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Calcium-induced conformational changes in the regulatory domain of the human mitochondrial ATP-Mg/Pi carrier

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ABSTRACT

The mitochondrial ATP-Mg/Pi carrier imports adenine nucleotides from the cytosol into the mitochondrial matrix and exports phosphate. The carrier is regulated by the concentration of cytosolic calcium, altering the size of the adenine nucleotide pool in the mitochondrial matrix in response to energetic demands. The protein consists of three domains; (i) the N-terminal regulatory domain, which is formed of two pairs of fused calcium-binding EF-hands, (ii) the C-terminal mitochondrial carrier domain, which is involved in transport, and (iii) a linker region with an amphipathic α-helix of unknown function. The mechanism by which calcium binding to the regulatory domain modulates substrate transport in the carrier domain has not been resolved. Here, we present two new crystal structures of the regulatory domain of the human isoform 1. Careful analysis by SEC confirmed that although the regulatory domain crystallised as dimers, full-length ATP-Mg/Pi carrier is monomeric. Therefore, the ATP-Mg/Pi carrier must have a different mechanism of calcium regulation than the architecturally related aspartate/glutamate carrier, which is dimeric. The structure showed that an amphipathic α-helix is bound to the regulatory domain in a hydrophobic cleft of EF-hand 3/4. Detailed bioinformatics analyses of different EF-hand states indicate that upon release of calcium, EF-hands close, meaning that the regulatory domain would release the amphipathic α-helix. We propose a mechanism for ATP-Mg/Pi carriers in which the amphipathic α-helix becomes mobile upon release of calcium and could block the transport of substrates across the mitochondrial inner membrane.

1. Introduction

The mitochondrial ATP-Mg/Pi carrier (APC) is a member of the mitochondrial carrier family of transport proteins. Mitochondrial carriers are typically located in the mitochondrial inner membrane and fulfil the vital role of shuttling nucleotides, amino acids, inorganic ions, keto acids, fatty acids and cofactors between the mitochondrial matrix and the cytosol [1]. APC is involved in the import of cytosolic adenine nucleotides into the mitochondrion and the export of inorganic phosphate from the mitochondrial matrix [2–4]. Mitochondria also have ADP/ATP carriers, a related but functionally distinct member of the mitochondrial carrier family [5]. In contrast to APC, ADP/ATP carriers catalyse the equimolar exchange of ADP from the cytosol for ATP synthesised in the mitochondrial matrix, meaning that their activity does not influence the size of the adenine nucleotide pool [5]. The unequal exchange of substrate catalysed by APC does have the potential to influence the total matrix adenine nucleotide pool. Therefore, APC has the important role of altering the mitochondrial adenine nucleotide pool in order to adapt to changing cellular energetic demands [6–8].

In yeast only one APC ortholog exists (*Sal1p*) [9], whereas in humans four genes encode full-length APC paralogs; *SLC25A24*, *SLC25A25*, *SLC25A23* and *SLC25A54* encoding for the protein short calcium binding mitochondrial carrier (SCaMC) isoform 1 (APC-1), SCaMC-2 (APC-3), SCaMC-3 (APC-2) and SCaMC-1L, respectively [3,10,11]. Another gene, *SLC25A41*, encodes for SCaMC-3L protein [12], which is shorter in sequence than the other APC isoforms (for a review see Satrústegui & Pardo, 2007) [13]. Mitochondrial carriers are fundamental to cellular metabolism, and have been implicated in many severe human diseases [14]. There is experimental support for the notion that up-regulation of human SCaMC-1 (HsAPC-1) expression in cancerous tissues may help these cells to evade cell death [15].

APC has a three-domain structure. The N-terminal domain of APC forms a calcium-sensitive regulatory domain [3,10]. The consequence of calcium binding to the regulatory domain of APC is a stimulation of the substrate transport activity of the carrier [2,4,7]. A structure of the regulatory domain of human APC isoform-1 has recently been solved [16], confirming the presence of four EF-hands, each of which is occupied by a bound calcium ion in a canonical pentagonal bi-pyramidal fashion. The EF-hands group together into two pairs, forming two

Abbreviations: APC, ATP-Mg/Pi carrier; SCaMC, short calcium binding mitochondrial carrier; LMNG, lauryl maltose neopentyl glycol; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; RMSD, root-mean-squared deviation; SEC, size exclusion chromatography; RD, regulatory domain.

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lobes connected by a long central α-helix, in a similar fashion to the four EF-hands of calmodulin [16].

The C-terminal domain of APC is a membrane protein with the characteristic structural fold of mitochondrial carrier proteins [17]. The carrier fold is composed of three repeats [18], each containing two membrane-spanning α-helices connected by a matrix α-helix, making a three-fold pseudo-symmetrical protein structure [19,20]. Atomic structures of both bovine [19] and yeast ADP/ATP carriers [20] provide the basis for our understanding of the structure/function relationship between key sequence elements conserved across the carrier family. Based on the identification of a conserved central substrate binding site [21] flanked by two salt bridge networks on either side [22], an alternating access mechanism has been proposed for substrate transport through carrier proteins [20,22].

The linker region between the N-terminal regulatory domain and the C-terminal carrier domain contains an amphipathic α-helix of unknown function. In the structure this α-helix is bound to EF-hand pair 3 and 4, analogous to the binding of a calmodulin recognition sequence motif to the hydrophobic pockets in the EF-hands of calmodulin [16].

Another member of the carrier family of proteins, the aspartate/glutamate carrier (AGC), also displays a calcium-dependence for activity and has an N-terminal regulatory domain that contains EF-hands. Structures of both the calcium-bound and calcium-free states of the regulatory domain of the human AGC have recently been solved [23]. The structures reveal eight EF-hand motifs, only one of which is involved in calcium binding. Most mitochondrial carriers are monomeric [24], but AGC was found to be dimeric [23]. EF-hands four to eight are recruited to form an extensive interface for homo-dimerisation that is critical for the observed calcium-induced conformational changes within the regulatory domain of AGC [23]. Thus both APC and AGC have N-terminal regulatory domains consisting of EF-hands. Although no significant sequence similarity exists between these regulatory domains, there is the possibility that they share a common mechanism of calcium-regulation, potentially based on a homo-dimer arrangement. The protein construct used in the previous study of the HsAPC-1 regulatory domain was mutated at the extreme N-terminus to replace a cysteine residue at position 15 with a serine [16], raising the possibility that a native dimer could have been disrupted [25].

NMR nuclear Overhauser effects have shown that the regulatory domain of HsAPC-1 is more dynamic and less structured in the calcium-free state [16]. Binding studies with surface plasma resonance have indicated that the regulatory domain interacts with the carrier domain in the absence of calcium [16]. A mechanism was proposed in which the regulatory domain binds to the carrier domain in the absence of calcium, capping it in order to block substrate transport [16], however that mechanism does not explain why the bound calcium-free regulatory domain would be more dynamic. Therefore, the mechanism that couples calcium binding in the regulatory domain to substrate transport in the carrier domain has not been resolved.

Here, an independent structural determination of the native human APC-1 regulatory domain was completed, which was combined with an investigation into the oligomeric state of the full-length human APC-1 protein. Furthermore, an extensive comparison of the conformations of EF-hand proteins in the calcium-free, calcium-bound and peptide-bound state was undertaken, which has allowed us to propose a mechanism for the regulation of APC that is consistent with all available experimental data.

2. Materials and methods

2.1. Expression and purification of full-length HsAPC-1

A construct of HsAPC-1 (Uniprot: Q6NUK1) was designed to include an eight-histidine tag followed by a Factor Xa cleavage site (IEGR) at the N-terminus, creating a cleavable purification-tag. The gene was codon-optimized for expression in *S. cerevisiae* by GenScript and cloned into a pYES3/CT expression plasmid (Invitrogen), with the inducible galactose promoter being replaced by the constitutive promoter for the yeast mitochondrial phosphate carrier, as described previously [26]. The expression plasmid was transformed into *S. cerevisiae* haploid strain W303-1b [27], using established methods [28].

S. cerevisiae was cultured, and HsAPC-1 was expressed following established methods [20], with the following modifications: pre-cultures of yeast were set up in synthetic-complete tryptophan-dropout medium (Formedium) supplemented with 2% glucose, and the main cultures were carried out in an Applikon bioreactor with 100 L YEPD medium. Mitochondria were prepared using established methods [29], flash frozen in liquid nitrogen, and stored under liquid nitrogen until use.

HsAPC-1 was solubilised in lauryl maltose neopentyl glycol (LMNG), and separated from insoluble protein by ultracentrifugation. Solubilised protein was passed through nickel sepharose affinity resin (GE Healthcare) to selectively bind full-length HsAPC-1, and washed with 50 column volumes of buffer A (20 mM Tris pH 7.4, 150 mM NaCl, 20 mM Imidazole, 0.1% (w/v) LMNG, 0.1 mg mL^{-1} tetra-oleoyl cardiolipin) and 30 column volumes of buffer B (20 mM Tris pH 7.4, 50 mM NaCl, 0.1% (w/v) LMNG, 0.1 mg mL^{-1} tetra-oleoyl cardiolipin). Factor Xa protease (New England BioLabs) was used to specifically cleave the HsAPC-1 protein from the affinity resin. After Factor Xa cleavage, HsAPC-1 was spun through an empty Proteus midi-spin column (Generon) to remove affinity resin. Protein concentrations were determined using the bicinchoninic acid assay [30] against a bovine serum albumin standard curve (Pierce). Purified protein was used immediately for analysis using SEC.

2.2. Expression and purification of HsAPC-1 regulatory domain

Primers were design in order to create a construct of HsAPC-1 to include just the regulatory domain and the linker region (residues 14 to 174) of the protein (Forward primer: GAAGGTAGAACCTCCGAAGA. Reverse primer: CCTAGGTCTAGACTCGAGTCATTATTATATATCAATACCTGT GGAATGTTT). The inter-domain loop was not included in this construct. The construct was cloned into the *Lactococcus lactis* expression plasmid pNZ8048, and the plasmid transformed into electrocompetent *L. lactis* NZ9000 using established methods [31]. Expression of the construct was carried out as described previously [23]. Purification of HsAPC-1 was achieved using a three-step chromatography protocol. First, clarified *L. lactis* lysate was passed over Ni sepharose resin (GE Healthcare). The resin was washed with 50 column volumes of purification buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT) containing 50 mM imidazole and eluted in purification buffer containing 250 mM imidazole. Second, recovered protein was concentrated, and desalted using a PD10 column (GE Healthcare). The purification tag was removed from the construct by digestion with Factor Xa protease (Supplementary Fig. 1). Finally protein was loaded onto a Superdex 200 pg 16/60 SEC column (GE Healthcare) in a running buffer of 20 mM Tris pH 7.5, 20 mM NaCl. Peak fractions were collected and concentrated to approximately 10 mg mL^{-1} for crystallography.

2.3. Experimental analysis of oligomeric state

Superdex 200 pg 16/60 SEC columns (GE Healthcare) were calibrated with molecular weight standards from both the high and low molecular weight standards kits (GE Healthcare), and unknown protein weights were estimated using the manufacturers recommended protocol. The SEC buffer used for analysis of the HsAPC-1 RD protein was purification buffer with either 5 mM calcium or 5 mM EGTA, with or without 1 mM DTT added as stated. The SEC buffer used for analysis of the full-length HsAPC-1 was 20 mM Tris pH 7.4, 50 mM NaCl, 0.03% (w/v) LMNG and 0.03 mg mL^{-1} tetra-oleoyl cardiolipin. The buffers used to run the molecular weight standards in each case matched the buffers being used in the analysis of each protein.