some inhibition by arginine, may argue for a rather unspecific transport of Cr into mitochondria, e.g. by an amino acid transporter or alike. Most recent data using high-resolution magic angel spinning (MAS) 1H-NMR indicate the existence of a mitochondrial pool of Cr forming a metabolic compartment (Bollard *et al*. 2003). However, this mitochondrial Cr is largely NMR invisible, that is, little Cr signal is seen in intact mitochondria, but upon extraction of the same mitochondria, the Cr signal suddenly appears [89]. Recent work from Saks *et al*. with skinned muscle fibers indicate that this Cr is not held inside mitochondria upon skinning [90]. Therefore, it can be envisaged that this Cr may be in the intermembrane compartment where also mitochondrial mtCK is located.

Experimental procedures

Materials

If not otherwise stated all chemicals were purchased from Sigma Chemical Co. (USA). Male Wistar rats (250–300 g) were purchased from BRL (Switzerland).

Tissue extracts and isolation of mitochondria

Male Wistar Rats (3–4 month of age) were anesthetized with diethyl ether and killed by cervical dislocation. Tissue of liver, skeletal and cardiac muscle, kidney, brain, spleen and testis were taken and immediately transferred to ice-cold buffer. Liver, brain, and kidney tissues were homogenized by a teflon/glass potter (Braun-Melsungen, Germany), whereas skeletal and heart muscle was homogenized by a Polytron mixer in 40 ml HEPES-sucrose buffer containing 250 mM sucrose, 10 mM HEPES-HCl pH 7.4, 0.5% BSA (essentially free of fatty acids) and 1 mM EDTA. The homogenate was centrifuged for 10 min at 700 × g to remove heavy debris as platelets and nuclei. An aliquot from the supernatant was taken for further analysis as the total tissue extract. The supernatant was centrifuged for 10 min at 7,000 × g and the resulting supernatant was stored for subsequent analysis as the soluble cytosolic fraction, while the pellet containing mitochondria was resuspended in 60 ml 250 mM sucrose, 10 mM Tris/HCl pH 7.4, 100 μM EGTA, 25% Percoll™ (Amersham Pharmacia Biotech, Sweden) and centrifuged for 35 min at 100,000 × g. Percoll™ fractions containing highly purified mitochondria were washed twice with 250 mM sucrose, 10 mM HEPES-HCl pH 7.4, 100 μM EGTA by centrifugation at 7,000 × g for 10 min. Washed mitochondria were then recovered from the pellet and resuspended in 200 μl of the washing buffer.

Western blotting

Extracts were separated in 10-12 % polyacrylamide SDS-gels and transblotted onto a nitrocellulose membrane (Schleicher and Schuell, Germany; Geneworks, Australia). The membrane was blocked with 5% fat-free milk powder in TBS(T) buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, (0.05% Tween)) for 1 h at room temperature. After washing for 30 min, membranes were incubated with 1:1,000–5,000 diluted anti-CrT peptide antibody in TBST buffer for 2 h at room temperature. After washing with TBS(T) buffer, the blot was incubated again with a 1:10,000 dilution of goat HRP-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Sweden; Silenus, Australia). The immunoreactive

bands were visualized using the Renaissance Western Blot Chemiluminescence Reagent Plus Kit (NEN, USA). Images were collected and analysed using a Kodak 1D Image Station.

Isolation of outer and inner membrane from rat liver mitochondria

The isolation of the mitochondrial membranes was done as previously described [63]. Briefly, rats were anaesthetized with diethyl ether and killed by cervical dislocation. The liver was taken and immediately transferred to ice-cold homogenization buffer (250 mM sucrose, 10 mM Hepes-KOH pH 7.4, 0.5% BSA, 1 mM EDTA) and freed from fat and connective tissue. The tissue was homogenized using a glass-teflon potter in homogenization buffer at 0°C. Nuclei and cell debris were pelleted by centrifugation at 700 × g for 10 min, and crude mitochondria were pelleted from the post-nuclear supernatant by centrifugation at 7,000 × g for 10 min. Enriched mitochondria were resuspended in 250 mM sucrose, 10 mM Hepes-KOH, pH 7.4, 0.1 mM EGTA and purified in a 25% Percoll™ density gradient. Highly enriched mitochondria were carefully collected from the gradient and washed twice in sucrose/Hepes buffer. Protein determination was performed with the BCA Kit (Pierce, USA). Fifty mg of mitochondria were then resuspended in 6 ml of 10 mM KH_2PO_4 buffer, pH 7.5 at 0°C. After 15 min to allow swelling, 6 ml of 10 mM KH_2PO_4 containing 30% sucrose, 30% glycerol, 10 mM $MgCl_2$, 4 mM ATP was added. After 60 min of incubation at 0°C to allow shrinking the mitochondrial suspension was treated with sonic oscillation using a Brandson sonicator. A first crude inner membrane fraction was pelleted at 12,000 × g for 10 min. The pellet was resuspended in 3 ml 10 mM KH_2PO_4 buffer. Pellet and supernatant were layered onto a discontinuous sucrose gradient consisting of 51, 37 and 25% sucrose and centrifuged in a swinging bucket rotor (SW 40) at 100,000 × g for at least 12 h at 4°C. The clear top of the gradient contained the soluble protein fraction, the 25–37% interphase contained the light outer membrane subfraction, and the 37–51% interphase contained the pure inner membrane subfraction. The membrane fractions were collected carefully from the gradient, diluted 1:10 in sucrose/Hepes buffer, and pelleted at 100,000 × g, 4°C for 1 h. The pellets were solubilized in sucrose/Hepes and analyzed.

Succinate dehydrogenase (SDH) enzyme assay

The specific SDH activity was measured indirectly via the increase in the absorption of reduced cytochrome c at 550 nm (ε = 19 mM^{-1} cm^{-1}). Ten µl sample was incubated in 1000 µl 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 7.4, 100 µM cytochrome c, 1 mM KCN, 2.5 mM succinate as described [64].

Plasma membrane giant vesicles preparation

Plasma membrane giant vesicles were prepared from rat (~ 100 mg – soleus, SOL; ~ 500 mg white gastrocnemius; WG and mixed muscle) and human (~ 70–130 mg) skeletal muscle samples as previously described [65]. All procedures were undertaken at room temperature, unless otherwise indicated. Freshly extracted samples were rinsed in KCl-Hepes buffer (140 mM KCl, 10 mM Hepes, pH 7.4) and finely cut lengthwise. Muscle pieces were placed in digestion solution (400 µl × 100 mg^{-1} tissue KCl-Hepes buffer with 80 ml collagenase; 1 ml protease inhibitor cocktail; 5 ml phenylmethylsulfonyl fluoride) and incubated at 34°C for 60–90 min. Collagenase activity was ceased by the addition of KCl-Hepes-EDTA (10 mM) and vesicles collected with 2–4 subsequent rinses with KCl-Hepes-EDTA buffer, until 10 ml (human and SOL) or 30 ml (WG and mixed) were collected. A solution of 11% KCl (1.4 M) in Percoll (Amersham Pharmacia Biotech, Sweden) was prepared and added to the muscle solution (2.2 ml × 10 ml^{-1} muscle solution) and mixed by inversion. The mixture was aliquoted into plastic tapered centrifuge tubes (~ 6 ml × $tube^{-1}$), and ~ 2 ml Nycodenz (4% in KCl-Hepes buffer) was carefully layered on top, followed by ~ 1 ml KCl-Hepes-EDTA buffer. Samples were then spun (60 × g, 45 min, 23°C with the brake off). Vesicles were collected from the Nycodenz layer and washed with an equal volume of KCl-Hepes buffer and spun (900 × g, 10 min, 23°C). The supernatant was aspirated from the pellet of vesicles, and the integrity of the vesicles confirmed under a microscope. Vesicles were then resuspended in a small volume of storage buffer (4% SDS in 10 mM Tris, 1 mM EDTA) and stored at –20°C until the Western blots were performed.

Red blood cell preparation

Blood (~ 8 ml) was collected into heparinized tubes and gently mixed before being aliquoted into 1.5 ml microfuge tubes and spun (~ 14,000 × g, 10 min, 4°C). The supernatant was removed and the pellet resuspended in 500 µl isotonic buffer (5 mM Na_2HPO_4 in 0.9% NaCl, pH 8), spun as above, and the supernatant removed. This washing step was repeated 3 times. 200 µl aliquots of whole red blood cells (RBC) were then collected from beneath the surface of the pellet, suspended in 500 µl solubilisation buffer (150 mM NaCl, 20 mM Hepes, pH 8, 1 mM EDTA, 0.1% Triton-X100) and shaken vigorously for 15–25 min at room temperature to release the membrane proteins. The samples were then spun as above and the supernatant collected and stored at –80°C until analyses.

Measurement of Cr transport into mitochondria

Cr uptake assays were performed using highly enriched, Percoll™ gradient purified mitochondrial preparations (adjusted to 10 mg × ml^{-1} protein concentration). The reaction was started by the addition of 10 µl of the mitochondria suspension to 90 µl transport buffer (10 mM Tris/HCl, pH 7.4, supplemented with 250 mM sucrose, 20 mM Cr, and 5 µCi × ml^{-1} [^{14}C]-Cr (American Radiolabeled Chemicals, USA), 10 µCi × ml^{-1} [^{3}H]-sucrose, 5 mM succinate/Tris, 2 µM rotenone, 2 mM $MgCl_2$, 10 mM Pi/Tris, 100 µM EGTA and 2 mM ADP) at RT. The pellet was solubilized in 100 µl of 1% SDS and counted in 4 ml scintillation cocktail 'Ultima Gold XR' (Packard) in a Packard 1500 Tri-Carb™ liquid scintillation counter. Double-isotope measurement settings were 0–18 eV for the [^{3}H]-isotope and 18–256 eV for the [^{14}C]-isotope. The amount of Cr uptake was calculated as the difference of total Cr subtracted by the Cr present in the space that was also accessible to sucrose. In the experiments with the anti-CrT antibodies, mitochondria (100 µg/mg mitochondrial protein) were preincubated for 1 h at 22°C either in 250 mM sucrose ± 100 µg digitonin × mg^{-1} protein alone or in 250 mM sucrose ± 100 µg digitonin × mg^{-1} protein together with anti-CrT_{COOH} or preimmune serum (at 1:100 final dilution). Subsequently, mitochondria were washed 3 times with 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA and finally incubated with Cr transport buffer and uptake measured as described above. Mitochondrial volume was estimated with the distribution of tritium labelled water and ^{14}C labelled sucrose in mitochondrial pellets, treated in parallel as described above. The final Cr concentration within mitochondria was estimated with the volume measured as 2 µl mg^{-1} mitochondrial protein.

Two dimensional gel electrophoresis

Isoelectric focusing (IEF) was performed according to [66] and the manufacturer's instruction of the IPGphor (Amersham Pharmacia Biotech, Switzerland). Precipitated protein samples were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 60 mM DTT). Samples were sonified in a water bath for 5 min at 30°C, mixed 30 min at 30°C, and centrifuged for 1 min at 13,000 × g. The IPG strip (Immobiline™DryStrip, 18 cm, Amersham Pharmacia Biotech, Switzerland) was transferred to the focusing tray containing the resuspended sample, and covered with 1–2 ml mineral oil (Amersham Pharmacia Biotech, Switzerland). Isoelectric focusing (IEF) was performed by a five step program: 50 V for 10 h, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 8,000 V for 12 h resulting in 92,000 Vh (20°C, 50 µA/Strip). Upon completion of IEF, the IPG strip was incubated for at least 15 min in equilibration buffer (50 mM Tris/HCl pH 8, 8.6 M urea, 30% glycerol, 2% SDS, 60 mM DTT). The second dimension was done in a similar manner to that described under SDS-PAGE. SYPRO® Ruby stained gel spots were cut out of the 2D gel and washed in 80 µl of 0.1 M NH_4HCO_3, for 5 min. An equal volume of 100% acetonitrile (ACN) was added. The gel particles were washed by that procedure 3 times. The gel pieces were dehydrated with 100 µl ACN, and dried in a Speed Vac for 5 min. For reductive alkylation, gel particles were rehydrated in 10 mM DTT in 0.1 M NH_4HCO_3, and incubated for 30 min at 56°C. The excess solution was removed, and the particles were dehydrated with ACN, 5 min. After removing ACN, gel particles were incubated for 20 min in 55 mM iodoacetamide in 0.1 M NH_4HCO_3 in the dark at room temperature to alkylate the proteins. After removing iodoacetamide solution, the particles where washed with 200 µl of 0.1 M NH_4HCO_3, 15 min. The particles were shrunken with ACN, dried under vacuum for 5 min, and put on ice.

In-gel trypsinisation

Samples were rehydrated in the trypsin solution (12.5 ng/µl Trypsin (Promega, Switzerland) in 50 mM NH_4HCO_3) for 30–45 min at 4°C. The remaining trypsin solution containing excess trypsin was removed, and the particles were briefly washed with 50 mM NH_4HCO_3. Subsequently, 50 mM NH_4HCO_3 was added to the gel pieces, just enough to keep them covered during the over night digestion (16–20 h at 37°C). The 'digest solution' containing some tryptic peptides was transferred to a fresh 0.5 ml tube and saved. For basic extraction of peptides 15 µl of 25 mM NH_4HCO_3 was added again to the gel pieces, and incubated for 15 min at 37°C while shaking. After spinning down, an equal volume of ACN was added. After shaking for 15 min at 37°C and sonication for 5 min in a sonication bath at 37°C, liquid was spun down and collected. Fifty µl of 5% formic acid was added to the gel particles, and these were incubated for 15 min at 37°C while shaking. After spinning down, an equal volume of ACN was added. After shaking for 15 min at 37°C and sonication for 5 min in a sonication bath at 37°C, liquid was spun down and pooled together with the other extracted peptides. Pooled samples were centrifuged at 10,000 × g for 10 min, and the supernatant was frozen in liquid nitrogen. Samples containing the collected extracted tryptic peptides were dried in a Speed Vac, dissolved in 7 µl of 0.5% acetic acid, and applied to the Liquid Chromatograph.

Mass spectroscopy

Liquid-Chromatograph-Electro-(nano)Spray-Ionization-MS/MS (LC-ESI-MS/MS) with Ion Trap technique was performed

at the Functional Genomic Center Zurich (Switzerland) using a LCQ DECA XP (Thermo Finnigan, USA). Data derived from LC-ESI-MS/MS spectrometry were used in Sequest®, a database screening program accessible only in the Functional Genomic Center Zurich (Switzerland). MALDI-TOF (Applied Biosystems, USA) was performed in the Protein Service Lab at the ETH Hoenggerberg. Dried peptides were dissolved in 3 µl ACN : 0.1 TFA, 2:1 (trifluoro acetic acid, Fluka, Switzerland). 2-, 5-dihydrobenzoic acid (DHB, Fluka, Switzerland) served as matrix, which was dissolved in 100 µl of ACN: 0.1 TFA, at 2:1. Two µl matrix solution were mixed with 1 µl of peptide solution and crystallized. Masses derived from MALDI-TOF-MS spectrometry were analysed in *Mascot* and in *Pep*MAPPER, (www.expasy.ch) to identify the different spots.

Phase partitioning

Phase partitioning was preformed as previously described [67]. To achieve this, 72 µg of mitochondrial protein was dissolved in 6 ml 2% Triton X-114 in PBS on ice. After 10 min of sonication and incubation for 1 h on ice, the sample was centrifuged at 14 000 × g for 10 min at 4°C. Heavy debris was sucked off. The supernatant was transferred to a new tube and incubated for 10 min at 37°C. After centrifugation for 10 min at 14,000 × g at RT, the aqueous supernatant and the detergent pellet were precipitated by TCA in H_2O, separated and analyzed by SDS-PAGE.

Membrane washing

Membrane washing was performed as previously described [68, 69]. After 10 min of sonication rat heart mitochondria (1 mg/ml) in 100 mM Na_2CO_3, pH 11.0, and incubation for 30 min on ice, membranes were centrifuged for 30 min at 15,000 × g at 4°C. The supernatant was precipitated with 10% TCA in H_2O, and both pellets together were separated and analyzed by SDS PAGE.

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