

Ca²⁺ Activation Kinetics of the Two Aspartate-Glutamate Mitochondrial Carriers, Aralar and Citrin

ROLE IN THE HEART MALATE-ASPARTATE NADH SHUTTLE*

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Ca²⁺ regulation of the Ca²⁺ binding mitochondrial carriers for aspartate/glutamate (AGCs) is provided by their N-terminal extensions, which face the intermembrane space. The two mammalian AGCs, aralar and citrin, are members of the malate-aspartate NADH shuttle. We report that their N-terminal extensions contain up to four pairs of EF-hand motifs plus a single vestigial EF-hand, and have no known homolog. Aralar and citrin contain one fully canonical EF-hand pair and aralar two additional half-pairs, in which a single EF-hand is predicted to bind Ca²⁺. Shuttle activity in brain or skeletal muscle mitochondria, which contain aralar as the major AGC, is activated by Ca²⁺ with S_{0.5} values of 280–350 nM; higher than those obtained in liver mitochondria (100–150 nM) that contain citrin as the major AGC. We have used aralar- and citrin-deficient mice to study the role of the two isoforms in heart, which expresses both AGCs. The S_{0.5} for Ca²⁺ activation of the shuttle in heart mitochondria is about 300 nM, and it remains essentially unchanged in citrin-deficient mice, although it undergoes a drastic reduction to about 100 nM in aralar-deficient mice. Therefore, aralar and citrin, when expressed as single isoforms in heart, confer differences in Ca²⁺ activation of shuttle activity, probably associated with their structural differences. In addition, the results reveal that the two AGCs fully account for shuttle activity in mouse heart mitochondria and that no other glutamate transporter can replace the AGCs in this pathway.

Mitochondrial carriers, MCs,³ are integral proteins of the mitochondrial inner membrane that function in the shuttling of

metabolites, nucleotides, and cofactors between the cytosol and mitochondria (1), a subset of which are the Ca²⁺ binding mitochondrial carriers (CaMC) (2). CaMCs fall into two groups: the aspartate/glutamate carriers (AGC) (2–5) and the ATP-Mg/inorganic phosphate (P_i) carriers (ATP-Mg/P_i carriers), also named SCaMC (for short CaMC) (6, 7). The two mammalian AGCs, aralar (2), also named aralar1 (AGC1) (3) and citrin (AGC2), are members of the malate-aspartate NADH shuttle (MAS) and citrin, the liver AGC2, is also a member of the urea cycle (8). The transport reaction catalyzed by the AGCs is the only irreversible step in MAS (8, 9) and a main point of regulation. The activity of both types of CaMCs is stimulated by Ca²⁺ acting on the external face of the inner mitochondrial membrane (5, 12, 13).

Ca²⁺ signaling in mitochondria is important for regulating mitochondrial function in response to intra- and extracellular cues. The main mechanism whereby Ca²⁺ modulates mitochondrial function involves Ca²⁺ entry in mitochondria via the Ca²⁺ uniporter (CU) or rapid uptake mode (RAM) mechanisms (14–16) followed by the activation by Ca²⁺ of three dehydrogenases (pyruvate, isocitrate, and α -ketoglutarate) in the mitochondrial matrix (17). This causes an increase in the mitochondrial NADH/NAD ratio. The CaMCs emerge as new, CU-independent pathways to transmit calcium signals in mitochondria by way of increases in the mitochondrial NADH/NAD ratio through the AGC-MAS pathway or through increases in mitochondrial ATP upon stimulation of the ATP-Mg/P_i carriers (18, 19).

Ca²⁺ regulation of CaMCs is provided by their long N-terminal extensions, which harbor a number of EF-hand motifs and face the intermembrane space (2–4, 6, 18). Indeed, the N-terminal extensions of AGCs (2, 3) and those of the rabbit SCaMC-1 ortholog Efinal (20) and rat SCaMC-2 ortholog MCSC (21) have been shown to bind Ca²⁺. However, beyond the presence of EF-hand binding motifs, the N-terminal extensions of AGCs and SCaMCs are quite different. Those of SCaMCs contain four EF-hand motifs and have high homology

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³ The abbreviations used are: MC, mitochondrial carrier; α KGDH, α -ketoglutarate dehydrogenase; AGC, aspartate/glutamate carrier; CaM, calmodulin; CaMCs, Ca²⁺ binding mitochondrial carriers; CU, calcium uniporter; DH,

dehydrogenase; EAAT1, excitatory amino acid transporter type I; GOT, glutamate-oxaloacetate transaminase; HMM, hidden Markov models; IMM, isolation media for muscle mitochondria; IMH, isolation media for heart mitochondria; MAS, malate-aspartate NADH shuttle; MSA, multiple sequence alignment; MSK, mannitol-sucrose-K⁺ medium; MDH, malate dehydrogenase; OGC, oxoglutarate-malate carrier.

to calmodulin (CaM) and CaM-related proteins (6). Unlike SCaMCs, the N-terminal extensions of AGCs (2, 3) are not closely related to CaM or any of the known members of the CaM superfamily (23). In the human proteome, the closest related protein is calcium binding atopy-related autoantigen, CBARA1 (24), with 23% identity and 43% similarity with aralar, and 24 and 40% with citrin, respectively. The AGCs catalyze an electrogenic 1:1 exchange of aspartate for glutamate plus a proton (8, 9, 25–28). Brain mitochondria have aralar as the only AGC isoform, and we have recently shown that MAS activity in brain mitochondria is stimulated by extramitochondrial Ca²⁺ with an $S_{0.5}$ for Ca²⁺ activation of around 320 nM (29), *i.e.* below the Ca²⁺ concentrations at which the calcium uniporter is known to be active. This opened up the possibility that neuronal MAS might be activated by cytosolic Ca²⁺ signals below the threshold of the calcium uniporter. Indeed, with the use of two photon microscopy imaging of mitochondrial NAD(P)H and neuronal cultures derived from aralar-deficient mice (30), we have shown that small Ca²⁺ signals that do not reach the mitochondrial matrix were able to activate NADH accumulation in mitochondria from control but not aralar-deficient neurons (29) under conditions of lactate utilization.

Aralar and citrin have differences in primary sequence, especially along their N-terminal halves but a very high identity along their MC homology sequence (3), which explains the very similar transport activity of both isoforms (5). In the present work, we have studied whether the differences in their N-terminal sequences could lead to differences in calcium regulation. By studying Ca²⁺ activation of MAS activity in tissues expressing the individual isoforms, we conclude that citrin has a smaller capacity to be activated by calcium than aralar, with an $S_{0.5}$ of about 100–150 nM.

We have next used this information to study the role of aralar and citrin in heart MAS activity. MAS is the dominant NADH shuttle in heart, (31–36), including human heart (37). Unlike skeletal muscle, which expresses only aralar, the heart shows high levels of both isoforms, with a preferential enrichment of aralar in atria (38). The role of MAS activity in adult heart is somewhat controversial. It is lower in adults than in neonates, and this has been attributed to a postnatal decrease in the levels of the oxoglutarate-malate carrier (OGC) observed in pig and rabbit, which could limit shuttle function in adult heart (36, 39). This contrasts with long standing evidence that AGC rate limits MAS function (8). Moreover, recent reports have introduced added complexity to heart MAS activity. Ralphe *et al.* (40, 41) have proposed that an isoform of the excitatory amino acid transporter type 1 (EAAT1) is present in heart mitochondria where it acts as a glutamate carrier within MAS and is responsible for the up-regulation of MAS by thyroid hormone.

To get a better understanding of the role of aralar and citrin in MAS heart activity and regulation by calcium, we have studied the effects of the selective deficiency of the two isoforms, aralar and citrin by employing mice with targeted disruption of each of the two genes. The results underscore a predominant role of aralar as the isoform that confers Ca²⁺ regulation to heart MAS activity and strongly suggest that no other carrier can substitute for the AGCs in MAS.

EXPERIMENTAL PROCEDURES

Prediction of EF-hands in Aralar/AGC1 and Citrin/AGC2 Sequences—A multiple sequence alignment (MSA) of known EF-hands was extracted from the Pfam data base (42) and used to build hidden Markov models (HMMs) libraries. The HMMs were then used to search the N-terminal sequences of the aralar-like subfamily of proteins previously obtained from the Uniprot data base (43), using a methodology similar to that employed by Truong and Ikura (44) to study the distribution of EF-hand protein superfamilies. The putative structure of discovered EF-hands was contrasted with the secondary structure prediction of MSAs of aralar and citrin sequences to ensure structural compatibility.

Materials—Fura2-pentapotassium salt and CalciumGreen-5N were from Molecular Probes. MDH was from Boehringer, digitonin from Fluka, bovine serum albumin (fraction V) from Intergen, and the rest of the reagents were from Sigma.

Animals—The mouse strains used were C57BL/6, C56BL/6xSv129, and Sv129 obtained from Harlan. Aralar^{+/+}, Aralar^{+/-}, and Aralar^{-/-} mice were generated as described previously (30) and maintained in a C57BL/6xSv129 mixed background.

Mice with targeted disruption of the citrin gene were obtained by gene trapping at Lexicon Genetics (The Woodlands, TX) in SVJ129 ES cells using the insertion vector method that was based on the gene trap technology of Lexicon (45) as described previously for Aralar-deficient mice (30). Slc25a13^{+/-} ES cells were injected into C57BL blastocysts, and chimeric mice were bred to C57BL (albino) wild-type mice. Slc25a13^{+/-} mice with a SVJ129xC57BL/6 inbred genetic background were backcrossed to C57BL/6 mice for at least 8 generations. The resulting slc25a13^{+/-} (Citrin^{+/-}) offspring were interbred to produce slc25a13^{-/-} (Citrin^{-/-}) mice and Citrin^{+/+} mice in a C57BL/6 genetic background.

All mouse strains and male Wistar rats were housed with a 12-h light cycle and fed ad libitum on a standard chow. Animals were sacrificed by cervical dislocation, the tissue of interest quickly dissected and kept on ice-cold media for mitochondrial isolation carried out at 4 °C. Rats were 3-months old. Unless indicated otherwise, mice were used at 15 days to allow comparisons between strains, as mice from one of the strains used, Aralar^{-/-}, do not survive beyond 20 days (30). All animal procedures were approved by the Committee for Animal Experimentation, Kagoshima University and European guidelines.

Genotyping—Aralar-deficient mice were genotyped as described previously (30). After determining the insertion site of the trap vector, the citrin genotype was analyzed by the duplex-PCR method. To this end, the sense primer -mCit Ex7F (5'-CgCTCCTTAACAACATggAAC-3')/mCit 3'LTR-F3 (5'-gTTCTCTAgAAACTgCTgAgg-3') within the exon 7/vector, and common antisense primer-mCit int 7B1 (5'-TCTgAT-CAAggTCTggAggAC-3') within intron 7 were used to amplify both the wild-type and mutant alleles, and the PCR products were visualized following separation on a 2% agarose gel.

Brain and Liver Mitochondria—Whole brain or liver were washed, minced, and homogenized in IM (mM: 250 sucrose, 25

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Hepes, 10 KCl, 1 EDTA, 1 EGTA, 1.5 MgCl₂, 1 dithiothreitol, 1 phenylmethylsulfonylfluoride, 1 iodoacetamide, and 0.1% bovine serum albumin, pH 7.4). Nuclei and cell debris were first removed by 10 min of centrifugation at 700 × *g*, and mitochondrial fractions were then spun down (15 min, 10,000 × *g*), and washed in MSK (mM: 75 mannitol, 25 sucrose, 5 potassium phosphate, 20 Tris-HCl, 0.5 EDTA, 100 KCl, and 0.1% bovine serum albumin, pH 7.4). Mitochondria were resuspended in MSK and kept on ice until used. Brain mitochondrial fractions correspond to crude preparations containing synaptosomes and free mitochondria (46).

Skeletal Muscle Mitochondria—Skeletal muscle mitochondria were obtained as described by Rolfe *et al.* (47) with minor modifications. Skeletal muscle was obtained from the four limbs, washed, and minced in IMM (mM: 100 sucrose, 9 EDTA, 1 EGTA, 100 Tris-HCl, 46 KCl, pH 7.4). After 10 min of incubation with Nagarse (0.4 mg/ml IMM; stirring on ice), the tissue was homogenized and processed as described for brain and liver.

Heart Mitochondria—Hearts were treated the same way as skeletal muscle, except that the medium for isolation was IMH, mM: 230 manitol, 70 sucrose, 1 EDTA, 5 Tris-HCl, pH 7.4 (48). Proteins were measured by the Bradford method.

Reconstitution of the Malate-Aspartate NADH Shuttle Activity in Mitochondria—The reconstitution of the malate-aspartate NADH shuttle was based on published procedures (49–51), modified as described in Pardo *et al.* (29) and Jalil *et al.* (30). Mitochondrial fractions (0.1–0.15 mg of protein, liver, and brain or 0.020–0.030 mg of protein, heart and muscle) were suspended in 3 ml of MSK (and 100 μM digitonin, in the case of brain preparations), and the shuttle was reconstituted as described (29, 30). MAS activity was started by the addition of 5 mM glutamate, and was determined from the decay in NADH fluorescence at 37 °C under constant stirring. To correct for any possible changes in free calcium concentration along the assay, the experiments have been also carried out in EGTA-calcium-buffered MSK medium, in which EDTA was replaced with 0.5 mM EGTA.

Free Calcium Calibration—The free calcium concentrations were determined fluorimetrically with Fura-2 (below 1 μM free calcium) and Calcium-Green (above 1 μM free calcium) as described (29). The concentrations of Fura-2 (*K*_d = 224 nM; excitation, 340 and 380 nm; emission, 510 nm) and Calcium-Green (*K*_d = 14 μM; excitation, 506 nm; emission, 532 nm) were 5 and 0.1 μM, respectively. The free calcium concentration was obtained by established procedures for ratiometric or non-ratiometric probes (52, 53).

Western Blot Analysis—Proteins were subjected to SDS-PAGE (8%), transferred onto nitrocellulose membranes, and dyed with Ponceau Red, to assess for protein load. The membranes were blocked and incubated for 2 h with antibodies raised against either aralar (2) (1:10,000) or citrin (3) (1:2000). An antibody against β-F1ATPase (54) (1:20,000, a gift from Prof. J. M. Cuezva) was used as control for mitochondrial protein load. After extensive washing, membranes were incubated for 45 min with a goat anti-rabbit IgG conjugated with horseradish peroxidase (1:10000, Bio-Rad), and the signal was

detected by chemiluminescence (ECL, PerkinElmer), as described earlier (38).

RESULTS

EF-hands in Citrin and Aralar—Using an HMM-based methodology, similar to those used by Truong and Ikura (44) in the superfamily of EF-hands-containing proteins, we found a surprising amount of EF-hand compatible motifs in the sequence of both aralar (AGC1) and citrin (AGC2). Up to nine different EF-hands were predicted using this procedure, all located in the first 330–340 amino acids of members of this family of proteins (Fig. 1), the ninth EF-hand (positions 302–330 in human aralar), being not equally consistent. The sequence of these long N-terminal extensions in aralar and citrin, and the particular spacing of the set of EF-hands have no known homolog within the large family of calcium-binding proteins.

The analysis of sequence signatures of known Ca²⁺-binding proteins (22) compared with those of the proposed eight EF-hands of aralar and citrin, yields a number of predictions about their functional characteristics (Fig. 1). The first EF-hand, EF1 (residues 13–46 in human aralar), is predicted to be active both in aralar and citrin. In fact, the presence of Glu or Asp residues in all signature positions 1, 3 (Asn in citrin), 5, and 12 suggests an enhanced Ca²⁺ coordination capability of EF1. A similar situation, but showing a more classical signature with Thr in coordination position 3, corresponds to EF2 (residues 54–84 in human aralar). EF4, EF6, and EF7 (residues 127–154, 194–223, and 228–259 in human aralar) appear to be nonfunctional, both in aralar and citrin.

Interestingly, EF3 (corresponding to residues 88–117 and 89–118 in human aralar and citrin, respectively) and EF5 (positions 159–189 (alarar) and 159–190 (citrin)) are predicted to differ in terms of Ca²⁺ binding, the canonical signature being present in aralar, but not in citrin (Ref. 22 and Fig. 1). Prediction of functionality of EF8 in aralar (residues 267–295) and citrin (residues 268–296) based on the presence of the Ca²⁺ binding signature gave an unclear result. This structure lacks aspartic or glutamic residues located at position 1, being substituted by Asp²⁷⁶ (alarar) or Asp²⁷⁷ (citrin) located in motif position 0. Whether or not this variant EF-hand structure could maintain functional ion binding properties cannot be deduced from sequence analysis.

The contribution to Ca²⁺ binding of the canonical EF-hand motifs in aralar and citrin, respectively, is unknown. However, the sequence differences between the two isoforms may be associated with differences in Ca²⁺ activation among the two AGCs.

Calcium Activation of the Malate-Aspartate NADH Shuttle in Liver, Brain, and Skeletal Muscle Mitochondria—MAS activity in rat brain mitochondria is activated around 3-fold by Ca²⁺, with an *S*_{0.5} for activation of around 320 nM (29). Calcium activation of mitochondrial dehydrogenases, such as isocitrate DH, αKGDH, and FAD-linked glycerol-3-phosphate DH, leads to an increase in substrate affinity, with no changes in *V*_{max} (see Refs. 19 and 55 for reviews). In contrast with this mechanism, the results in Fig. 2, *A* and *B* show that Ca²⁺ activation of MAS in mouse brain mitochondria does not result in changes in the

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nounced, resulting in a 3-fold increase in activity (from 26.7 ± 2.64 to 86.18 ± 5.2 nmol NADH min⁻¹ mg prot⁻¹), with an $S_{0.5}$ of 324 ± 57.4 nM (29). Ca²⁺ activation of MAS activity and $S_{0.5}$ values did not vary when assays were performed in calcium-buffered media (results not shown). We have also studied Ca²⁺ activation of MAS activity in mouse brain and skeletal muscle

mitochondria (with aralar as single AGC) and mouse liver mitochondria (citrin as major single AGC) (2, 3, 4, 38). Ca²⁺-stimulated MAS activity in all tissues (Fig. 3) and the $S_{0.5}$ for activation were higher in brain and skeletal muscle, which have aralar as AGC, compared with liver, which has citrin (316 ± 68 nM brain mitochondria, 280 ± 26 nM for muscle mitochondria, and 120 ± 20 nM liver), in agreement with the results obtained in rat (Fig. 2C). On the other hand, the total activation was the same in all tissues, about 2.5-fold.

The higher $S_{0.5}$ value for Ca²⁺ activation in tissues with aralar instead of citrin as the major AGC isoform suggests that the structural differences among isoforms may be related to their differences in Ca²⁺ activation.

Ca²⁺ Activation of Heart Malate-Aspartate NADH Shuttle—MAS is the dominant NADH shuttle in heart (31–36, 56, 57), and the two isoforms aralar and citrin are expressed in rat and mouse heart (2–4, 38). However, the role of each isoform in MAS function remains unknown. Recent reports have suggested that the AGCs are only one of the possible players in the heart malate-aspartate shuttle at the step of glutamate uptake in mitochondria, and that the EAAT1, a plasma membrane glutamate carrier from brain, is localized to heart mitochondria and functions as a glutamate carrier within MAS (40, 41). Therefore, it is necessary to clarify the contribution and role of each of the two AGC isoforms to understand MAS function in heart.

To address this point we have studied MAS activity in mouse strains with disrupted citrin or aralar genes. Citrin-deficient mice were generated by gene trapping, the gene trap vector inserted in intron 7 of *slc25a13* gene (Fig. 4, A and B), and have a dose-dependent reduction in citrin mRNA and citrin protein levels in liver (Fig. 4, C and D). As observed for a different strain of citrin-deficient mice (58), disruption of the citrin gene also results in a dose-dependent reduction in citrin mRNA and protein levels in kidney and heart without any compensatory change in aralar levels (Fig. 4, C and D). Aralar-deficient mice were also generated by gene trapping as described previously (30). Aralar-deficient and citrin-deficient mice have different backgrounds (hybrid C57BL/6xSv129 and pure C57BL/6, respectively), that are named aralar wild-type and citrin wild-type for simplicity.

Extramitochondrial Ca²⁺-activated MAS in heart mitochondria from both wild-type strains as shown in Fig. 5 and Table 1. The $S_{0.5}$ for activation was 323 ± 44 nM in wild-type aralar and

367 ± 77 nM in wild-type citrin strains. MAS activity in heart mitochondria from Aralar^{+/-} mice decreased to about one-half, with no change in Ca²⁺ activation (Fig. 5A), and it was drastically reduced in heart mitochondria from Aralar^{-/-} mice, which have only citrin as the AGC isoform (from 207 ± 31 to 54 ± 13 nmol NADH min⁻¹ mg prot⁻¹ as V_{max} values in wild-type and Aralar^{-/-}, respectively). Strikingly, Ca²⁺ activation of MAS was almost lost in Aralar^{-/-} mice, the

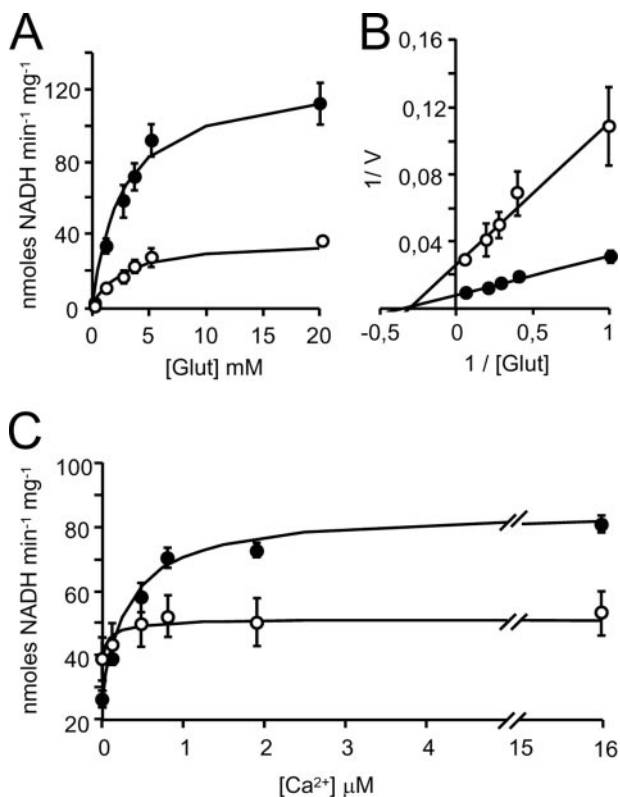


FIGURE 2. Characterization of the malate-aspartate NADH shuttle. MAS was reconstituted with brain mitochondria from 3-month-old mice, and activity was followed by the decrease in NADH fluorescence after glutamate addition. A and B, variation of MAS activity with glutamate concentration at two free calcium concentrations: 0 μ M (i.e. below 22 nM) calcium and 10 μ M calcium (open and closed circles, respectively). The corresponding Lineweaver-Burk plot is shown in B. C, kinetics of calcium activation of MAS in liver mitochondria (open circles) or brain mitochondria (closed circles) from 3-month-old rats. The data obtained were fit by nonlinear regression to the following equation: $V = V_0 + \{((V_{max} - V_0) \times [Ca^{2+}]) / (S_{0.5} + [Ca^{2+}])\}$, where V is MAS activity at each $[Ca^{2+}]$, V_0 is basal activity at no (0 nM) $[Ca^{2+}]$, V_{max} is maximal activity, and $S_{0.5}$ is the calcium concentration, which generates half-maximal activation), using Sigma Plot v.9 (Jaendell Scientific). The overall goodness of each fit was evaluated in terms of coefficient of correlation ($R^2 > 0.9$). Results are mean \pm S.E. of three independent experiments performed in triplicate.

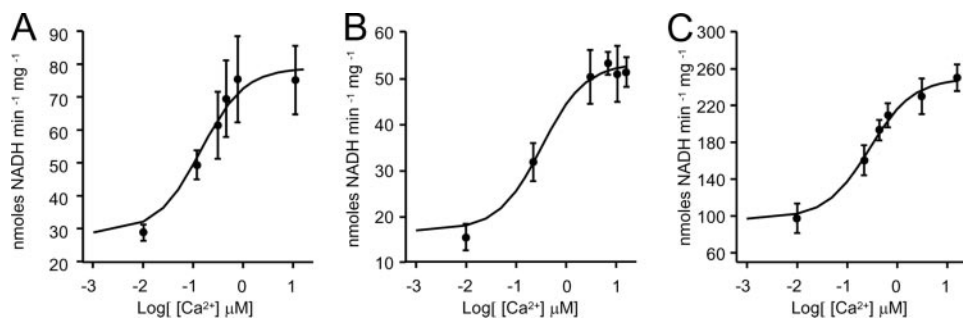


FIGURE 3. Ca²⁺ stimulation of MAS activity in mouse tissues. MAS was reconstituted with liver (A), brain (B), or skeletal muscle (C) mitochondria derived from 15-day-old mice, in the presence of Ruthenium Red (200 nM) at different free calcium concentrations. Data are mean \pm S.E. of three experiments performed in triplicate.

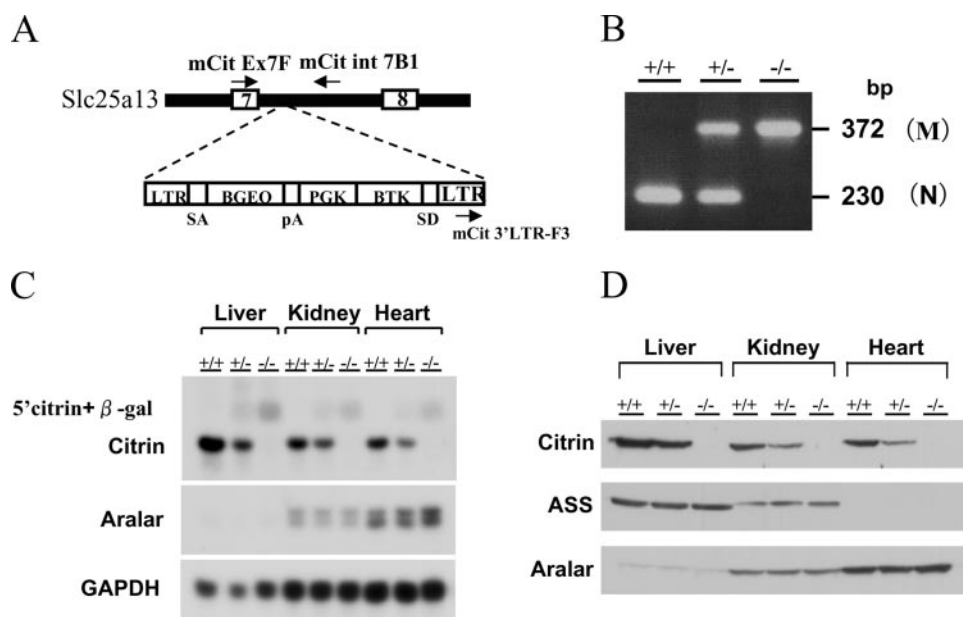


FIGURE 4. Generation of Citrin^{-/-} mice. A, characterization of the gene trap vector insertion site in the mutant *slc25a13* gene. LTR, long terminal repeat; SA, splicing acceptor site; BGEO, β -galactosidase + neomycin-resistant gene cassette; pA, polyadenylation sequence; PGK, phosphoglycerate kinase-1 promoter; BTK, Brutton tyrosine kinase cassette, SD, splicing donor site. B, genotyping of citrin-deficient mice. A representative litter containing Citrin^{+/+} (+/+), Citrin^{+/-} (+/-), and Citrin^{-/-} (-/-) mice is shown. For the duplex-PCR genotyping method, sense primer-mCit Ex7F/mCit 3'LTR-F3 within the exon 7/vector, and common antisense primer-mCit int 7B1 within intron 7 (see A) were used to amplify both the wild-type (N: 230 bp) and mutant (M: 372 bp) alleles, and the PCR products were visualized following separation on a 2% agarose gel. C, Northern blot analysis. 20 μ g of total RNA per lane were used. Citrin, fusion gene (5'-Cit + β -galactosidase), aralar, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were detected. D, Western blot analysis of citrin, ASS, and aralar. 4 μ l of 10% homogenate per lane were resolved on a 10% SDS-polyacrylamide gel and immunoblotted with anti-citrin, anti-ASS, and anti-aralar antibodies.

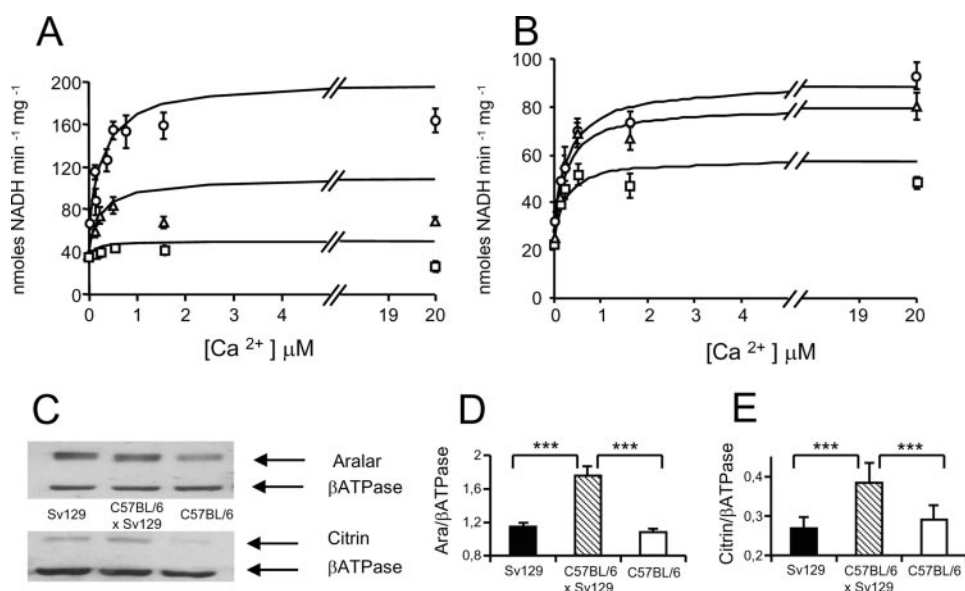


FIGURE 5. Ca²⁺ activation of MAS in heart mitochondria. MAS activity was determined in heart mitochondria isolated from 15-day-old mice. A, kinetics of Ca²⁺ activation in mitochondria from Aralar^{+/+} (circles), Aralar^{+/-} (triangles), and Aralar^{-/-} (squares) mice in C57BL/6xSv129 background. B, kinetics of Ca²⁺ activation in mitochondria from Citrin^{+/+} (circles), Citrin^{+/-} (triangles), and Citrin^{-/-} (squares) mice in C57BL/6 background. C, isolated heart mitochondria from wild-type mice of different backgrounds were probed by Western blotting against aralar (1:10000) or citrin (1:2000) and with antibodies against β F1-ATPase (1:20000). D and E, histograms representing the ratio of aralar/ β F1-ATPase and citrin/ β F1-ATPase, respectively as means \pm S.E. (n = 4). Significant differences to the C57BL/6xSv129 background is denoted by *** (p < 0.005).

maximal increase in activity being only 1.3-fold (Fig. 5A), with an $S_{0.5}$ of 94 ± 28 nM. This suggests that in heart mitochondria, Ca²⁺ activation is mainly conferred by the presence of aralar.

mixed C57BL/6xSv129 and pure C57BL/6 strains. As shown in Table 2, one dose of aralar or citrin contributes an activity of about 76.5 or 27 nmol NADH min⁻¹ mg protein⁻¹, respec-

MAS activity in heart mitochondria from Citrin^{+/-} and specially Citrin^{-/-} mice was reduced with respect to the wild-type (V_0 and V_{max} 34 ± 5 and 90 ± 9 nmol NADH min⁻¹ mg prot⁻¹, respectively, in the wild type, versus V_0 and V_{max} 24 ± 1.6 and 66 ± 4.3 nmol NADH min⁻¹ mg prot⁻¹, respectively, in Citrin^{-/-}) but still activated by Ca²⁺ with an $S_{0.5}$ of 270 ± 38 nM; essentially the same as the wild-type strain (Fig. 5B). Because aralar is the only AGC isoform in the heart of Citrin^{-/-} mice, this result further confirms that calcium activation of MAS in heart mitochondria is conferred mainly by aralar.

Strain-dependent Variations in Aralar and Citrin Levels—Unexpectedly, MAS activity in aralar and citrin wild-type mice was quite different, about 2-fold higher in aralar wild-type animals (note the different scales on the y-axis in Fig. 5, A and B and Table 1). As indicated above, the genetic backgrounds of the two types of null animals were different. Aralar wild-type mice, a C57BL/6xSv129 mixed strain (30), whereas citrin-deficient mice, a pure C57BL/6 strain. To study the cause of increased MAS activity in the mixed background, aralar and citrin levels were determined by Western blotting using the β -subunit of F1-ATPase as the mitochondrial marker in pure Sv129 and C57BL/6 and mixed C57BL/6xSv129 backgrounds (Fig. 5C). Both citrin and aralar were significantly increased in heart mitochondria, from the mixed background with respect to either of the pure backgrounds (Fig. 5, D and E). A similar increase in citrin levels was noted in liver mitochondria (results not shown). However, brain aralar levels were the same in the three backgrounds (results not shown). From these results, we have calculated the contribution of single doses of aralar and citrin to MAS activity in heart mitochondria from

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TABLE 1

MAS activity in mouse strains

MAS was reconstituted with heart mitochondria from 15-day-old mice of C57BL/6xSv129 or C57BL/6 strains and different aralar and citrin genotypes. Data are mean ± S.E. of three independent experiments performed in triplicate. Significant differences with respect to Aralar^{+/+} (Student's *t* test) are indicated.

Strain	Genotype	S _{0.5} [Ca ²⁺] _{free}	V _{max}	Activation fold
		<i>nM</i>	<i>nmol NADH min⁻¹ mg prot⁻¹</i>	
C57BL/6xSv129	Aralar ^{+/+}	323 ± 44	207 ± 31	2.9
	Aralar ^{+/-}	223 ± 44	110 ± 22 ^a	2.9
	Aralar ^{-/-}	94 ± 28 ^b	54 ± 13 ^b	1.36
C57BL/6	Citrin ^{+/+}	367 ± 77	90 ± 9 ^a	2.7
	Citrin ^{+/-}	269 ± 4	81 ± 4,6 ^a	3.1
	Citrin ^{-/-}	270 ± 38	66.07 ± 4.3 ^a	2.7

^a *p* < 0.05.

^b *p* < 0.005.

TABLE 2

Calculated contributions of individual AGC isoforms to MAS activity in heart mitochondria

The contribution of one dose of Aralar to MAS activity in C57BL/6xSv129 mice was calculated as the difference in MAS activity between Aralar^{+/+} and Aralar^{-/-} mice divided by two. The contribution of one dose of citrin in the C57BL/6 strain was calculated in a similar way from the activities in the Citrin^{+/+} and Citrin^{-/-} mice. In C57BL/6xSv129 mice, the contribution of one dose of citrin was calculated as half of the MAS activity in Aralar^{-/-} mice, and a similar procedure was used to calculate one dose of aralar in C57BL/6 citrin^{-/-} mice. The V_{max} numbers are shown. Total MAS activity in each background was calculated as the sum of four independent equations ($V = V_0 + \{((V_{max} - V_0) \times [Ca^{2+}]) / (S_{0.5} + [Ca^{2+}])\}$), each one corresponding to one of the four possible AGC doses, in which V₀ and V_{max} were the calculated values, S_{0.5} for aralar was that obtained in the Citrin^{-/-} mouse (Table 1), and the S_{0.5} for citrin was that obtained in the Aralar^{-/-} mouse (Table 1). The total MAS values thus generated were fit by nonlinear regression to a single equation. Calculated S_{0.5} for MAS activity in heart mitochondria from Aralar^{+/+} (C57BL/6xSv129) and Citrin^{+/+} (C57BL/6) mice obtained from the fit are shown. Aralar/βATPase and Citrin/βATPase ratios for each background are from the data in Fig. 5.

	Calculated activity per dose of aralar	Calculated activity per dose of citrin	Calculated S _{0.5} in AGC ^{+/+} mice	Aralar/βATPase	Citrin/βATPase
	<i>nmol NADH min⁻¹ mg prot⁻¹</i>	<i>nmol NADH min⁻¹ mg prot⁻¹</i>	<i>nM</i>		
C57BL/6xSv129	76.5	27	217	1.75 ± 0.11	0.38 ± 0.05
C57BL/6	33	12	249	1.07 ± 0.04	0.29 ± 0.03
Ratios	2.3	2.2		1.6	1.3

tively, to heart MAS activity in C57BL/6xSv129 mice, but only about half those values in C57BL/6 strains. The difference in activities between strains (about 2-fold each) agrees with the increase in aralar and citrin protein levels in the mixed C57BL/6xSv129 background observed in Western blots (about 1.4-fold) (Table 2). Moreover, the kinetics of Ca²⁺ activation in Aralar^{+/+}, Aralar^{+/-}, Citrin^{+/+}, and Citrin^{+/-} mice closely matches that obtained, assuming that individual doses of each AGC contribute independently to the activity and Ca²⁺ regulation of MAS (compare calculated S_{0.5} values for Aralar^{+/+} and Citrin^{+/-} mice in Table 2 with experimental S_{0.5} values in Table 1). In summary, the results suggest that MAS activity in mouse heart mitochondria can be fully accounted for by the two AGCs, aralar and citrin, as glutamate carriers, because the disruption of each results in a residual MAS activity, which agrees with the level of the undisrupted AGC isoform. Moreover, each isoform appears to contribute independently to MAS activity and Ca²⁺ regulation. Thus, while other mitochondrial glutamate carriers may be present in heart mitochondria (the glutamate/hydroxyl carrier (59) or the plasma membrane EAAT1 reported to be present in rat heart mitochondria (40, 41)), our results do not support that glutamate carriers that are different from the AGCs function in mouse heart MAS.

DISCUSSION

We report that the AGC members of the CaMC subfamily belong to a novel family of EF-hand Ca²⁺-binding proteins. These proteins contain four pairs of EF-hands, and a single nonfunctional hypothetical EF9. Most EF-hand Ca²⁺ binding motifs occur in pairs, and the two-EF-hand domain is considered to be the functional unit (60, 61). In aralar and citrin, only one of these pairs, made up of EF1 and EF2, is predicted to be

functional, and deletion studies suggest that in citrin, Ca²⁺ binding is mostly conferred by the EF1-EF2 pair (3), suggesting that the remaining EF-hands do not play a major role in the absence of EF1-EF2. However, aralar contains two additional EF half-pairs, *i.e.* two pairs of EF-hands, EF3-EF4 and EF5-EF6, in which only one EF is predicted to bind Ca²⁺, occupying the odd position. It is possible that these extra half-pairs contribute to modulate the calcium affinity of the EF1-EF2 pair. Thus, the presence of these two additional EF half-pairs in aralar may result in Ca²⁺ binding properties that are different than those of citrin.

Indeed, we have found a consistent difference between Ca²⁺ activation kinetics of MAS in mitochondria containing either aralar or citrin as the only AGC isoform. The S_{0.5} for activation in liver, which contains only citrin, is about 100–150 nM (rat and mouse liver: 142 nM and 120 nM, respectively), but it is significantly higher, 280–350 nM in brain and skeletal muscle mitochondria, which contain only aralar (rat and mouse brain 324 nM and 316 nM, respectively; skeletal muscle 280 nM). Although these results cannot exclude that interactions with other yet unknown proteins in the intermembrane space are involved in defining the Ca²⁺ activation values, they suggest that the structural differences among isoforms are associated with their different sensitivity to Ca²⁺.

A more straightforward proof of the role of structural differences between aralar and citrin in explaining the difference in Ca²⁺ regulation arises from the study in the heart, an organ where both isoforms are expressed at high levels, using mice deficient in the two isoforms. While the loss of citrin results in MAS activity with similar Ca²⁺ activation kinetics as the wild-type animals, and an S_{0.5} close to the aralar values (280–350

nM), the loss of aralar, which results in MAS activity exclusively dependent on citrin, is associated with a drastic loss of Ca²⁺ activation of MAS in heart mitochondria and an $S_{0.5}$ for activation corresponding to the citrin values (100–150 nM). This proves that the Ca²⁺ activation properties of citrin and aralar when tested in one tissue, the heart, are indeed quite different; a result that strongly emphasizes the notion that the differences between the two AGCs are caused by their structural differences rather than to tissue-specific interactions.

On the other hand, the variations of Ca²⁺ activation values for the same AGC in different tissues (citrin in rat and mouse liver and aralar-deficient mouse heart, aralar in brain, and skeletal muscle or citrin-deficient heart), may reflect tissue-specific interactions with yet unknown proteins in the intermembrane space, or the formation of homodimers and tetramers in some of these tissues as has been recently reported (62).

What is the functional role of these differences in Ca²⁺ activation between isoforms? It is self-evident that an $S_{0.5}$ close to resting cytosolic Ca²⁺ levels will maintain AGC fully active in the resting state. The AGC-dependent changes in MAS activity will merely reflect the changes in substrates and products, *i.e.* follow the changes in mass action ratio, but it is doubtful that calcium activation plays any significant role. This is the probable situation with citrin, at least in those tissues, such as rat liver, where the $S_{0.5}$ for calcium activation is lowest. On the other hand, higher $S_{0.5}$ values, as those found in mitochondria where aralar is the only isoform (like brain), are better suited to provide Ca²⁺ activation of MAS in response to small Ca²⁺ signals, under conditions where the Ca²⁺ uniporter is not operating, or at the beginning and end of each miniature increase in mitochondrial Ca²⁺ or Ca²⁺ mark (63). In serving as the forefront of the mitochondrial response to a Ca²⁺ signal, aralar may prime mitochondria to respond to Ca²⁺ by prolonging mitochondrial energization beyond the duration of the Ca²⁺ mark (19). This is the situation found in CNS neurons (29).

The role of the two isoforms, aralar and citrin, present together in heart is intriguing. We have previously shown that aralar is enriched in atria (38), while citrin appears to be enriched in ventricles,⁴ suggesting a differential localization of the two isoforms within the heart as a whole. Moreover, it is possible that these isoforms are distributed within specialized subsets of mitochondria or even within different subcompartments in the inner mitochondrial membrane (64). It is known that atrial cell mitochondria are heterogeneous with regards to their responses to excitation contraction coupling (65). Unlike ventricular myocytes, that have a well developed t-tubule system with voltage-operated Ca²⁺ channels (VOCs) juxtaposed to ryanodine receptors (RyR) in the neighboring sarcoplasmic reticulum, in atrial cells VOCs are only present on the outer membrane surrounding the cell, and only small numbers of RyR are located close to the VOCs (65–67). These “junctional RyR” located at the periphery of the cell respond to the opening of VOCs originating a sub-sarcolemmal Ca²⁺ signal that does not propagate into the center of atrial myocytes. Mitochondria are located both at the periphery and center of atrial myocytes, but

only those located at the periphery, close to the junctional RyR, are involved in calcium uptake and buffering under normal pacing conditions (65, 68). Whether aralar and citrin have a differential distribution within these specialized mitochondria and participate in shaping the mitochondrial response to Ca²⁺ signals are key questions requiring further investigation.

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