FEBS Letters 583 (2009) 782–786

FEBS Letters

journal homepage: www.FEBSLetters.org

# A single amino acid residue is responsible for species-specific incompatibility between CCT and α-actin

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ARTICLE INFO

*Article history:*
Received 8 January 2009
Accepted 19 January 2009
Available online 5 February 2009

Edited by Michael R. Bubb

*Keywords:*
Actin
ACT1
Muscle
CCT
Chaperonin
Protein folding

ABSTRACT

**Actin is dependent on the type-II chaperonin CCT (chaperonin containing TCP-1) to reach its native state. In vitro, yeast CCT folds yeast and also mammalian cytoplasmic (β/γ) actins but is now found to be incapable of folding mammalian skeletal muscle α-actin. Arrest of α-actin on yeast CCT at a folding cycle intermediate has been observed by electron microscopy. This discovery explains previous observations in vivo that yeast mutants expressing only the muscle actin gene are non-viable. Mutational analysis identified a single specific α-actin residue, Asn-297, that confers this species/isoform folding specificity. The implications of this incompatibility for chaperonin mechanism and actin–CCT co-evolution are discussed.**


## 1. Introduction

It is well established that actin, the key structural component of the cytoskeleton, is unable to fold autonomously and requires interaction with the cytosolic chaperonin CCT (chaperonin containing TCP-1) to attain its native conformation. Chaperonin dependence of a protein typically arises when autonomous folding is kinetically or thermodynamically unfavourable or when a folding intermediate is aggregation-prone. This is alleviated by folding within or around the chaperonin cavity which alters the substrate free energy landscape and prevents intermolecular interactions. The mechanism by which type-II chaperonins operate and specifically how CCT folds actin is not yet properly understood, although the requirement of actin for CCT is thought to be both aggregation-preventing and thermodynamic in origin [1].

In higher eukaryotes several isoforms of actin exist, α-actins which form sarcomer units with myosin filaments in muscle tissue and β and γ isoforms which form cytoskeletal networks. Mutations in α-actin lead to clinically severe inherited muscular diseases [2].

Yeast has been used as an effective model to study the in vivo effects of β- and γ-actin mutations. However, it has not been possible to study mutant muscle actins in this way as α-actin is incompatible with yeast viability [3]. Here we demonstrate that the basis of this incompatibility lies at the protein folding stage rather than being a function of native state interactions. Further investigation has revealed that the folding process is disrupted on yeast CCT, and is caused by a single amino acid difference (N297) present in α-actin with interesting implications for both folding mechanism and the co-evolution of CCT and actin, one of its primary substrates.

## 2. Materials and methods

### 2.1. Purification of CCT from the yeast Saccharomyces cerevisiae and CCT–actin complexes

Yeast CCT was purified using a calmodulin binding peptide inserted into an internal loop in the apical domain of the CCT3 subunit as an affinity tag, as described previously [4]. Rabbit skeletal muscle α-actin was isolated and purified as described [5]. His-tagged yeast Plp2 was expressed in *Escherichia coli* and purified over a Talon column (McCormack et al., unpublished data). For EM visualization we used CCT-ANC2 (3CBP), harbouring the CBP-tag in subunit CCT3 and mutation G345D in subunit CCT4. Mutation G345D has been shown to exhibit reduced folding kinetics without affecting the actin folding or actin–CCT interaction due to perturbed ATP-allostery [6]. CCT–actin complexes were formed

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0014-5793/$34.00 
doi:10.1016/j.febslet.2009.01.031

by unfolding α-actin in EDTA for 10 min prior to incubation with CCT-ANC2(3CBP) and Plp2 in the presence of ATP and $MgCl_2$ for 30 min at 30 °C. Complexes were separated over a Superose 6 gel filtration column (GE Healthcare) in GF buffer (10 mM HEPES, pH 8, 150 mM KCl, 1 mM DTT, 0.01% LDAO, 10% glycerol) supplemented with ATP and $MgCl_2$, and peak fractions were concentrated to 6.4 mg/ml.

### 2.2. EM grid preparation and data analysis

CCT–actin complex preparation was diluted 1 in 175 in buffer containing 4% glycerol. Typically, droplets of 3 μl were applied to carbon coated quantifoil 1.2 grids, and stained with uranyl acetate. The grids were examined in a FEI-T12 at 200 kV and 42 000× magnification. Photos were taken with a Kodak SO163 at a typical defocus of 1 μm, equivalent to a first zero at 18.5 Å on average. These were digitised on a Nikon scanner, using a summing factor of 3, giving a Å/pixel ratio of 4.632. The data was filtered at 100 Å with no low-pass filter, normalised and masked using Imagic [7]. 2D-alignment against a chosen reference set was performed in SPIDER [8], followed by multi-statistical analysis (MSA) in Imagic. This was repeated until no further improvement was observed. The data was then split into side views and no-side views, the latter containing typical top views but also the less well defined oblique views.

### 2.3. Actin mutation construction, in vitro protein expression and folding assays

Controlled CCT–actin folding assays [4] and in vitro translation assays [9] were performed as described previously. Yeast CCT folding assays were performed in 60 μl volumes at 30 °C with the following order of addition: folding assay buffer (20 mM HEPES (pH 8), 150 mM KCl, 20% glycerol), ATP/ADP mix to final concentration of 1 mM/0.1 mM CCT (1 μl stock solution concentration between 5 and 20 mg/ml) and finally the in vitro translated actin substrate (generally 10% or 15% of the final reaction volume). Human α-actin cDNA, ACTA1, and yeast ACT1 were translated in vitro in $^{35}$S-methionine containing EcoPro™ T7 coupled transcription/translation system made from fractionated *E. coli* extract (Novagen). The TNT®-T7 coupled rabbit reticulocyte lysate system (Promega) was used for the mammalian CCT folding experiments. For both the yeast and mammalian assays, aliquots were taken at set reaction time-points and stored on ice until loading of the entire time-course onto a single, pre-cooled native 6% (w/v) acrylamide gel with 1 mM ATP added to the gel mix and 0.1 mM ATP added to the running buffer (25 mM Tris, 190 mM glycine). Site directed mutagenesis of the plasmid DNAs was achieved using mutant single stranded primers (MWG) and the QuikChange™ kit and protocol (Stratagene).

## 3. Results

### 3.1. Skeletal muscle α-actin is not re-folded by yeast CCT

Actin species that are nascent/unfolded, folded or CCT bound can be distinguished on the basis of their differing mobilities under non-denaturing polyacrylamide gel electrophoresis. This has been exploited to investigate in vitro CCT-mediated folding of actin by producing radiochemically labelled nascent actin via incorporation of $^{35}$S-methionine into the elongating polypeptide chains [4,9,10]. Translation in eukaryotic cell extract allows investigation of the interaction between nascent actin and the endogenous CCT, whereas a prokaryotic translation system must be supplemented with purified CCT (e.g. from yeast). Human α skeletal muscle gene, ACTA1 (which encodes identical amino acid sequence to rabbit skeletal muscle α-actin) was expressed in the yeast CCT enriched *E. coli* extract system. Nascent α-actin was found to bind yeast CCT but surprisingly, folded product was not released, unlike yeast actin which is bound, folded and released (Fig. 1). This is unexpected as yeast CCT folds human β-actin under these same conditions [4]. Furthermore, a range of other actin isoforms and species-specific variants have been expressed in yeast strains and therefore must be able to be folded by yeast CCT in vivo [11]. To test whether an additional cytosolic component is required to assist the folding and release of α-actin by yeast CCT, rabbit reticulocyte lysate was added to the EcoPro-yeast CCT folding assay, after allowing the α-actin to bind yeast CCT. No folding activity was observed (data not shown), establishing that a sequence-specific effect intrinsic to the actin–CCT interaction is responsible for the inability of yeast CCT to fold α-actin. This discovery explains longstanding observations that *Saccharomyces cerevisiae* strains expressing α-actin are non-viable [3].

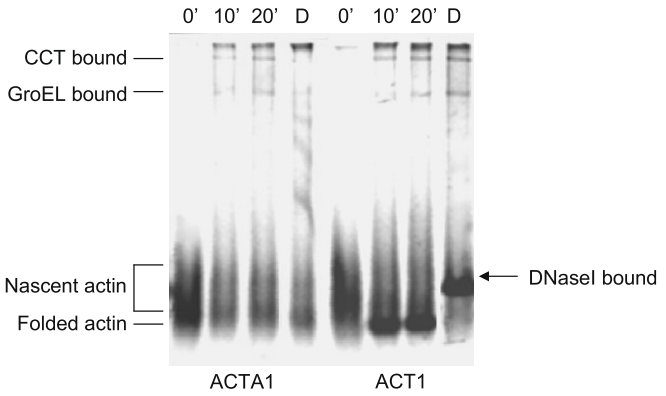


**Fig. 1.** Interaction of in vitro expressed yeast and human actin with yeast CCT. Yeast CCT in vitro folding assays of ACT1, yeast actin, and ACTA1, human skeletal α-actin. After 20 min, 1 μg DNaseI was added and the reaction incubated for 10 min at 30 °C (D). Yeast actin binds and is folded by yeast CCT while α-actin binds CCT but is not folded.

### 3.2. Imaging of rabbit actin bound to yeast CCT

To establish whether α-actin binds yeast CCT in the expected conformation [12], α-actin–yeast CCT complexes were imaged by negative stain electron microscopy. Complexes were formed by incubating EDTA unfolded actin with yeast CCT in the presence of ATP and co-factor Plp2 [10,13]. Plp2 is a phosducin-like protein that is known to interact with CCT [10,13]. Plp2 increases the amount of actin bound to CCT (McCormack et al., unpublished results). A total of 9423 particles were selected and classified. These actin–CCT preparations showed a high percentage of oblique views over side and top views. The orientations of CCT bound to EM-grids is known to be dependent on ATP-status, and it is likely that the presence of substrate also has an effect. One hundred and forty classes were calculated (40 side views, 19 top views and 81 oblique views) consisting of ~60 particles each. The number of classes was not reduced further to accommodate the known heterogeneity of CCT particles [14]. Fig. 2 shows six classes of typical top views, revealing density inside the CCT cavity that is asymmetrically distributed with respect to the eight subunit CCT ring. Within the density inside the cavity one can always distinguish two halves that are either in line or at a slight angle with respect to each other. The similarity of these CCT top views with previously published images of nucleotide-free mouse testis CCT–rabbit α-actin complexes [12] suggests that rabbit α-actin binds the yeast CCT ring in a similar conformation.