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ORIGINAL PAPER

Small heat shock proteins prevent aggregation of citrate synthase and bind to the N-terminal region which is absent in thermostable forms of citrate synthase

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Abstract Citrate synthase (CS) is often used in chaperone assays since this thermosensitive enzyme aggregates at moderately increased temperatures. Small heat shock proteins (sHsps) are molecular chaperones specialized in preventing the aggregation of other proteins, termed substrate proteins, under conditions of transient heat stress. To investigate the mechanism whereby sHsps bind to and stabilize a substrate protein, we here used peptide array screening covering the sequence of porcine CS (P00889). Strong binding of sHsps was detected to a peptide corresponding to the most N-terminal α-helix in CS (amino acids Leu_{13} to Gln_{27}). The N-terminal α-helices in the CS dimer intertwine with the C-terminus in the other subunit and together form a stem-like structure which is protruding from the CS dimer. This stem-like structure is absent in thermostable forms of CS from thermophilic archaebacteria like *Pyrococcus furiosus* and *Sulfolobus solfatacarium*. These data therefore suggest that thermostabilization of thermosensitive CS by sHsps is achieved by stabilization of the C- and N-terminae in the protruding thermosensitive soft-spot, which is absent in thermostable forms of the CS dimer.

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Introduction

The small heat shock proteins (sHsps) are ubiquitous stress-induced chaperones involved in protection against heat stress-induced inactivation and aggregation of proteins (Horwitz 1992; Jakob et al. 1993; Ehrnsperger et al. 1997; Buchner et al. 1998a; Van Montfort et al. 2001; Haslbeck et al. 2005).

The sHsps are large oligomeric proteins (the name refers to the fact that the subunits are small, approx. 20 kDa), constitutively expressed or induced at certain developmental stages or in response to stress. All organisms, from bacteria to man, have sHsps. One of the most well characterized bacterial sHsps is the *E. coli* inclusion body binding protein IbpA/B often exploited to improve the production of recombinant protein (Han et al. 2004; Jiao et al. 2005; Lethanh et al. 2005; Matuszewska et al. 2005). The human genome encodes ten different sHsp homologues (Kappe et al. 2003), with important roles in human disease (Clark and Muchowski 2000). One of the most highly expressed and well-characterized human sHsps is αB-crystallin (Horwitz 2003), which plays an important role in avoiding cataract and desmin-related myopathy, and is upregulated in a number of neurological diseases. In plants, sHsps form an especially pronounced part of the stress response. Several different sHsp genes (25 in *Arabidopsis thaliana*) are expressed in different cellular compartments, such as cytosol, mitochondria and chloroplasts (Waters and Vierling 1999). The chloroplast-localized sHsp, Hsp21, protects plants against heat and light stress (Harndahl et al. 1999). In all cells the sHsps

play a complex role in cellular processes such as differentiation and apoptosis (Arrigo 2005).

The molecular mechanism whereby the sHsps interact with and prevent aggregation of other proteins is not fully understood. The structural information of sHsp–substrate protein complexes is poor, based mainly on low-resolution electron microscopy imaging of substrates bound to yeast Hsp26 (Haslbeck et al. 1999). The substrate-binding region in sHsps has been mapped in a plant sHsp (Lee et al. 1997) and in mammalian α-crystallin sHsps (Sharma et al. 2000) based on approaches such as peptide binding and ANS-fluorescence. The structure has been resolved to atomic resolution for three different sHsps (an archaebacterial sHsp, Kim et al. 1998; a plant cytosolic sHsp, van Montfort et al. 2001; and a parasitic flatworm sHsp, Stamler et al. 2005). From these structures, crystallized in absence of bound substrate, in can be concluded that in each sHsp subunit there are sites that may act as substrate-binding surfaces when exposed to a substrate protein but otherwise hidden as contact surfaces between the subunits in the assembled sHsp oligomer (van Montfort et al. 2001). Whereas some information is thus available on sHsp–substrate protein interactions concerning which parts of the sHsp interacting with the substrate proteins, there is yet little known about which parts of the substrate proteins interacting with the sHsp.

We therefore set out to characterize the sHsp–substrate protein interaction with emphasis on a substrate protein. As substrate protein, porcine citrate synthase (CS) was chosen since it is one of the most widely used in chaperone assays (Buchner et al. 1998a, b). In its cellular context, CS is a mitochondrial, regulated enzyme catalysing the first step in the citric acid cycle, the condensation of oxaloacetate and acetyl-CoA to form citrate and CoA. As sHsps, recombinantly expressed *Arabidopsis thaliana* Hsp21 and human αB-crystallin were used, both previously shown by light scattering measurements capable to prevent temperature-induced aggregation of CS and other model substrate proteins (Gustavsson et al. 2001; Harndahl et al. 2001).

Here we have used peptide array screening (Frank 1992, 2002; Hultschig and Frank 2004) for probing what parts of the model substrate protein CS that are involved in the substrate–chaperone interaction and identified a region with presumed sHsp-binding that is absent in thermostable CS species.

Materials and methods

Stock solutions of proteins

Porcine CS (10 mg/ml) was obtained from Roche (Mannheim, Germany). Desalting and buffer exchange into 12.5 mM Tris–HCl pH 7.0, 75 mM NaCl, 10 mM DTT was performed using disposable protein desalting columns (Pierce, Rockford, IL) to yield a stock solution of 2.1 mg/ml. Recombinant proteins were obtained as described for Hsp21 below, and elsewhere for αB-crystallin and Hsp27 (Boros et al. 2004). All protein stock solutions (concentrations 2–5 mg/ml) were stored in –20°C until use.

Purification of Hsp21

Hsp21 was recombinantly expressed in *E. coli* as described previously (Harndahl et al. 2001), and further purified by urea-induced monomerization and unfolding (Blennow et al. 1995) in order to remove trace amounts of bound bacterial proteins. The preparation of Hsp21 oligomers (1 ml, 2–3 mg/ml) was incubated in urea-buffer (4 M urea, 25 mM Tris/HCl, 150 mM NaCl, 10 mM DTT, pH 7.0) on ice for 20 min, and loaded to a size exclusion chromatography column (Pharmacia HiLoad/Superdex 200 HR 16/60) equilibrated with urea buffer. The predominant peak contained the Hsp21 monomers. Collected fractions from this peak were subjected to dialysis (4°C, 15–20 h, one buffer change) with urea-free buffer (12.5 mM Tris, 75 mM NaCl, 10 mM DTT, pH 7) using 12–14 kDa cut off Spectra/Por membranes (Spectrum medical Industries Inc, Houston, TX) to permit re-assembly of Hsp21 oligomers. The dialysed sample was concentrated with Macrosep (30 K cut off, PALL Life Sciences, SPG Media, London, UK) for 1 h at 7,100*g*. Any Hsp21 monomers, which had not re-assembled into oligomers were thus discarded. Finally, protein concentration to 2–5 mg/ml was performed with Microsep (100 K cut off, PALL Life Sciences), 30 min at 2,600*g*.

CS aggregation–protection assay

CS aggregation and Hsp21 protection was determined as in (Basha et al. 2004). CS (1 μM) was incubated with or without Hsp21 (12 μM) for 20 min at 47°C, in a total volume of 100 μl, followed by separation of soluble protein and aggregated protein by centrifugation (10 min at 14,000*g* at 4°C). Supernatant was withdrawn and concentrated to dryness in a Speed-Vac (Labex, Helsingborg, Sweden), and the pellet washed once by resuspension in 1 ml urea-free buffer and pelleted once more. Supernatant and pellet fractions were thereafter resuspended in loading buffer [0.0625 M Tris/HCl, 25% glycerol (v/v), 2% SDS (w/v), 5% *β*-mercaptoethanol (v/v), 1.25% of 1% bromophenol blue (v/v)], and incubated at 95°C for 5 min. The gels (Ready gels, 12% Tris/HCl, BioRad Laboratories AB, Sundbyberg, Sweden) were run in Laemmli buffer system at 200 V, 100 mA. Protein corresponding to 12.5 and

2.5 µg Hsp21 and CS, respectively, were loaded per lane, and the gel was stained with collodial CBB.

Synthesis of peptides and covalent coupling to cellulose membranes

Peptide arrays with synthetic peptides corresponding to the whole sequence of the porcine CS (P00889) covalently attached to a cellulose membrane were obtained by spot synthesis according to (Frank 1992) with a modified Gilson 222/401 Spotting robot, using a novel amino-PEG-cellulose as a solid support. F-moc derivatives of amino acids and further reagents for the peptide synthesis were obtained from Bachem (Bubendorf, Switzerland), Calbiochem (Bad Soden, Germany), and Alexis (Grünberg, Germany). Amino-PEG-cellulose membranes were obtained from AIMS Scientific Products GbR (Braunschweig, Germany). Organic solvents were obtained from Lancaster (Mühlheim, Germany) and SDS (Peypin, France).

Binding assays with peptide array membranes

Incubation of sHsps with peptide arrays

Binding assays was performed as described in (Koch et al. 2002). The amount of sHsps bound to the peptide array was determined directly on the cellulose membrane, without transfer of bound proteins to a nitrocellulose membrane. Therefore false positive labelling due to weak and unspecific binding of sHsps to the membrane was avoided, probing only high-affinity sHsp target sequences. False positive labelling due to unspecific binding of antibodies used for immunoquantification of bound sHsps were assessed by a separate incubating of the peptide array membrane without prior incubation with the corresponding sHsp; these values were used to subtract the background labelling. Each binding experiment was repeated at least twice and in all cases the standard deviation was less than 10%. The peptide array membrane was first washed, 3×5 min with T-TBS (20 mM Tris/HCl, 140 mM NaCl, pH 7.6 with 0.1% (v/v) Tween-20), thereafter incubated for 2 h, or overnight, with a casein-based blocking buffer, prepared by dilution five times with T-TBS a concentrate from Sigma-Genosys (Cambridge, UK; Cat. No. SU-07-250) and addition of sucrose (5%, w/v). After washing with T-TBS, 3×5 min, incubation was performed with one of the sHsp proteins (1 mg in 20 ml blocking solution) for 1 h at 23°C on a rocking table. Unbound protein was washed away with T-TBS, 3×5 min.

Detection of bound sHsps

The amount of sHsp protein bound to the peptide spots on the peptide array membrane was detected with primary antibodies specific for either of the two sHsps. For Hsp21, a polyclonal rabbit antiserum raised against recombinant Hsp21 (Innovagen AB, Lund, Sweden) was used at a 1:500 dilution in blocking buffer. For detection of αB-crystallin, a mouse monoclonal antibody was used (Boros et al. 2004). Incubation 1 h with primary antibody was followed by T-TBS washing (3×5 min), incubation 1 h with alkaline phosphatase-conjugated secondary antibodies (goat–anti-rabbit, 1:3,000, Bio-Rad, Sundbyberg, Sweden or goat–anti-mouse, 1:5,000, Promega, Madison, WI), followed by T-TBS washing (3×5 min).

Quantification of sHsp binding

Visualization of the bound proteins was achieved by determining the alkaline-phosphate catalyzed fluorescence of ECF substrate (Amersham Pharmacia Biotech, Uppsala, Sweden) by scanning the membrane on a Storm 860 workstation (Molecular Dynamics, Sunnyvale, CA), and quantification performed using the ImageQuant software.

Regeneration of membrane for repeated use

The peptide array membrane was regenerated after use by stripping off all bound proteins by washing [3×10 min with each of water, Buffer A (8 M urea, 1% SDS (w/v), 0.5% (v/v) ß-mercaptoethanol), Buffer B (50% (v/v) ethanol, 10% (v/v) acetic acid] and ethanol.

Results

To ensure that the Hsp21-preparation could protect CS against temperature-induced aggregation in vitro just as αB-crystallin (Boros et al. 2004), an assessment was made of CS aggregation upon incubation at 47°C, in the absence or the presence of Hsp21. Soluble protein (supernatant, S) was separated from aggregated protein (pellet, P) showing that all CS aggregated and recovered in pellet in the absence of Hsp21, whereas all CS were maintained soluble in the presence of Hsp21, which thus prevented the CS aggregation (Fig. 1).

To evaluate which part of CS the sHsps bind to and thereby prevent its temperature-induced aggregation, a peptide array membrane was used, onto which synthetic peptides corresponding to the entire sequence of the CS were covalently attached. All synthesized peptides were 15 amino acids long with a 3 amino acids shift along the

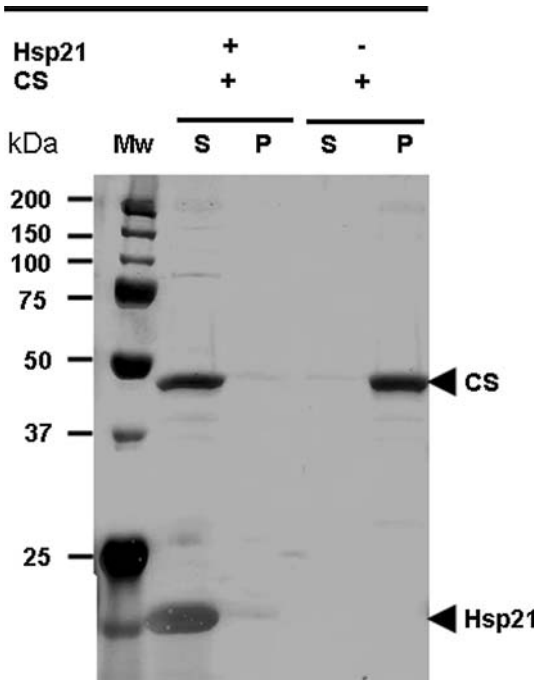

Fig. 1 Aggregation of CS is prevented by sHsps. CS (1 μM) was incubated for 20 min at 47°C in absence or presence of Hsp21 (12 μM), after centrifugation soluble protein (supernatant, *S*) was separated from aggregated protein (pellet, *P*). Protein corresponding to 12.5 and 2.5 μg was loaded per lane of Hsp21 and CS, respectively, SDS-PAGE gel stained with CBB

sequence between each peptide. The sHsp binding to the peptides in the various spots in the peptide array membrane was quantified after incubation with Hsp21 and αB-crystallin.

Binding of sHsps only occurred to amino acids representing the most N-terminal part of the CS. Figure 2a shows the first row on the cellulose-membrane, with peptides covalently attached in circular spots, and above the spots it is outlined how the spot numbers relate to the numbers of the 15-mer peptides These amino acid numbers refer to the complete CS sequence (P00889), including the 27 first amino acids which correspond to the signal sequence which is not present in the mature form of CS. The 15-mer peptide number 14 was strongly labelled, by Hsp21 (Fig. 2b) and αB-crystallin (Fig. 2c). The 15-mer peptide number 14 corresponds to the amino acid sequence L_{13}IPKEQARIKTFRQQ$_{27}$ in the N-terminal part of the mature CS sequence. For αB-crystallin binding also occurred to a neighbouring peptide with several overlapping amino acids, A_{19}RIKTFRQQHGNTVV$_{33}$. A third sHsp, human Hsp27, also labelled the peptide L_{13}IPKEQARIKTFRQQ$_{27}$ (data not shown). Thus three different sHsps bound strongly to the amino acid sequence L_{13}IPKEQARIKTFRQQ$_{27}$ in the N-terminal part of the mature CS sequence.

The strong binding of sHsps to the CS peptide L_{13}IPKEQARIKTFRQQ$_{27}$ corresponds to the most N-terminal α-helix in CS, the so-called helix A (Remington et al. 1982), which is surface exposed in the CS dimer. Remarkably, this part of the CS enzyme is absent in thermostable forms of CS, as outlined in the sequence alignments in Fig. 3, and in the CS structures in Fig. 4. The CS dimer from one angle resembles an oak tree with a stem-like structure comprised of helix A from each of the monomer, which is close to the C-terminus of the other monomer (Fig. 4a–c). This stem-like structure is absent in thermostable forms of CS from themophilic bacteria (Fig. 4d).

Discussion

To our knowledge no evidence has hitherto been provided concerning what parts of any substrate protein the sHsp-binding is targeted to. The here used substrate protein, CS, is one of the best-characterized proteins in terms of thermostability (Russell et al. 1994, 1997; Arnott et al. 2000). The here presented data can therefore be compared with detailed 3D-structures of CS in various forms with different thermostability. Thermosensitive porcine CS is a dimer of identical monomers, with each monomer composed of 20 α-helices distributed between a small catalytic domain (helices N–R) and a large domain (helices A–M, S–T) which forms most of the inter-subunit contacts. The dimer interphase, comprised of an eight α-helical sandwich of four antiparallel pairs of helices (F, G, L, M), harbouring the active site with ligands to both citrate/oxaloacetate and CoA/Ac being provided from each of the monomer subunits. Maintenance of the dimeric integrity at higher temperatures is therefore important, with monomerization preceding aggregation.

Thermostable forms of CS have been investigated in molecular detail to understand the difference in thermostability between CS from pig, *Thermoplasma acidophilum*, *Thermus aquaticus*, *Sulfolobus solfatacarius* and *Pyrococcus furiosus*, organisms that differ profoundly in terms of optimal growing temperatures (37, 55, 70, 85 and 100°C, respectively) (Russell et al. 1994, 1997; Arnott et al. 2000; Nordberg Karlsson et al. 2003). The gradual increase in thermostability for CS from the above-mentioned sources has been coupled to a number of structural differences, such as a reduction in the number and lengths of loops in the CS structure, and stabilization of the interactions between the four helices F, G, L and M that form the dimer interphase, either by an increased number of hydrophobic amino acids or an increased number of ion-pair forming amino acids. Apart from these differences,

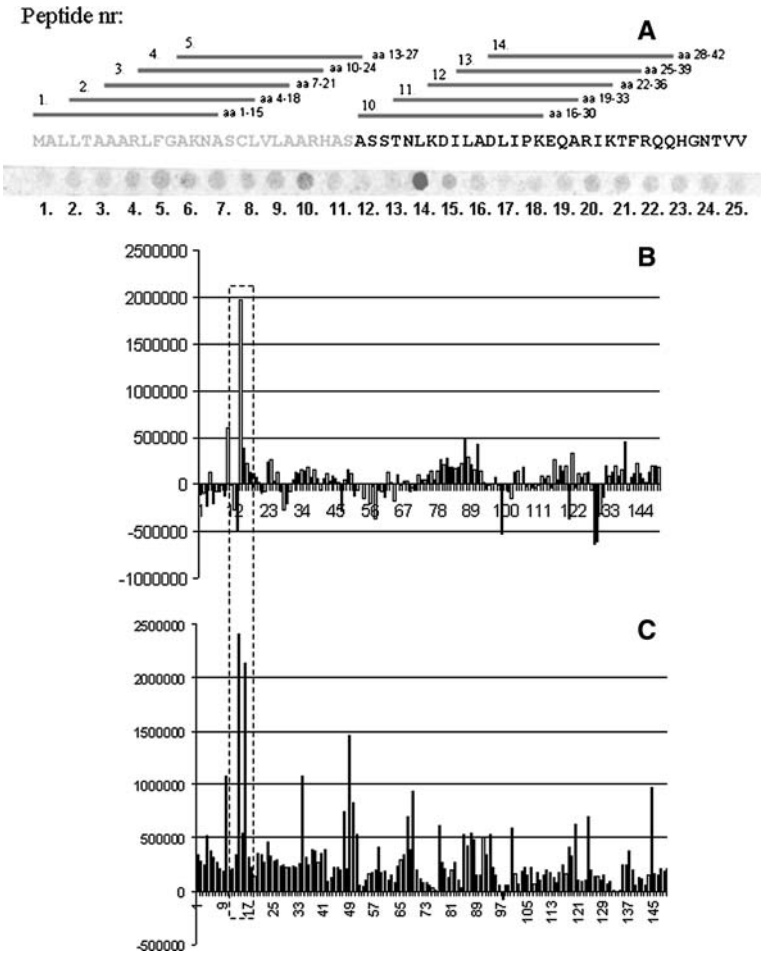

Fig. 2 Binding of sHsps to CS assessed by peptide array screening. Synthetic peptides, corresponding to the amino acids of the CS sequence (P00089) fixed in arrays onto a cellulose membrane were incubated with or without two different sHsps. Binding of sHsps was evaluated by immunodetection and fluorescence imaging. **a** The synthesized peptides were 15 amino acids long with a 3 amino acid shift along the sequence, with numbering referring to the numbering of peptides used in panel **b** and **c**. *Lower part* shows the corresponding first row on the cellulose-membrane, with peptides covalently attached in circular spots which are numbered according to the peptides. Note that the first 18 amino acids (*greyshaded* in sequence) belong to the mitochondrial presequence, which is not present in mature CS. **b** Quantification after fluorescence imaging of the binding of Hsp21 to CS peptide number 1–150. **c** Quantification after fluorescence imaging of the binding of αB-crystallin to CS peptide number 1–150. Background labelling without sHsps was subtracted

thermostable forms of CS differ from thermosensitive CS also in lacking the N-terminal part of the sequence (Fig. 3), and the stem-like structure in the porcine CS (Fig. 4d). In thermosensitive CS, the C-terminus in one monomer is close to helix A in the other monomer (Fig. 4c). In thermostable forms of CS where helix A is absent, the C-terminus of one monomer folds over and interacts with the other monomer resulting in a close monomer–monomer interaction promoting dimer integrity at higher temperatures (Russell et al. 1997). Another typical feature in thermostable CS is that the N-terminae in the two subunits are close to each other, in contrast to thermosensitive CS where the N-terminae are flexible and situated on each side of the dimer.

Thus sHsps could provide a means of dimer stabilization in presence of helix A, in its effect resembling the stabilization of the N- and C-terminae of thermostable CS, by locking up the otherwise flexible N- and C-terminae to the other monomer. Many proteins are degraded from the terminae and lowering their flexibility may increase thermostability and reduce the accessibility for proteases and degradation, which is indeed an effect recently reported for sHsps (Han et al. 2005).

In summary, our peptide array data suggest that sHsps stabilize the thermosensitive CS dimer by binding to a structure, which is lacking in thermostable forms of CS, comprised of the N-terminal region, which is close to the C-terminus in the other monomer.

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                                                          A
P00889|CISY_PIG     MALLTAAARLFGAKNASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGNTVV 60
Q9CZU6|CISY_MOUSE   MALLTAATRLLGAKNSSCLVLAARHASASSTNLKDVLSNLIPKEQARIKTFKQQHGKTVV 60
O75390|CISY_HUMAN   MALLTAAARLLGTKNASCLVLAARHASASSTNLKDILADLIPKEQARIKTFRQQHGKTVV 60
Q53554|CISY_PYRFU   ------------------------------------------------------------
P80148|CISY_SULSO   ------------------------------------------------------------
P21553|CISY_THEAC   ------------------------------------------------------------

                        B                                   C
P00889|CISY_PIG     GQITVDMMYGGMRGMKGLVYETSVLDPDEG-IRFRGYSIPECQKMLPKAKGGEEPLPEGL 119
Q9CZU6|CISY_MOUSE   GQITVDMMYGGMRGMKGLVYETSVLDPDEG-IRFRGYSIPECQKMLPKAKGGEEPLPEGL 119
O75390|CISY_HUMAN   GQITVDMMYGGMRGMKGLVYETSVLDPDEG-IRFRGFSIPECQKLLPKAKGGEEPLPEGL 119
Q53554|CISY_PYRFU   -MNTEKYLAKGLEDVYIDQTNICYIDGKEGKLYYRGYSVEELAELST---------FEEV 50
P80148|CISY_SULSO   ----MSVVSKGLENVIIKVTNLTFIDGEKGILRYRGYNIEDLVNYGS---------YEET 47
P21553|CISY_THEAC   -MPETEEISKGLEDVNIKWTRLTTIDGNKGILRYGGYSVEDIIASGAQ--------DEEI 51
                          . :  *:..:     .   :* .:* : : *:.: :     .          *
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Fig. 3 Alignment of the N-terminal part of the CS amino acid sequence from mammalia and thermophilic archaebacteria. The CS sequences represent enzymes in organisms growing optimally at 37 (pig, mouse, human), 55 (*Thermoplasma acidophilum*), 85 (*Sulfolobus solfataricus*), and 100°C (*Pyrococcus furiosus*) taken from Swiss-Prot and aligned with Clustal W. Above the porcine CS sequence the secondary structure elements are indicated, taken from the resolved structure for porcine CS (accession number in PDB: 4CTS). The mitochondrial presequence, which is not present in the mature protein, is indicated by *grey letters*. *Bold letters* in the sequence of porcine citrate synthase (P00889) indicates the amino acids 13–27 LIPKEQARIKTFRQQ representing the strongly labelled spot (peptide 14) in Fig. 2

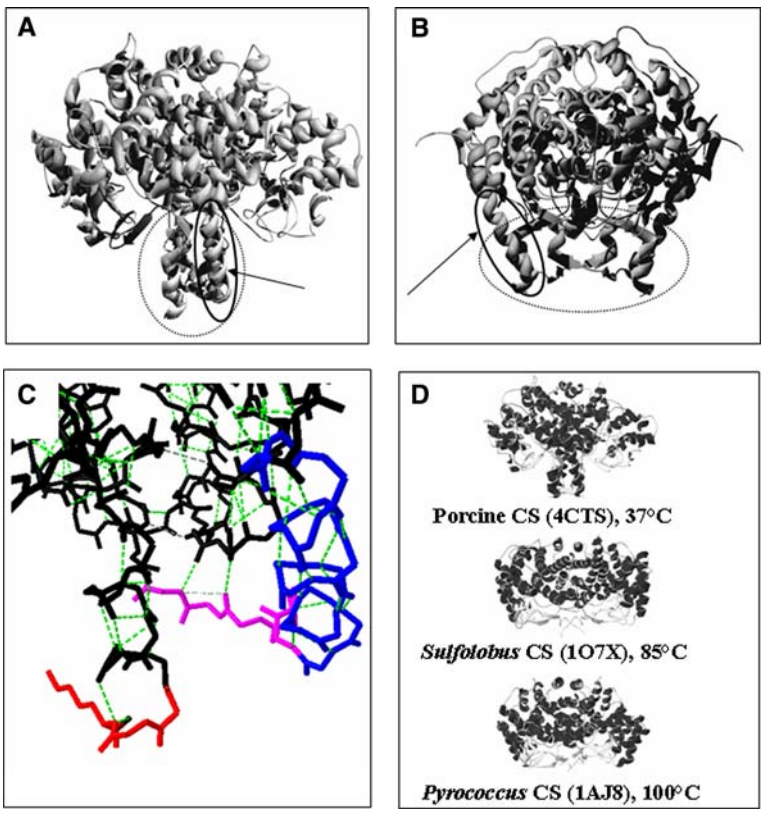

Fig. 4 The stem-like structure and helix A in thermosensitive forms of CS. Porcine CS (PDB: 4CTS), **a** sideview and **b** frontview, highlighting the appearance of the CS dimer as an oak tree with a stem-like structure (encircled with *dashed line*) formed by the N-terminal helix A (encircled, and pointed by *arrow*) from one monomer with the C-terminal part of the other monomer. **c** Close-up showing the amino acid sequence L_{13}IPKEQARIKTFRQQ$_{27}$ (α-helical) in *blue* and additional amino acids 28–33 in *purple*, and the C-terminus of the other monomer subunit in *red* (amino acids 435–437). **d** Comparison of ribbon presentations of CS structures from species with different growth optima: porcine CS (37°) which has the stem-like structure and helix A; PDB-file 4CTS, *Sulfolobus solfataricus* (85°C) CS; PDB-file 107X, and *Pyrococcus furiosus* (100°C) CS; PDB-file 1AJ8

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