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Uptake of creatine phosphate into heart mitochondria: a leak in the creatine shuttle

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Abstract

CrP uptake into isolated rat heart mitochondria was studied using silicone oil centrifugation. Further, the involvement of the mitochondrial adenine nucleotide translocase was examined by measuring CrP accumulation in mitochondria in the presence of substrates and inhibitors of the ATP/ADP-carrier and by investigating uptake kinetics in liposomes reconstituted with purified bovine heart adenine nucleotide translocase protein. CrP is accumulated in the matrix space of isolated rat heart mitochondria and mitoplasts. The uptake is inhibited by carboxyatractyloside, a specific inhibitor of the mitochondrial adenine nucleotide translocase, and by ADP, phosphoenolpyruvate, 3-phosphoglycerate and pyrophosphate, compounds which are able to bind to the carrier. It is not inhibited when the mitochondrial membrane potential is decreased. CrP is transported into reconstituted liposomes at a rate which is about 3 orders of magnitude lower than the rate for ATP uptake. The transport is sensitive to temperature change and to carboxyatractyloside. It is concluded that CrP is specifically taken up by heart mitochondria via the mitochondrial adenine nucleotide translocase. The transport in mitochondria in situ is facilitated by the close local and functional interaction of the mitochondrial creatine kinase and the adenine nucleotide translocase within contact sites between inner and outer mitochondrial membrane. A certain amount of CrP synthesized by the mitochondrial creatine kinase thus escapes its usage at cytosolic energy consuming processes.

Keywords: Creatine shuttle; Adenine nucleotide translocase; Heart mitochondria; (Rat)

1. Introduction

In heart cells the transfer of ATP from the mitochondria to the cytosolic energy consuming processes can be effectively mediated via the creatine shuttle [1]. In this pathway creatine is phosphorylated by the mitochondrial creatine kinase (Mi-CK). The Mi-CK, which is located in the intermembrane space, uses ATP from oxdiative phosphorylation, supplied via the mitochondrial adenine nucleotide translocase (ANT).

Abbreviations: ANT, adenine nucleotide translocase; $C_{12}E_8$, octethylenglycolmonododecylether; CAT, carboxyatractyloside; CrP, creatine phosphate; DTT, dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid; Hepes, *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]; Mi-CK, mitochondrial creatine kinase; PEP, phosphoenolpyruvate; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Tris, tris[hydroxymethyl]aminoethane; Tricine, *N*-tris[hydroxymethyl]methylglycine.

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Thereby a high ADP-concentration is generated in the intermembrane space which continuously stimulates the regeneration of ATP in oxidative phosphorylation without decrease of the cytosolic phosphorylation potential [2]. Creatine phosphate (CrP) leaves the intermembrane space by diffusion and reaches the cytosolic creatine kinases; there it is used for the rephosphorylation of cytosolic ADP.

The thermodynamically unfavorable phosphorylation of creatine is facilitated in mitochondria due to the close coupling of the Mi-CK reaction to oxidative phosphorylation [3]. According to current knowledge both reactions are coupled by the ANT which is associated with the Mi-CK within contact sites of the inner and outer mitochondrial membrane [4]. Mi-CK has thereby preferential access to ATP generated in the mitochondria.

CrP permeates the outer membrane through porin channels. The permeability of porin channels to anions is low at a high transmembrane potential and vice versa [5]. Therefore, CrP might accumulate within the intermembrane space when oxidative phosphorylation is active and the electrical field perceived by the porin is relatively high. High amounts of CrP were found inside the mitochondria of stimulated rat heart [3] and skeletal muscle [6]. This finding is surprising in view of the fact that no mitochondrial transport system for CrP is known.

We supposed that the ANT might be responsible for CrP uptake into the mitochondria, due to the close association of Mi-CK and ANT within the contact sites [3,6]. Thus, it has been reported that phosphoenolpyruvate, which has a chemical structure similar to that of CrP, is taken up by rat liver mitochondria in a carboxyatractyloside sensitive manner [7]. We, therefore, studied creatine phosphate uptake in isolated rat heart mitochondria and in liposomes with reconstituted ANT.

2. Materials and methods

2.1. Materials

All enzymes and coenzymes were either from Boehringer (Mannheim, Germany) or from Sigma Chemie (München, Germany), chemicals were from Merck (Darmstadt, Germany) and were of highest purity available. γ-^{32}P-ATP (10 MBq/10 μl) was from Hartmann Analytik (Braunschweig, Germany).

2.2. Isolation and incubation of heart mitochondria

Rat heart mitochondria were isolated essentially according to Jacobus and Saks [8] in a medium containing 0.25 M sucrose, 10 mM Tris, 5 mM KH_2PO_4, 20 mM KCl, 0.2 mM EDTA, pH 7.2, using 1 mg of trypsin for three hearts.

Mitoplasts were prepared by incubation of the mitochondrial pellet in 20 mM TES for 10 min (modified from [9]). Where 125 mM KCl + valinomycin was added, the osmolarity was maintained by decreasing the sucrose concentration.

Mitochondria and mitoplasts (1 mg/ml) were incubated at pH 7.2 in the medium: 170 mM sucrose, 70 mM HEPES, 5 mM KH_2PO_4, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$ and 2.5 mM glutamate + 2 mM malate as respiratory substrates. The incubation temperature was 37°C for the measurement of respiration and room temperature for metabolite uptake determinations using silicone oil centrifugation. Additional substrates as well as inhibitors were added as indicated in Table 1, Tables 2 and 3 and in Fig. 1.

The mitochondria had respiratory control ratios of 8–15 (6–8) in the absence (presence) of 5 mM $MgCl_2$, with 2.5 mM glutamate + 2 mM malate as respiratory substrates. The rate of respiration was about 200 nmol O_2/min per mg protein at 37°C. Mitoplasts had respiratory control ratios of about 4 in the absence of magnesium.

Beef heart mitochondria were prepared from fresh beef hearts from the slaughterhouse according to the method of Smith [10]. Mitochondria (10 mg/ml) were frozen in portions of 1 ml and thawed immediately before the use.

2.3. Determination of metabolite uptake into rat heart mitochondria by silicone oil centrifugation [11]

Mitochondria were incubated for 2 min as described above with substrates added as indicated in the Section 3. 200 μl of the incubation mixture was then transferred to a coleman tube containing 70 μl silicone oil layered on the top of 20 μl of 1.2 M perchloric acid. Separation of mitochondria from

medium was achieved by centrifugation of the mitochondria through the silicone layer into perchloric acid. Since mitochondria always transfer a certain amount of medium into the pellet, it was estimated in parallel samples, containing ^{14}C-sucrose and $^{3}H_2O$, from the amount of radioactivity found in the pellet [12]. It was determined that 2 μl of the extramitochondrial incubation medium was taken over to the sediment with 1 mg of mitochondrial protein. Therefore, a correction for extramitochondrial creatine phosphate in the pellet was made. CrP was determined enzymatically in perchloric acid extracts of pellet and supernatant, respectively [13].

2.4. Isolation of the ANT from beef heart mitochondria and reconstitution into liposomes

The ANT was solubilized and purified from beef heart mitochondria by using $C_{12}E_8$ (Fluka AG, Buchs, Germany) as detergent [14]. This procedure was chosen in order to avoid any contamination by copurified porin. Liposomes were prepared by sonicating 100 mg/ml turkey egg yolk phosphatidylcholine (Sigma, München, Germany) in 400 mM sucrose, 100 mM Tricine, pH 7.2, until the suspension was clear (10–15 min) using a Branson sonifier W 250 with microtip. The sonication vessel was cooled in ice during the preparation of liposomes and the solution was flushed with argon. Cardiolipin (5.2 mg/ml, ethanolic solution) and ATP (or ADP, as indicated in the corresponding experiments), were added to the solubilized carrier protein. After mixing with liposomes and detergent (phospholipid 12 mg/ml, detergent 18 mg/ml, protein 0.12 mg/ml), the mixed micelles were incubated for 5–10 min at 0°C and formation of proteoliposomes was achhieved by repeated hydrophobic chromatography on Amberlite columns [15] applying 15 column passages. In order to remove external substrate (ATP or ADP) the reconstituted proteoliposomes were separated by size-exclusion chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) equilibrated in buffer solution (100 mM Tricine, pH 7.2), and sucrose in concentrations to provide conditions isoosmolar to the interior of the proteoliposomes. The concentration of ANT was ca. 1 μg protein per ml proteoliposomal solution, 5 μl of the vesicular internal volume corresponded to 1 ml of proteoliposome solution.

2.5. Preparation of creatine-^{32}-phosphate

Radioactively labelled CrP was prepared from γ-^{32}P-ATP (4 MBq/ml) by incubation of 1 ml medium (62 mM Tris, pH 8.5, 5 mM total ATP, 5 mM creatine, 14.000 U creatine kinase from rabbit muscle (Boehringer, Mannheim, Germany)) for 1h at room temperature. The reaction was stopped by perchloric acid and neutralized by 1 M triethanolamine/5 M KOH. CrP was separated from ATP, ADP, AMP and creatine by HPLC [16]. The eluate was incubated with apyrase (1.25 U/ml) Grade VII, from Sigma (München, Germany) to remove all remaining adenine nucleotides. Then the reaction was terminated by perchloric acid and the medium neutralized as described above. The concentration of creatine phosphate was determined enzymatically [13] as 85 μM and the radioactivity measured by liquid scintillation counting as 8500 dpm/μl.

3. ATP and CrP-uptake into liposomes [17]

3.1. ATP-uptake

140 μl liposomes were incubated for 1–6 min at room temperature with 10 μl mix containing 1 mM ATP and 0.37 MBq ^{3}H-ATP. The process was stopped by addition of 10 μl 600 mM pyridoxalphosphate in 1M imidazol. External nucleotides were removed on Dowex columns (size 2×10 cm): 125 μl liposome suspension was applied to the column and eluted with 1ml 500 mM sucrose. The radioactivity in the eluate was determined by liquid scintillation counting and the rate of uptake evaluated from the initial velocity [17].

3.2. CrP-uptake

140 μl liposomes were incubated for 1–25 min at room temperature with 60 μl Cr^{32}P-solution (8500 dpm/μl) and 10 μl unlabelled CrP, 14 mM, to yield a final concentration of 2 mM CrP. The reaction was stopped at corresponding time and the mixture treated as described above. The radioactivity of the eluates was determined by liquid scintillation counting and plotted against the time of reaction. The rate of uptake was calculated from the initial velocity of the

process. In some experiments, where liposomes were incubated for 25 min in the absence of labelled creatine phosphate, the amount of CrP in the eluate was determined as 100 ± 20 μM using enzymatic analysis.

4. Results and discussion

It has been shown that mitochondria of intact rat and skeletal muscle accumulated CrP and that the incorporated amount depended on the oxidative activity of the muscle [3,6]. CrP is synthesised by the Mi-CK located at the outer site of the inner mitochondrial membrane and released into the intermembrane space. Diffusion of CrP into the cytosol is regulated by the porin channel of the outer mitochondrial membrane [18,19]. It is feasible that, CrP might accumulate in the intermembrane space at a high phosphorylative activity of mitochondria, and might be transferred by the mitochondrial adenine nucleotide translocase into the mitochondrial matrix. The aim of the present study was, therefore, to investigate whether the ANT could be reponsible for the uptake and which factors influence CrP transport.

4.1. CrP-uptake by mitochondria and mitoplasts

Table 1 shows that CrP was found in the mitochondrial pellet obtained by centrifugation of the mixture through silicone oil. It may be argued that CrP, which is generated in the intermembrane space, is retained there to a certain extent due to the limited diffusion through the porin channels. In this case only minute amounts of CrP should be detected in mitoplasts. However, the comparison between mitoplasts and mitochondria shows the similar level of the incorporated CrP which testifies for accumulation in the matrix but not in the intermembrane space.

Table 1
CrP-uptake in rat heart mitochondria

Additions	Creatine phosphate in:	
	Supernatant (μM)	Mitochondria (nmol/mg)
Mitochondria:		
1 mM ATP, 10 mM Cr	647 ± 54	3.3 ± 0.2
+ CAT 10 μM	320 ± 37	0.3 ± 0.1
+ valinomycin 1 μM, KCl 125 mM	141 ± 24	2.6 ± 0.2
+ FCCP 0.3 μM	483 ± 31	3.1 ± 0.4
Mitoplasts:		
1 mM ATP, 10 mM Cr	530 ± 50	4.2 ± 0.3

CrP in supernatant and mitochondria was determined after separation by silicone oil centrifugation (see Section 2).
$n = 4$–6 duplicates $\pm$ S.E.M.

Table 2
Influence of ADP on CrP-uptake into rat heart mitochondria (see Table 1)

Additions	Creatine phosphate in:	
	Supernatant (μM)	Mitochondria (% of control)
1 mM ATP, 10 mM Cr	647	100
+ 2 mM PEP, 150 U PK	1124 ± 179	– [a]
+ 1 mM ADP	837 ± 82	120 ± 17
+ 2 mM ADP	720 ± 26	131 ± 18
+ 3 mM ADP	581 ± 85	55 ± 10
0.5 mM CrP	417 ± 23	85 ± 6
+ 10 mM ADP	471 ± 44	– [a]
1 mM CrP	1052 ± 80	76 ± 6
+ 10 mM ADP	750 ± 115	– [a]

[a] Below detectable level; absolute values for control (1 mM ATP, 10 mM creatine), see Table 1; control values in the sediment were set 100%).

4.2. Influence of substrates and inhibitors of the ANT on CrP-uptake

The ANT in energized mitochondria exports ATP^{4-} in exchange for cytosolic ADP^{3-}. The electrogenic nature of the exchange imposes the asymmetry of the transport process at a high mitochondrial membrane potential [20]. The transport is specific for adenine nucleotides but certain other phosphorylated compounds are also transported with low efficiency. Thus, it has been reported that phosphoenolpyruvate, pyrophosphate and 2-phosphoglycerate are taken up by rat liver mitochondria in a carboxyatractyloside-sensitive manner [7,21,22]. The ANT is also inhibited by carboxyatractyloside.

Our experiments show that in heart mitochondria CrP uptake is inhibited in the presence of carboxyatractyloside (Table 1), and also in the presence of ADP, which is the physiological substrate for the ANT (Table 2). Further, CrP-uptake is suppressed in

the presence of the phosphorylated compounds phosphoenolpyruvate, pyrophosphate and 2-phosphoglycerate (Fig. 1). The inhibitory effect of various ANT-binding ligands as well as of a specific inhibitor of ANT is a strong indication for the involvement of the ANT in CrP uptake into the mitochondria.

CrP has three negative and one positive charge so that its net charge is 2-fold negative. Therefore, a presumed uptake of CrP^{2-} in exchange for ATP^{4-} or (ADP^{3-}) should be somehow dependent on the membrane potential in a way that CrP is accumulated in the mitochondria due to the negative potential inside. About tenfold accumulation was observed in all cases. However, the observed gradient $[ATP^{4-}/CrP^{2-}]_{cyt}/[ATP^{4-}/CrP^{2-}]_{mit}$ or $[ADP^{3-}/CrP^{2-}]_{cyt}/[ADP^{3-}/CrP^{2-}]_{mit}$ was far from the expected one according to the membrane potential and in fact, would correspond to electric potentials around zero (not shown). Consequently, compounds which lower the mitochondrial membrane potential and the protonmotive force, such as potassium in the presence of valinomycin, as well as FCCP, would not affect the accumulation of CrP in the mitochondria. This was in fact observed in our experiments (Table 1). These findings are not surprising in view of the extremely low rate of CrP-transport (see below) compared to the activity of the

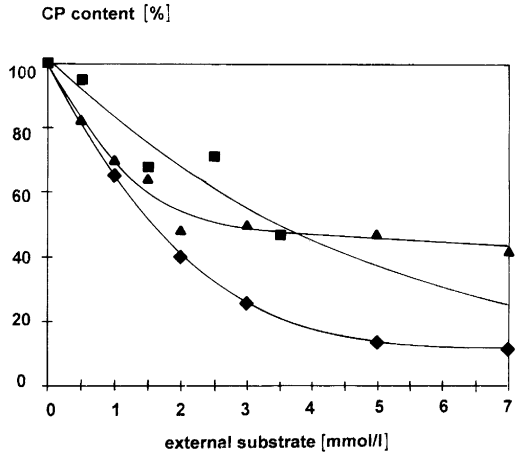

Fig. 1. Inhibition of CrP-uptake into isolated rat heart mitochondria by phosporylated compounds. 2-Phosphoglycerate (♦), phosphoenolpyruvate (■) and pyrophosphate (▲). Incubation as in Table 1, with varying inhibitor substrate concentrations. The experimental points are means of six experiments. Control is assigned to be 100%.

Mi-CK reaction and to the diffusion rate of CrP out of the intermembrane space. Earlier investigations on rat liver mitochondria[20], where it has been demonstrated, that the ATP/ADP-exchange is electrogenic were performed in the presence of oligomycin to avoid competition between adenine nucleotide synthesis and transport. The observed accumulation of CrP in the matrix space can also be explained in terms of kinetic control. According to the higher concentrations of ATP and ADP in the mitochondria (millimolars, compared to micromolars in the medium) nucleotide competition should decrease the probability of exit for CrP from the matrix. On the other hand, the low extramitochondrial concentration of these compounds facilitated the entrance of CrP into the mitochondria. The result of these processes will be a certain accumulation of CrP inside the mitochondrial matrix.

The effect of ADP on CrP accumulation is of particular interest: High concentrations of ADP inhibited the intramitochondrial accumulation of externally added CrP, or of CrP produced in the intermembrane space by Mi-CK (Table 2). However ADP is also necessary for the CrP-uptake. In fact, when phosphoenolpyruvate together with pyruvate kinase were added to the incubation medium as a sink for external ADP, CrP − accumulation in the sediment was completely abolished (Table 2). It was restored upon addition of ADP but again inhibited when external ADP exceeded 3 mM. In the presence of phosphoenolpyruvate/pyruvate kinase a high external ATP and low ADP not only inhibited ATP-export but consequently also the uptake of all molecules which can be imported in exchange for mitochondrial ATP.

Taken together, these experiments show that CrP is taken up by isolated heart mitochondria and that this uptake is sensitive to substrates and inhibitors of the ANT. The uptake probably occurs in exchange for mitochondrial ATP (or ADP, see below).

4.3. CrP-transport by ANT reconstituted in liposomes

Studies using the reconstituted ANT should unequivocally demonstrate the ability of the ANT to transport CrP. Since the reconstituted rat heart ANT appeared to be less active and more sensitive to inactivation than beef heart ANT, we used ANT isolated from beef heart mitochondria for studying

the CrP-uptake. The concentration of CrP in liposomes preloaded with 5 mM ATP and incubated in the presence of 2 mM CrP (see Section 2) was determined enzymatically and radioactively as 100 ± 20 μM at steady state. This was too low to perform an accurate determination of the uptake kinetics by enzymatic analysis; therefore, ^{32}P-CrP was used for uptake rate measurements.

The liposomes did not incorporate any radioactively labelled ATP or CrP in the absence of ANT-protein (not shown). The rates of ATP uptake initiated by reconstituted ANT (Table 3) were comparable to the rates found in the literature under similar conditions [23]. The process was only partially inhibited (~ 70%) by carboxyatractyloside. This is in line with the fact that a significant portion of the carrier after reconstitution is oriented in the opposite direction [24], thus being inaccessible to this inhibitor.

The CrP-uptake rate was 500–700-fold lower than the uptake rate of ATP, sensitive to CAT and increased at higher temperature (Table 3). Although the amount of CrP incorporated into reconstituted liposomes as well as the rate of its uptake were very low, the process was specific and was not caused by binding to the phospholipid vesicles or to the translocase protein: Control liposomes (without ANT) showed no uptake at all and the number of CrP molecules found in the liposomes exceeded the binding capacity of the ANT molecules by the factor 15 (17.5 pmol ANT molecules/ml incubation medium, with a binding capacity of 1, versus 290 pmol CrP/ml). This ratio may actually have been significantly higher taking into account, that only around 10% of the incorporated ANT is functionally active [25].

Table 3
^{3}H-ATP and ^{32}P-CrP uptake rates by reconstituted bovine heart adenine nucleotide translocase

Liposomal concentration and incubation temperature	v (μmol·min^{-1}·mg ANT protein^{-1})
ATP-uptake:	
5 mM ATP, 20°C	1.44 ± 0.14
5 mM ATP, 150 μM CAT, 20°C	0.46 ± 0.05
CrP-uptake:	$v \cdot 10^{-3}$
5 mM ATP, 20°C	2.48 ± 0.31
5 mM ATP, 150 μM CAT, 20°C	– [a]
5 mM ADP, 20°C	3.04 ± 0.28
5 mM ADP, 150 μM CAT, 20°C	0.30 ± 0.02
5 mM ADP, 37°C	4.34 ± 0.42

$n = 4 \pm$ S.E.M.
[a] Not detectable.

According to our present knowledge, there is no physiological role for mitochondrial matrix creatine phosphate. This does not exclude, however, that it is taken up by mitochondria under certain conditions. The presence of creatine in the mitochondria has been described by several groups earlier [3,6,26,27]. The present data clearly show, that the mitochondrial ANT transports CrP, though with low efficiency, but sufficiently to accumulate a certain amount within the mitochondria. The results obtained with mitoplasts exclude the possibility that CrP found in mitochondria is exclusively associated with the intermembrane space.

Finally it should be noted that the finding of CrP uptake into mitochondria, which is mediated by the ANT, might be an additional argument for the close interaction of the Mi-CK and the ANT at the inner mitochondrial membrane and thus for the action of a creatine shuttle [1]. In the context of the latter, it would represent a leak in the transfer of energy rich phosphate from mitochondria to the myofibrils.

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