Novel Mitochondrial Creatine Transport Activity

IMPLICATIONS FOR INTRACELLULAR CREATINE COMPARTMENTS AND BIOENERGETICS*

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Immunoblotting of isolated mitochondria from rat heart, liver, kidney, and brain with antibodies made against N- and C-terminal peptide sequences of the creatine transporter, together with in situ immunofluorescence staining and immunogold labeling in the mitochondrial inner membrane. In addition, a novel creatine uptake via a mitochondrial creatine transport activity was demonstrated by [14C]creatine uptake studies with isolated mitochondria from rat liver, heart, and kidney showing a saturable low affinity creatine transporter, which was largely inhibited in a concentration-dependent manner by the sulphydryl-modifying reagent NEM, as well as by the addition of the above anti-creatine transporter antibodies to partially permeabilized mitochondria. Mitochondrial creatine transport was to a significant part dependent on the energetic state of mitochondria and was inhibited by arginine, and to some extent also by lysine, but not by other creatine analogues and related compounds. The existence of an active creatine uptake mechanism in mitochondria indicates that not only creatine kinase isoenzymes, but also creatine transporters and thus a certain proportion of the creatine kinase substrates, might be subcellularly compartmentalized. Our data suggest that mitochondria, shown here to possess creatine transport activity, may harbor such a creatine/phosphocreatine pool.

Creatine (Cr) and phosphocreatine (PCr) play fundamental roles in cellular energetics (for reviews, see Refs. 1–3). Cells that do not synthesize Cr, like skeletal and cardiac muscle, must take it up from the blood through an active Cr transport system (CRT) (4). cDNA and gene sequencing of the CRTs from rabbit, rat, mouse, human, and the electric ray (Torpedo) (5–10) have shown that CRTs are composed of 611–636 amino acid residues with a calculated molecular mass of ~70 kDa. The CRT sequences (Protein Data Bank accession number for rat CRT = P28570 and for human CRT = P48029) are most closely related to the γ-aminobutyric acid, taurine/betaine transporter subfamily (46–53% amino acid sequence identity), while the homology to the glyoxine, proline, catecholamine, and serotonin transporters is less pronounced (38–34%). Computational analysis revealed that these CRTs, like other neurotransmitter transporters, are integral membrane proteins containing 12 putative transmembrane domains (8). CRT expression has been studied by a few research groups (11–16). The presence of two different gene products expressed in various tissues corresponding to two major polypeptides of ~55 and ~70 kDa has been described. The two polypeptides are most likely generated by alternative splicing (12). This assumption is supported by the fact that antipeptide antibodies generated against the N- and C-terminal region of the cDNA-derived CRT polypeptide sequence, all recognize the same two proteins with molecular masses of ~55 and ~70 kDa on Western blots (11–16). Furthermore, the existence of CRT splice variants has recently been suggested, based on genetics studies using rapid amplification of cDNA ends methods (17). Recent immunofluorescence studies have indicated a high degree of intracellular CRT localization (16), which is consistent with a mainly mitochondrial localization (15). This finding is in contrast to the general view that CRT, represented here by two major ~55- and ~70-kDa CRT-related proteins, is localized exclusively in the plasma membrane. Instead, it was also recently reported that only a minor CRT protein species with an intermediate apparent molecular mass of ~58 kDa is located in the plasma membrane (15). This ~58-kDa polypeptide has been identified by surface biotinylatation of intact cardiomyocytes, followed by Western blotting with anti-CRT antibodies, or alternatively by Western blotting of highly enriched plasma membrane fractions (15). Also in contrast to the prevailing view that mitochondria do not contain Cr, PCr uptake into isolated rat heart mitochondria had been reported earlier (18) and was attributed, however, to the activity of adenine nucleotide translocase. The same authors also studied changes in the subcellular distribution of ATP, ADP, Cr, and PCr depending on the physiological state in tochrome oxidase core complex; PBS, phosphate-buffered saline; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; β-GPA, β-guanidinopropionic acid.
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rat fast twitch gastrocnemius and slow twitch soleus muscles by fractionation of freeze-clamped and freeze-dried tissue in non-aqueous solvents (19). It was found that during isotonic contraction of gastrocnemius muscles, the mitochondrial content of total Cr and PCR decreased with a parallel increase in extramitochondrial total Cr, indicating a net transfer of Cr across the mitochondrial membranes. In line with the above observation, in vivo isotope tracing studies with labeled Cr have shown that creatine kinase does not have access to the entire cellular Cr and PCR pools (20), 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 31P NMR magnetization transfer studies (21, 22), as well as with recent 31H NMR spectroscopy data (23), 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 (23). From these studies, it was also concluded that the total PCR/Cr pool must be divided into physical compartments, or chemical entities, without fast exchange, and the authors even mentioned that increased flux through mitochondria could provide an explanation for their experimental results (23). It is, however, important to emphasize that by the above experimental approaches, no specific information neither on the nature or the direction of Cr shuttling pathways nor on the identity of such putative Cr compartments could be inferred. These findings led us to search for a potential mitochondrial Cr transport activity in muscle and non-muscle tissues, which would be associated with corresponding CRT protein(s), by using cell fractionation techniques, confocal immunofluorescence, immunoelectron microscopy, as well as substrate transport studies. Here we show that the mitochondrial inner membrane possesses active CRT activity, which seems to be associated with distinct CRT-related polypeptides.

**EXPERIMENTAL PROCEDURES**

**Materials**

If not otherwise stated all chemicals were purchased from Sigma. Male Wistar rats (250–300 g) were purchased from Invitrogen BRL, (Fullinnsdorf, Switzerland). The characterization of our rabbit anti-CRT peptide antibodies has been described earlier (12).

**Immunofluorescence of Sections from Rat Ventricle**

Freshly excised rat ventricles were fixed for 3 h at room temperature in PBS containing 3% paraformaldehyde. Tissues were dehydrated and embedded in paraffin by standard techniques. Ten-μm slices were cut with a microtome, paraffin was removed with xylene, and sections were washed with 70% ethanol and stored in PBS. For immunofluorescence microscopy, tissue sections were permeabilized first with 0.2% Triton X-100 for 15 min, then with 0.1% SDS for 30 s and subsequently washed in PBS for 30 min. The sections were blocked in 5% goat serum albumin and 1% bovine serum albumin in PBS. Primary antibodies (rabbit anti-CRT peptide antibody 1:200, mouse anti-cytochrome oxidase subunit IV (COX, Molecular Probes) 1:200, both diluted in PBS containing 0.1% Tween) for 20 min, followed by incubation with a goat-anti-rabbit IgG conjugated with 10 nm colloidal gold, for 45 min with goat-anti-rabbit IgG conjugated with 10 nm colloidal gold, washed with buffer 1 again, and finally with double distilled H2O. After contrasting sections with 2% uranyl acetate and 2% lead citrate, pictures were taken by a transmission electron microscope JEOL200 at 100 kV.

**Western Blotting**

Extracts were separated in 10% polyacrylamide SDS gels and trans-blotted onto a nitrocellulose membrane (Schleicher & Schuell, Bottmingen, Germany). The membrane was blocked with 5% fat-free milk powder in TBS buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.4) for 1 h at room temperature. After washing for 30 min, membranes were incubated with 1:5,000 diluted anti-CRT peptide antibodies in TBS buffer for 2 h at room temperature. After washing with TBS buffer, the blot was incubated again with a 1:10,000 dilution of goat peroxidase-conjugated anti-rabbit secondary antibody (Amersham BioSciences). The immunoreactive bands were visualized using the Renaissance Western blot chemiluminescence reagent plus kit (PerkinElmer Life Sciences).

**Isolation of Outer and Inner Membrane from Rat Liver Mitochondria**

The isolation of the mitochondrial membranes was done according to Ref. 41. Briefly, rats were anesthetized with diethyl ether and killed by cervical dislocation. Tissue of liver, skeletal and cardiac muscle, kidney, and brain were taken and immediately transferred to ice-cold buffer. Liver, brain, and kidney tissues were homogenized by a Teflon/tissue glass potter (Braun, Melsungen, Germany), whereas skeletal and heart muscle was homogenized by a Poltron mixer in 40 ml HEPES-sucrose buffer containing 250 mM sucrose, 10 mM HEPES-HCl, pH 7.4, 0.05% bovine serum albumin (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 7000 × 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 of 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 100 μM EGTA, 25% PercollTM (Amersham Biosciences) and centrifuged for 35 min at 100,000 × g. PercollTM 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 7000 × g for 10 min. Washed mitochondrial pellets were then recovered from the pellet and resuspended in 200 μl of the washing buffer.
Mitochondrially suspended transport pellets were solubilized in 100 mM Cr, respectively, and fitted to an Eadie-Hofstee plot (Fig. 6). Mitochondria were washed three times in 250 mM sucrose, 10 mM anti-CRT or preimmune serum (at 1:100 final dilution). Subsequently, these mitochondrial suspensions were transferred onto a carbon-coated electron microscopy grid. The reaction was started by the addition of 100 nM FCCP. Cr transport assays were performed under standard conditions but without succinate and ADP. Next, we examined whether CRT was immunofluorescence pattern (Fig. 1, α-COX). Intracellular Location of CRT in Rat Heart—Indirect immunofluorescence staining of rat heart sections with antibodies directed against a 15-mer C-terminal peptide of CRT revealed a predominantly intracellular localization of CRT-related protein in rat heart by confocal microscopy. Sections of 10 μm of paraffin-embedded rat left ventricle, after fixation and permeabilization (see "Experimental Procedures"), were stained for 1 h at room temperature with polyclonal rabbit anti-CRT peptide antibodies, followed by incubation for 1 h with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and double stained by a monoclonal mouse anti-COX antibody (α-COX) followed by a Cy3-conjugated donkey anti-mouse secondary antibody. The presence of CRT in mitochondria was verified by merging both fluorescence channels (merge). Sections were analyzed with a Leica TCS SP laser confocal microscope with a He Ne/Ar laser and Leica scanning electronics and software. Image processing was done on a Silicon Graphics Iris 4D/25 workstation, using Imaris (Bitplane AG) software.

RESULTS

Intracellular Location of CRT in Rat Heart—Indirect immunofluorescence staining of rat heart sections with anti-CRT antibodies directed against a 15-mer C-terminal peptide of CRT revealed a predominantly intracellular localization of CRT-related protein (Fig. 1, α-COX). The spotted pattern of the immunofluorescence signal suggests that the distribution of the protein within the cell is not homogeneous and might be associated to intracellular organelles. The remarkably ordered and regular alignment of small, anti-CRT-positive spots along the contractile apparatus suggests a periodic association with regular structures of the myofibrillar apparatus that is typical for mitochondria in muscle. Co-staining with the mitochondrial marker COX, a mitochondrial transmembrane protein and thus a marker for mitochondria (24), indeed displayed an identical immunofluorescence pattern (Fig. 1, α-COX), as indicated by the co-localization of anti-CRT and anti-COX staining (Fig. 1, merge). Essentially the same immunolocalization was obtained also with antibodies directed against a 15-mer N-terminal synthetic peptide of CRT (12) (not shown). To precisely identify the site(s) of intracellular CRT localization, we performed immunoelectron microscopy studies on sections of the adult rat myocardium, treated with anti-CRT antibodies followed by colloidal gold-conjugated secondary antibodies. Fig. 2A clearly shows the specific labeling of mitochondria by anti-CRT antibodies that are evenly distributed within the muscle fibers, with some non-mitochondrial and otherwise very low background staining, as compared with staining with preimmune serum (Fig. 2B).
expressed in mitochondria of different tissues by Western blotting. In the experiments reported in Fig. 3, protein extracts of mitochondria (m) from brain, heart, kidney, and liver were separated in SDS-PAGE together with the corresponding cytosolic fractions (c) and total tissue extracts (t), transferred to nitrocellulose membranes and probed with two different anti-CRT antisera raised against synthetic peptides corresponding to the C-terminal (A) and N-terminal (B) sequences of the cDNA-derived CRT sequence (12). Mitochondrial purification was assessed in parallel with an anti-COX antibody (C). The result clearly demonstrates that the ~55- and ~70-kDa CRT-related proteins are both recognized by both the anti-N-terminal, as well as the anti-C-terminal, CRT antibodies (12) and that both of these immunoreactive polypeptides are predominantly localized in mitochondria of all tissues tested, where they are highly enriched relative to the total extracts.

**Intramitochondrial Location of CRT-related Proteins**—To assess the distribution of the CRT protein within mitochondria, purified rat liver mitochondria were ruptured by osmotic swelling and ultrasonic treatment. Heavy (inner) and lighter (outer) mitochondrial membranes, as well as soluble, non-membrane-associated proteins were separated in sucrose step gradients, and the corresponding protein extracts were finally analyzed by anti-CRT Western blots (Fig. 4). The experiments shown in this figure revealed that both CRT-related polypeptides were highly enriched in the heavy (inner) membrane fraction (lane MIM), while the light (outer) membrane fraction (lane MOM) and the soluble matrix fraction (lane MX) contained virtually no immunoreactive signal. The relative enrichment of the CRT proteins in the heavy fraction can be easily appreciated from a comparison of the signal in the total liver homogenate (Fig 4A, lane liver) and in the inner membrane fraction (Fig. 4, lane MIM). Mitochondrial marker antibodies against COX, as well as against voltage-dependent anion channel (VDAC) were used to probe for mitochondrial inner and outer membrane, respectively. To confirm the submitochondrial localization of CRT with an independent approach, its accessibility to anti-CRT antibody was determined before and after osmotic rupture of the outer membrane. Mitochondria were incubated with the anti-CRT antibody either in iso-osmolar (250 mM sucrose) or hypo-osmolar medium (50 mM sucrose), followed by antibody detection with protein A-gold (10 nm), using a negative staining technique on whole mitochondria. As shown in Fig. 5, hypo-osmotic treatment caused the formation of peripheral vesicles of outer membrane (arrowheads in A and B), while the inner membrane cristae were still conserved. Significant anti-CRT antibody labeling was seen only under hypo-osmotic conditions and was particularly prominent at the inner membrane (A). Labeling was specific, since it was not observed after treatment with preimmune serum (B), while under iso-osmolar conditions only a few gold particles were seen on the surface of mitochondria (C). Quantification and statistical analysis of the number of gold grains confirmed that significant mitochondrial staining was only seen under hypo-osmotic conditions (D).

**Cr Uptake Assay with Isolated Mitochondria**—Heart, liver, and kidney mitochondria were isolated and carefully purified with Percoll™ density gradients to minimize contamination with plasma membrane vesicles and other membranes or organelles. These mitochondria were tested for their ability to accumulate Cr by incubating them in sucrose buffer containing 14C-labeled Cr in a total concentration of 20 mM Cr, which is close to the physiological range for working muscle (2, 3). Fig. 6 shows that heart, liver, and kidney mitochondria take up Cr with similar kinetics, in a process that leveled off after about 5 min (A). Cr association with the mitochondrial pellet reflected a true transport process, because Cr was sequestered into a sucrose-inaccessible space, as assessed by inclusion of 3H-labeled sucrose into the incubation buffer. The absolute amounts of Cr taken up by heart (Fig. 6A, filled squares), liver (open circles), and kidney mitochondria (open triangles) were about 12, 16, and 19 nmol × mg⁻¹ mitochondrial protein. Cr uptake followed saturation kinetics with an apparent K_m and V_max for Cr transport of 15.90 (± 1.32) mM and 11.79 (± 1.15) nmol ×
that is, energized rat heart mitochondria (5 mM succinate) showed ~20% higher Cr transport activity as compared with partially uncoupled mitochondria (1 mM ADP), whereas the addition of the uncoupler, FCCP, which completely abolishes the mitochondrial membrane potential, led to a ~35% decrease in Cr transport activity, as compared with control rates. Mitochondrial Cr transport activity was significantly inhibited by the related guanidino compound, arginine, as well as by the amino acid, lysine, but not so by other Cr analogues or amino acids (see Table I). Interestingly, no inhibition of mitochondrial CRT was seen with β-GPA that is known to significantly inhibit sarcolemmal CRT (12, 15).

Finally, the link between the CRT mitochondrial proteins and Cr transport was addressed in the experiments shown in Fig. 8, where incubation of mitochondria with anti-CRT antibody completely inhibited Cr transport in hypo-osmotically treated mitochondria (B, a-CRT column), while uptake was unaffected in iso-osmotic sucrose media (A). These experiments complement the subcellular and submitochondrial localization studies by immunofluorescence and immunoelectron microscopy, respectively, and strongly support the suggestion that mitochondrial Cr transport is likely to be mediated by the ~55 and ~70 CRT-related polypeptides residing in the inner mitochondrial membrane.

DISCUSSION

In this paper, we have shown (i) that the two major CRT-related protein species of ~55 and ~70 kDa, which have been independently identified earlier by various groups (11–16), are associated with the inner mitochondrial membrane, as demonstrated here by immunofluorescence and immunoelectron mi-

Fig. 4. Submitochondrial localization of CRT-related protein by fractionation of mitochondrial membranes. Rat liver mitochondria were ruptured by a repeated swelling and shrinking procedure followed by ultrasonic treatment according to Ref. 41. Soluble matrix proteins, lighter (outer), as well as heavy (inner), mitochondrial membranes were separated in discontinuous sucrose density gradients and analyzed. The Western blot (10 μg of protein per lane) shows an anti-CRT immunoblot of protein extracts from rat liver total homogenate (liver), soluble cytosolic proteins (cytopl), rat liver mitochondria (mito), mitochondrial outer membrane (MOM), soluble mitochondrial matrix proteins (MX), as well as mitochondrial inner membrane (MIM), indicating the strongest anti-CRT signal in this mitochondrial inner membrane fraction. As controls, anti-COX and anti-voltage-dependent anion channel (α-VDAC) antibodies were used to identify mitochondrial inner and outer membrane, respectively.

Fig. 5. Intramitochondrial localization of CRT-related protein by immunolectron microscopy. Rat liver mitochondria (1 mg/ml) were incubated for 1 h at room temperature at different osmolarities with polyclonal rabbit anti-CRT peptide antibody (1:200 dilution) or preimmune serum. Mitochondria were washed three times and incubated for 1 h with protein A-labeled colloidal gold (10 nm) and washed again three times. Mitochondria were then negatively stained with 2% ammonium molybdate, pH 7.4. A, 50 mosmol/kg with anti-CRT peptide antibody; B, 50 mosmol/kg with preimmune serum; C, 250 mosmol/kg with anti-CRT peptide antibody; D, means of gold grains counted per mitochondrion, with 20 mitochondria from two experiments of each condition, were analyzed and plotted accordingly.
The amount of Cr uptake is expressed as nmol × mg⁻¹ mitochondrial protein. The graphs correspond to mitochondria from heart (filled black squares), liver (open circles), and kidney (open triangles). Initial rates of Cr uptake were measured for 3 min as a function of Cr concentration at room temperature at 0.53, 1, 2, 10, and 20 mM Cr, respectively. Values are means ± S.E. of measurements from two individual animals in each of which Cr transport was measured in triplicate. Cr uptake values were fitted to Eadie-Hofstee plot (B). Mean (± S.E.) $K_m$ and $V_{max}$ values fitted from each individual uptake curve were 15.90 (± 1.32) mM and 11.79 (± 1.15) nmol × mg⁻¹ mitochondrial protein × min⁻¹, respectively. C illustrates inhibition by NEM of Cr uptake into isolated rat heart mitochondria measured for 15 min. Aliquots of mitochondria were preincubated for 10 min with increasing concentrations of NEM, followed by Cr uptake measurements. The amount of Cr uptake is expressed as nmol of Cr × mg total mitochondrial protein⁻¹ × 15 min⁻¹.

**Table I**

Inhibition of Cr uptake by structurally related compounds

| Compound     | Average ± S.E. | Significance | n   |
|--------------|----------------|--------------|-----|
| Control      | 19.0 ± 4.9     | p > 0.3      | 14  |
| PCr          | 17.1 ± 6.6     | p > 0.3      | 8   |
| Creatinine   | 22.2 ± 7.9     | p > 0.3      | 3   |
| Cycle-creatine| 17.7 ± 1.0     | p > 0.3      | 3   |
| β-GPA        | 19.9 ± 2.2     | p > 0.3      | 5   |
| Arginine     | 14.4 ± 4.4     | p < 0.03     | 14  |
| Lysine       | 15.2 ± 3.8     | p < 0.04     | 13  |
| Proline      | 21.6 ± 2.9     | p > 0.3      | 11  |
| Glutamine    | 18.6 ± 6.3     | p > 0.3      | 10  |
| Ornithine    | 16.7 ± 2.0     | p > 0.3      | 3   |
| Citrulline   | 20.0 ± 1.7     | p > 0.3      | 3   |
| GABA         | 16.2 ± 5.8     | p > 0.3      | 4   |

nmol Cr × mg⁻²

Inhibition of uptake of radioactive creatine into mitochondria by various creatine analogues, related guanidino compounds, and amino acids was measured. The Cr uptake assays were performed at room temperature using highly enriched, Percoll-grade gradient-purified mitochondrial preparations in transport buffer containing 10 mM Tris/HCl, pH 7.4, supplemented with 250 mM sucrose, 2 mM Cr, and 5 μCi × ml⁻¹ [¹⁴C]Cr, 10 μCi × ml⁻¹ [³²P]sucrose, 2 μM rotenone, 2 mM MgCl₂, 10 mM P/Tris, and 100 μM EGTA (serving as control) and in the presence of 1 mM GABA, γ-aminobutyric acid.

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These mitochondrial protein(s), as well as protein sequencing of the immunoreactive polypeptides, our results have established that Cr is transported in mitochondria through a specific carrier system. The presented data also indicate that mitochondrial Cr uptake is dependent at least in part on the energetic state of mitochondria and that this Cr transport can be competitively inhibited by arginine and to a lesser extent also by lysine, but not by other Cr analogues or related compounds. This new data provide an explanation for several intriguing
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FIG. 8. Inhibition of mitochondrial Cr uptake by anti-CRT antibodies. Mitochondria were pretreated for 1 h at 22 °C in 250 mM (iso-osmotic condition) (A) or 50 mM sucrose (hypo-osmotic condition) (B) together with rabbit anti-CRT C-terminal peptide serum, preimmune serum (PIS), or with the same volume of the corresponding sucrose buffer only (control). Subsequently, mitochondria were washed three times with 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA and Cr transport assays performed as described in the legend to Fig. 6.

findings in the literature and have important implications for our current understanding of intracellular compartmentation of high energy compounds. Indeed, our results suggest that mitochondria may participate in energy metabolism by regulation of the intracellular distribution of Cr.

CRT Isoforms—The classical plasma membrane CRT that is responsible for high affinity uptake of Cr into cells has recently been shown to represent only a minor CRT isoform, in quantitative terms, with an apparent molecular mass of 120 kDa, has also been recognized by our antibodies as a weak signal in total tissue homogenates (12, 15). The ~58-kDa polypeptides, in contrast to the two mitochondrial polypeptide species of ~55 and ~70 kDa referred to here, is hardly visible on Western blots of total tissue extracts, but can be enriched in preparations of purified plasma membranes, as well as in erythrocytes, but is absent in mitochondria (15). Besides this latter minor CRT species residing in the plasma membrane, we propose here that the two major CRT-related protein species of ~55 and ~70 kDa are residing inside mitochondria in the inner mitochondrial membrane (Fig. 9).

Mitochondrial Location of the Two Major CRT-related Protein Species—The results obtained from our immunofluorescence studies, as well as immunoelectron microscopic analysis, demonstrate a predominantly mitochondrial location of the two major CRT-related proteins of ~55 and ~70 kDa in heart (Figs. 1 and 2), consistent with earlier results obtained from immunofluorescence work on cross-sections of rat skeletal muscle and myocytes in culture (15). A mitochondrial localization of CRT is independently supported by the finding that slow type-I oxidative muscle fibers stained consistently stronger with anti-CRT antibodies as compared with fast type-II glycolytic fibers (16). This can be explained by the fact that mitochondrial content and volume fraction are significantly higher in type-I versus type-II muscle fibers. In addition, the Western blot studies presented here, using isolated mitochondria from rat heart, liver, kidney, and brain, clearly demonstrated an enrichment of both major CRT-related polypeptides in these organelles, which apparently are the major site of CRT accumulation. Since the results obtained with liver, brain, and kidney were qualitatively similar to those of cardiac and skeletal muscle, mitochondrial CRT expression is predominant not only in sarcomeric muscle but also in non-muscle tissues, including the liver, which itself is the major organ of Cr biosynthesis (2).

We could further demonstrate that CRT-related polypeptides are exclusively localized in the inner mitochondrial membrane as would be expected for a mitochondrial transporter. Indeed, immunogold labeling of mitochondria was only observed after the outer membrane was permeabilized by hypotonic buffers, and mitochondrial fractionation confirmed that both proteins were highly enriched in the heavy inner membrane fraction that also contained COX subunit I. Additionally, and as would be expected for an integral membrane protein, no CRT was detectable in the soluble fractions of cell homogenates and mitochondria. As judged from the sequence data, no mitochondrial pre- or leader sequence seems to be present in CRT such that the protein would have to find its way into mitochondria by internal sequences that are likely facilitating the import and insertion of CRT(s) into the inner mitochondrial membrane, as, for example, has been shown to be the case also for adenine nucleotide translocase (28), as well as for other mitochondrial membrane proteins (29).

Mitochondrial Transport of Cr—Consistent with the presence of an inner membrane Cr transporter, our studies with isolated mitochondria, using [14C]-labeled Cr, provide strong evidence that mitochondria are indeed able to accumulate Cr. The apparent \( K_m \) of ~15 mM may appear high, but it does in fact match the physiological range of intracellular Cr concentration in muscle (2, 3) (see Fig. 9). Assuming that 1 mg of
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Mitochondrial protein corresponds to a matrix volume of 1 μl, intramitochondrial Cr may reach concentrations of the order of 20 mM. Strong evidence that Cr transport is mediated by a carrier protein comes from the fact that Cr transport activity is inhibited by the sulphhydryl-modifying agent, NEM, which also inhibits a number of other mitochondrial carriers, including the P_{i} carrier (30). The fact that DTNB and DNFB turned out to be less inhibitory for mitochondrial Cr uptake than NEM may be explained by accessibility problems due to the larger molecular size and greater hydrophobicity of the former compounds compared with NEM.

Interestingly enough, our inhibition studies of mitochondrial Cr transport with creatine analogues, as well as related guanidino compounds, amino acids, and other substrates of the 12-membrane-spanning neurotransmitter transporter family, to which CRT belongs to, indicate that mitochondrial CRT, in contrast to sarcolemmal CRT, seems not to be entirely specific for Cr alone, since arginine and to some extent also lysine showed significant inhibition of Cr uptake. (Table I). The observed difference of inhibition by β-GPA between the plasma membrane CRT (see Refs. 12 and 15) and the mitochondrial CRT, the latter remaining unaffected by this Cr-analogue, indicates that these related CRT isoforms differ in their transport characteristics (K_{M} for Cr) (15), substrate specificity, and susceptibility toward inhibitors, like β-GPA (12, 15).

Evidence that the transport activity is mediated by the ~55/70-kDa CRT-related polypeptide species rests on the inhibitory effects of the specific polycyclical anti-CRT antibodies on Cr transport into mitochondria, where a complete blockage of Cr uptake was observed in mitochondria after the outer membrane had been permeabilized by preincubation of mitochondria under hypotonic conditions (Fig. 8), a finding that matches inner membrane staining by the same antibodies (Figs. 4 and 5). As mentioned above, the final molecular identification of the ~55/70-kDa polypeptide species as the bona fide mitochondrial Cr transporters must await purification, sequencing, and reconstitution of these minor mitochondrial protein. The present observations represent an essential step toward this goal.

Implications for Cr Compartmentation—These new results shed some light on the possible existence of an intramitochondrial pool of Cr and/or PCr (Fig. 9) and thus may account for a set of interesting earlier observations. For example, during recovery after exhaustive exercise in oxidative type-I, but not in glycolytic type-II muscle fibers (31), the overall PCr concentrations display an overshoot, which may be explained by an accumulation of Cr within mitochondria, which would be transphosphorylated to PCr via mitochondrial creatine kinase, suggesting the existence of a PCr/Cr compartment that may be displaced from the overall creatine kinase equilibrium, at least temporarily (32). Earlier observations, showing that mitochondrial Cr content differed considerably in resting as compared with fatigued muscle, suggested that there may be a traffic of Cr across the mitochondrial inner membrane (18). Recent data with skinned muscle fibers indicated that no further Cr can be specifically released from mitochondria of these fibers after their permeabilization with detergents (33). However, an accumulation of PCr in mitochondria is supported by experiments with cell cultures, where isolated mitochondria from cells, after growth factor withdrawal, showed an over 100-fold higher PCr content than control cells (34). Since our Cr uptake studies with isolated mitochondria were done in the presence of mitochondrial substrates under conditions favoring maximal respiration, it is entirely conceivable that Cr uptake, and possibly also the maintenance of a mitochondrial Cr pool within mitochondria, may depend on the energy charge of mitochondria, e.g. would only be observable in actively respiring mitochondria. This is corroborated by our data showing that mitochondrial Cr uptake into isolated mitochondria is significantly hampered after addition of uncoupling agents.

The existence of localized creatine kinase isoenzymes forming functionally coupled subcellular microcompartments with ATP-generating and ATP-utilizing processes, possibly involving distinct PCr/Cr pools (3), is also supported by studies on transgenic mice that lack both sarcomeric and mitochondrial creatine kinase (35), which no longer show Cr-stimulated mitochondrial respiration (36). The idea that CRT(s), as well as the creatine kinase substrates themselves, may be compartmentalized has recently gained additional support from in vivo experiments. [3H]Cr isotope infusion of fish under different metabolic conditions (resting, actively swimming, exhausted, and recovering), followed by freeze-clamping and analysis of the specific radioactivity of the Cr and PCr pools, showed that a significant fraction of cellular Cr is not freely and rapidly exchanging with exogenously added radioactive Cr and that creatine kinase may not have immediate access to the total pool of PCr and Cr (20), a finding that strongly suggests the existence of at least some separate intracellular Cr pool(s). Finally, the data presented in this work may provide a likely explanation for the unexpected and anomalous NMR behavior of Cr and of creatine kinase flux measurements in vivo, as obtained by 1H NMR (23) and 3P NMR (21, 22, 37), respectively. Finally, experiments with isolated mitochondria to which either PCr, Cr, or none of both had been added showed a rather high Cr background in the latter samples, which was referred to by the authors (46) as “unexplained interference,” which in hindsight was probably due to the presence of Cr in freshly isolated mitochondria (18, 19), which, however, was rapidly lost with time, as was probably also the case for chemically skinned muscle fibers, where after the rather lengthy skinning procedure, no more Cr could be released from mitochondria (33). In summary, all these findings appear to imply the presence of intracellular pools of Cr and/or PCr that are not entirely in equilibrium with one another, with one of them likely being mitochondrial origin.

Conclusions and Perspectives—A full understanding of the function and the purpose of mitochondrial Cr transport will obviously need a more detailed characterization of the process, as well as the proteins involved, e.g. by protein sequencing and thorough studies of the reconstituted CRT protein(s), work which is currently in progress. Open questions are also whether Cr transported into mitochondria is immediately recharged via mitochondrial creatine kinase to PCr (36, 38), e.g. for energetic purposes, or whether Cr, a highly abundant cationic compound in the cytosol, could fulfill some protective role as an osmolyte to guarantee mitochondrial integrity under conditions of cellular stress. Interestingly enough, Cr has been shown to exert marked protection against Ca^{2+}-induced mitochondrial permeability transition pore opening (39), an early event of cellular apoptosis. The importance of assessing the pathway(s) and cellular location of Cr transport is further highlighted by a description of the first patients with an X-linked genetic disease due to defects of the CRT gene (SLC6A8) (40). These patients have a very low concentration of cerebral and cerebellar Cr and display some of the typical symptoms of Cr deficiency, such as general developmental defects and severe speech impairment, hypotonia, intractable epilepsy, with a disease progression eventually leading to brain atrophy (40). Assessing whether impaired mitochondrial transport of Cr is part of the pathogenetic mechanism appears to be of great value for understanding this disease and for fully

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2 R. Balaban, personal communication.
appreciating the possible role of mitochondria in energy homeostasis beyond the strict requirement for ATP synthesis.

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