Amino Acids (2016) 48:2049–2055
DOI 10.1007/s00726-016-2206-3

# Structural correlates of the creatine transporter function regulation: the undiscovered country

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Received: 12 December 2015 / Accepted: 23 February 2016 / Published online: 7 March 2016
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**Abstract** Creatine (Cr) and phosphocreatine constitute an energy shuttle that links ATP production in mitochondria to subcellular locations of ATP consumption. Cells in tissues that are reliant on this energy shuttle, such as myocytes and neurons, appear to have very limited ability to synthesize creatine. Therefore, these cells depend on Cr uptake across the cell membrane by a specialized creatine transporter (CrT solute carrier SLC6A8) in order to maintain intracellular creatine levels. Cr supplementation has been shown to have a beneficial effect in numerous in vitro and in vivo models, particularly in cases of oxidative stress, and is also widely used by athletes as a performance enhancement nutraceutical. Intracellular creatine content is maintained within narrow limits. However, the physiological and cellular mechanisms that mediate Cr transport during health and disease (such as cardiac failure) are not understood. In this narrative mini-review, we summarize the last three decades of research on CrT structure, function and regulation.

**Keywords** Creatine · Creatine transport · Energy metabolism · Membrane transporter modulation

Handling Editor: T. Wallimann and R. Harris.

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## Introduction

Creatine (Cr) and phosphocreatine (PCr), together with creatine kinases (CK), constitute an energy shuttle that links ATP production in mitochondria to the locations of ATP consumption, e.g. contractile machinery and ion pumps such as the plasmalemmal $Na^+/K^+$ ATPase and the sarcolemmal $Ca^{+2}$ ATPase (Nash et al. 1994; Wallimann et al. 1992; Wyss and Kaddurah-Daouk 2000). Cells that are most reliant on the creatine energy shuttle, such as myocytes and neurons, appear to have very limited, if any, such biosynthetic ability for endogenous generation of their own Cr (Russell et al. 2014). Therefore, these cells depend on Cr uptake across the cell membrane by a specialized transporter, the creatine transporter (CrT), to maintain proper intracellular Cr concentrations.

CrT is a symporter that uses the energy accumulated in the sodium gradient across the membrane to drive the "uphill" transport of Cr into the cell. During the transport cycle a $Cl^-$ ion is also translocated into the cell with a stoichiometry of $2Na^+$:$1Cl^-$;1Cr. Kinetic analyses demonstrate that the requirement for sodium is absolute, whereas transport can still occur in the absence of chloride (Dai et al. 1999; Sora et al. 1994). Its substrate selectivity has been carefully examined (Dai et al. 1999; Guimbal and Kilimann 1993, 1994; Nash et al. 1994; Saltarelli et al. 1996; Sora et al. 1994). Cr structural analogues 3-guanidinopropionate (GPA), 3-guanidinobutyrate (GBA), guanidineacetic acid (GAA), cyclocreatine (cCr) are also transported, albeit with reduced efficiency compared to Cr, by CrT. Of particular importance, PCr, is not transported by CrT, nor does it compete with Cr for transport as it is the case for GPA, or GBA (Nash et al. 1994; Saltarelli et al. 1996). These properties of CrT, as regards to substrate selectivity

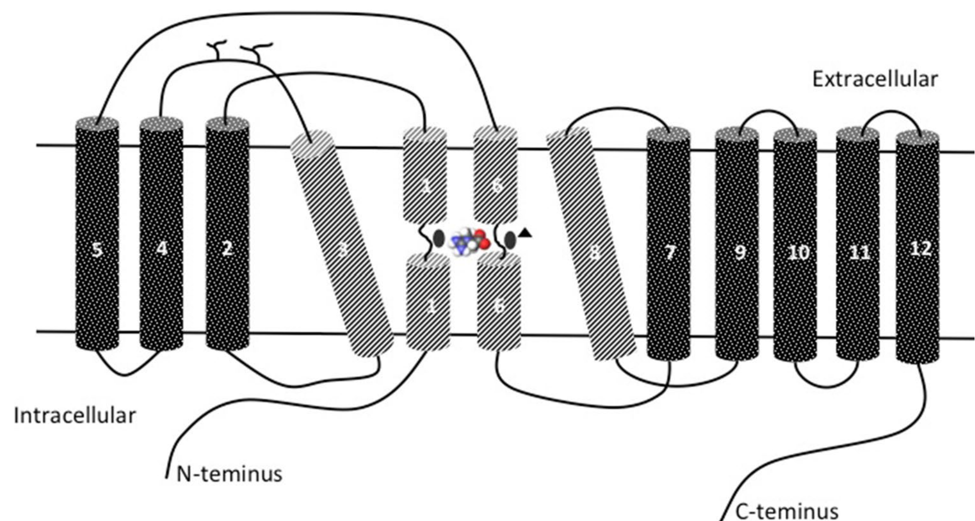


**Fig. 1** Schematic representation of the proposed organization of the CrT protein in the membrane. Adapted from models by Christie, and Parmod et al. (Christie 2007; Pramod et al. 2013). Transmembrane domains (TM) 1 ,3 ,6 ,8 (shaded in *grey*) contribute to the permeation pathway as indicated by a Cr molecule and sodium ions (*ovals*) and a single chloride ion (*triangle*). N-linked glycosylation sites are depicted in the extracellular loop connecting TM 4 and 3

are explained by the structural constraints of the permeation pathway as modeled using the available structural data (Christie 2007).

The gene encoding the creatine transporter protein (solute carrier, SLC6A8), has been cloned and sequenced (Dai et al. 1999; Nash et al. 1994; Sora et al. 1994) in humans. It predicts a membrane protein of 635 amino acids, which is highly conserved between species. Based on sequence homology, it has been classified as a member of the SLC6 family of $Na^+/Cl^-$-dependent plasma membrane neurotransmitter transporters, just like the transporters for taurine, proline and betaine (Kristensen et al. 2011). CrT is proposed to have 12 transmembrane (TM) domains (Fig. 1), with the N- and C-termini localized on the cytoplasmic side of the membrane. High resolution structural data for a bacterial leucine transporter belonging to the same family of membrane transporter proteins as CrT have shed light on the general organization of these membrane proteins within the lipid bilayer (Yamashita et al. 2005). Structure–function analysis using site-directed mutagenesis delineated amino acids within TM3 that are important for binding and permeation of Cr (Dodd and Christie 2001, 2005, 2007). These data in combination with sequence homology have been used to propose structural models, largely based on the structural framework of bacterial leucine transporter, and have been reviewed elsewhere (Christie 2007; van de Kamp et al. 2014).

The effects of lacking or suboptimal Cr transport function are most evident in patients suffering from inborn errors in the gene encoding CrT. These individuals have intellectual disabilities with severe speech delay (100 %), behavioral abnormalities (85 %), and seizures (59 %). The intellectual disabilities become more pronounced with age, and most adult patients have severe intellectual disabilities (van de Kamp et al. 2014). A murine model of CrT deficiency model has been developed that recapitulates some of the characteristics observed in patients afflicted by impaired CrT function. Transgenic CrT knock-out mice have impaired learning and memory capacity, and are thus considered a suitable model for testing therapies for creatine transporter deficiencies (Hautman et al. 2014; Skelton et al. 2011).

Additionally, decreased cardiac Cr content is observed in human heart failure, most prominently during its advanced stages, regardless of its initial cause. Studies performed in humans and animal models of cardiac failure demonstrate that a decrease in Cr levels antedates a decrease in ATP levels, the latter which is a hallmark of advanced cardiac failure (Maslov et al. 2010; Neubauer 2007).

There is ample evidence supporting the beneficial effects of dietary Cr supplementation in healthy individuals seeking to increase athletic performance (Lanhers et al. 2015), as an adjuvant therapy for individuals suffering debilitating neurodegenerative diseases (Gualano et al. 2012), and major depressive disorders (Lyoo et al. 2012), and in animal models of ischemia (Perasso et al. 2013) and neurodegenerative diseases (Beal 2011). The potential for therapeutic creatine augmentation in the setting of cardiac myopathies was demonstrated in mouse model of myocardial infraction (Lygate et al. 2012) and it has recently been reviewed elsewhere (Zervou et al. 2015). Cr supplementation has been reported to have a protective effect in neurons, myoblasts, and cardiomyocytes in culture when the cells are subjected to hypoxic and increased oxidative stressors (Adcock et al. 1999, 2002; Balestrino et al. 2002; Caretti et al. 2010; Santacruz et al. 2015a; Sartini et al. 2012). Similar positive results have been reported in studies of the effects of Cr supplementation in animal models of neonatal hypoxia (Allah Yar et al. 2015; Ellery et al. 2013), and in a study with human volunteers that evaluated the

effects of Cr supplementation on cognitive function during oxygen deprivation (Turner et al. 2015). The mechanisms mediating the beneficial effect of Cr supplementation are not clear, however, and seem to be in addition to, or independent of its role in the energy shuttle.

Given the role Cr plays in maintaining adequate energy reserves in metabolically active tissues, the reported benefits of oral Cr supplementation, and the observations indicating Cr levels are affected by disease, it is important to understand how Cr transport is regulated during health and disease. Although there has been progress characterizing Cr transport control, neither the structural components of this modulation, nor the cell signaling cascades involved, are fully understood. What follows is a summary of recently reported studies on the regulation of Cr transport.

## Cr transport modulation by substrate availability

Intracellular Cr content in muscle cells is tightly maintained, as demonstrated by earlier studies in L6 rat myoblasts (Loike et al. 1988) and, more recently, in cardiomyocytes (Darrabie et al. 2011). In these cells, Cr transport appears to be largely regulated by extracellular substrate availability. Increases in extracellular Cr decrease the rate of Cr uptake, whereas decreases in Cr content increase Cr transport by changing the $V_{max}$ of transport without significantly altering $K_m$. Interestingly, the negative feedback in response to elevated extracellular Cr levels appears to depend on de novo protein synthesis (Loike et al. 1988). Using an unbiased global gene array approach, thioredoxin interacting protein (Txnip) has been identified as important for both in vitro and in vivo negative feedback regulation by increased intracellular Cr (Zervou et al. 2013). Txnip is an α-arrestin with roles in redox regulation via inhibition of the denitrosylating enzyme, thioredoxin, which suggests a role for S-nitrosylation in the regulation of creatine transport. Further analysis in the murine heart has shown that in cardiac muscle Cr transport capacity modulation in response to creatine availability is primarily regulated by post-translational modifications of the CrT protein, rather than by changes in transcription of the gene encoding CrT (ten Hove et al. 2008).

## Cr transport is modulated by kinases and phosphatases

Previous studies of L6 myocytes (Loike et al. 1988) and *Xenopus* oocytes expressing CrT protein (Dai et al. 1999) showed that PKC activation by the phorbol ester β-PMA reduced the $V_{max}$ for Cr transport. This reduction in Cr transport was also observed in cardiomyocytes in culture (Santacruz et al. 2015b). In contrast, Cr transport was enhanced after phosphatase PP1a/PP2a activity was inhibited by Calyculin A (Santacruz et al. 2011), suggesting that the increase in Cr transport capacity in response to diminished Cr availability is mediated by changes in the phophorylation state of CrT and/or a yet to be identified partner.

Taken together, these observations suggest that S and/or T residues in the CrT protein are the target of phosphorylation by PKC isoform(s). A reduction in transport has also been reported upon PKC activation in other closely related $Na^+/Cl^-$ transporters, including GAT1, SERT, NET, and DAT. In these transporters, there is an increase in phosphorylation of the transporter proteins, which correlates with decreased substrate uptake and increased rates in transporter internalization (Kristensen et al. 2011). Our group used site-directed mutagenesis to eliminate high probability PKC phosphorylation sites individually and in groups (Santacruz et al. 2015b). The multisite CrT mutants included Y11, a residue that is predicted to be phosphorylated, although it is not a substrate of PKC. These amino acid substitutitons did not affect Cr transport regulation. Therefore, both the individual and multi-site CrT mutant proteins responded to PKC activation and substrate availability in a manner equivalent to the wild type CrT (Santacruz et al. 2015b). Previous reports of changes in serine or tyrosine phosphorylation regulated CrT function (Wang et al. 2002; Zhao et al. 2002) are questionable due to the use of a commercially available antibody that was later found to be non-specific.

AMP-activated protein kinase AMPK (AMPK) regulates cellular energy homeostasis by switching off energy-consuming pathways in favor of energy-generating processes (Hardie et al. 2012). This modulates Cr transport in a tissue-specific manner. In cardiomyocytes, AMPK activation reduces Cr transport by changing the $V_{max}$ for Cr transport, likely through signaling cascades that alter transporter protein content in the cell membrane, as also seen with GLUT4 transporter after cellular activation of AMPK (Lang and Foller 2014). In these cells, AMPK may serve as a positive and physiological regulator of Cr transport ensuring that sufficient quantities of Cr are available to support myocellular function when cardiomyocytes are stressed (Darrabie et al. 2011). On the other hand, in kidney epithelia, AMPK activation decreases CrT activity and expression in the apical membrane of kidney proximal tubule cells (Li et al. 2010). In these cells, AMPK may mediate a tissue-specific response that limits cellular metabolic demands by reducing apical $Na^+$ entry, mitigating the load on basolateral $Na^+/K^+$-ATPase activity and ATP consumption to maintain transcellular ionic gradients. It is not known if the CrT protein is a direct substrate of AMPK, and the mechanisms by which AMPK modulates CrT function in a cell/tissue-specific manner are yet to be revealed.

Cr transport is electrogenic, and the inward $Na^+$ currents ($I_{Crea}$) generated as a result of the translocation of Cr into

the cell can be recorded using the two-electrode voltage patch-clamp technique in Xenopus oocytes expressing CrT protein. Using this experimental approach, the effects of co-expression with kinases previously reported to modulate the function of other unrelated membrane transporters have been studied. CrT co-expression with Serum and Gluocorticoid Inducible Kinases SGK1 and SGK3 (Shojaiefard et al. 2005), Mammalian Target of Rapamycin-mTOR (Shojaiefard et al. 2006) or mammalian Phosphatidylinositol-3-phosphate-5-kinase PIKfyve (Strutz-Seebohm et al. 2007) increase Cr transport in *Xenopus* oocytes. On the other hand, co-expression with Janus-Activated Kinase 2 (JAK-2) (Shojaiefard et al. 2012) or SPS1-related Proline/Alanine-rich Kinase (SPAK) and the Oxidative Stress-Responsive Kinase 1 (OSR1) (Fezai et al. 2014) decrease $I_{Crea}$ in *Xenopus* oocytes. None of these studies established that CrT protein was phosphorylated by the kinases, and the authors clearly state that these kinases could phosphorylate and thus modify the function of other signaling molecules involved in the regulation of CrT.

## Other regulators of Cr transport

Co-expression of CrT protein with the β-glucoronidase Klotho (Almilaji et al. 2014) increased Cr transport in *Xenopus* oocytes. Klotho regulates Na-coupled phosphate transporters (Dermaku-Sopjani et al. 2011), Na+/K+ATPase (Sopjani et al. 2011), and excitatory amino acid transporters EAAT 3 and EAAT4 (Almilaji et al. 2013). Although the mechanism mediating the increase in CrT function is not understood, the authors speculate that Klotho stabilizes the carrier protein in the cell membrane. Interestingly, enhanced CrT function was also observed following treatment of the oocytes with physiological concentrations of the recombinant human Klotho protein, which appeared to depend on its glucorunidase activity.

The CrT protein is subjected to N-linked glycosylation at residues N192 and N197 (Fig. 1). Site-directed mutagenesis was used to replace the modified asparagine's with structurally similar aspartate residues. The resulting proteins retained creatine transport capacity, however they appeared to be inserted less efficiently into the cell membrane (Straumann et al. 2006). A similar result was reported for other membrane proteins, such as the Shaker potassium channel, where N-linked glycosylation sites were ablated (Santacruz-Toloza et al. 1994).

## Transcriptional and translational regulation of CrT

The majority of studies pertaining to the regulation of Cr transport capacity have focused on the modulation of the mature CrT protein's function. However, recent studies are beginning to shed light on regulatory mechanisms that alter the transcription and translation of the CrT gene. Consensus sites for Estrogen-related receptor-α (ERR α) have been identified upstream of the promoter region and within the first intron of the CrT gene (Brown et al. 2014). In skeletal muscle, ERR α partners with members of the peroxisome proliferator-activated receptor γ, coactivator-1 (PGC-1) PGC-1α and PGC-1β, to regulate the expression of genes involved in energy metabolism and substrate oxidation (Handschin and Spiegelman 2006). Brown et al. demonstrated that in L6 cells, PGC-1α and ERRα directly interact with the CrT gene and increase CrT mRNA and protein expression.

The RNA message encoding CrT is subject to alternative splicing, resulting in splice variants encoding truncated CrT proteins. In a recent study, it was demonstrated that two of these variants, while lacking Cr transport function, increase Cr transport through co-expression with the full-length CrT (Ndika et al. 2014). The mechanism mediating this increase involves enhanced trafficking during the biogenesis of the transporter, and perhaps an increase in the half-life of the mature CrT protein in the membrane, by "deflecting" ubiquitinilation and, therefore, proteosomal degradation.

## Concluding remarks

Current understanding of Cr transport modulation, although incomplete, clearly demonstrates that cells that depend on Cr transport to maintain intracellular Cr stores, control the creatine transporter very tightly. Some progress has occurred in the understanding of the regulation mechanisms and signaling cascades important for Cr transport regulation. However, this understanding is fragmentary. The findings to date are, for the most part, derived from in vitro cell culture systems. Thus, care should be taken when generalizing between cell types, since, although there is a unique CrT protein, its modulation is complex and tissue-specific. For example, very little is known about how different neurons modulate Cr transport. Elucidation of the precise mechanisms for CrT modulation will allow for better-designed supplementation protocols tailored to the specific Cr transport capabilities of a target tissue or organ system. Part of this approach should include metabolomic, proteomic, and transcriptosomic profile analysis in order to shed light on why, within a given cohort of individuals, some respond to Cr supplementation and others do not e.g., responders vs non-responders. This is particularly relevant in view of clinical trials showing no beneficial effects of Cr supplementation in the elderly (Alves et al. 2013; Lobo et al. 2015) and neurodegenerative diseases (Bender and Klopstock 2016) that were preceded by encouraging preclinical trials (Beal 2011). Especially disappointing is the

outcome of the phase III Clinical Trial on Creatine supplementation in early stage Parkinson Disease (http://parkinsontrial.ninds.nih.gov/netpd-LS1-study-termination.htm). Although the trial was halted because "the results of an interim analysis showed that it was futile to complete the study because longer patient follow-up was not likely to demonstrate a statistically significant difference between creatine and placebo," it demonstrated that long-term Cr oral supplementation is safe, with no adverse effects (Kieburtz et al. 2015). These negative outcomes underscore the need for a better understanding of Cr transport regulation as an essential guide to protocols that fully harness the therapeutic potential of Cr supplementation.

**Acknowledgments** The support of the University of Texas Medical Branch, Galveston, its Institute for Translational Sciences, and Department of Surgery. We gratefully acknowledge Enrique Toloza for his assistance in the preparation of this manuscript.

**Compliance with ethical standards**

**Conflict of interest** No conflicts of interest, financial or otherwise, are declared by the author(s).

**Research involving human participants and/or animals** Not applicable (No human subjects or animals were used in the preparation of this mini-review).

**Informed consent** Not applicable (The preparation of this mini-review did not involve research requiring informed consent).

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