Instituto Juan March
de Estudios e Investigaciones

115 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Chaperonins: Structure and Function

Organized by

W. Baumeister, J. L. Carrascosa and J. M. Valpuesta

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Introduction

J. M. Valpuesta, J. L. Carrascosa and W. Baumeister

The correct folding of proteins is a cellular process of paramount importance that is far from being completely understood. As first stated by Anfinsen several decades ago, the information necessary for the proper folding of a protein is encoded in its own amino acid sequence. Nevertheless, folding of proteins in the cell is faced with problems associated with the enormous macromolecule concentration, with the intermembrane transport of proteins or with stress situations. To cope with these problems, the organisms have generated a series of proteins, termed molecular chaperones, which assist in the folding of denatured polypeptides. Besides this fundamental role, molecular chaperones are also involved in a variety of cellular processes, ranging from prion-related protection, immunosuppressive function and anti-tumor immunization. Among this ever increasing family of proteins, the best characterised members are the chaperonins, molecular chaperones with a molar mass around 60 kDa. that are assembled into multimeric complexes composed of two rings, each one enclosing a cavity where folding takes place.

The chaperonins have been classified in two groups: the first one contains the chaperonins from eubacteria like GroEL from *E. coli* or those found in eukaryotic organelles (such as the mitochondrial Hsp60 or the Rubisco binding protein from chloroplasts), while Group II encloses all chaperonins from archaeobacteria and the eukaryotic cytosolic chaperonin (CCT). Both types of chaperonins share a similar morphology but they have differences both in structure and function. Some of the differences are related to the degree of oligomerization as well as the presence or absence of a cochaperonin that assists the chaperonin in its functional cycle.

This workshop has assembled scientists working in different aspects in the field of chaperonins, and it has provided a good opportunity to review some of the most exciting aspects of both structure and function of this family of proteins. Reports on the Group I chaperonins have been focused on GroEL. Several talks have dealt with detailed biochemical analysis of the folding activity of GroEL. New kinetic data have allowed to further characterize the allosteric mechanism governing the GroEL folding cycle. The discussion of the detailed structured of GroEL and its complex with GroES, obtained by X-ray diffraction, together with the electron microscopy reconstructions of different conformers, has shed new light on the mechanisms underlying the interaction with the substrate. The fine structural

analysis of GroEL mutants, together with the possibility of kinetic simulations, seems to be a very promising avenue for further analysis of the subtle details of the folding process.

The talks presented in the workshop have provided a boost in the field of Group II chaperonins. The great effort invested lately in this type of chaperonins has generated an exciting combination of structural, biochemical and genetic data that have revealed a more complex picture and different function and mechanism than those observed for GroEL. Atomic information obtained for the thermosome and several co-factors of CCT was presented, as well as electron microscopy three-dimensional reconstructions of the thermosome and the cytosolic chaperonin. The three-dimensional structures of substrate-bound CCT, and the structural transitions driven by the binding and hydrolysis of ATP were also discussed. These data, combined with detailed analysis using biochemical and mutational methods, have allowed to outline the basic features of the CCT functional cycle. The discussions during the workshop highlighted the different roles of every chaperonin type in the folding of either non-specific or specific proteins, and revealed exciting hypothesis on the evolution of these molecular machines, along with their substrates, during the generation of the different phila.

José M. Valpuesta, José L. Carrascosa and Wolfgang Baumeister

Session 1
Chair: Arthur L. Horwich

Mechanism of group I and group II chaperonins: Recent insights and open questions

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Group I chaperonins can transiently enclose unfolded polypeptide in a cage that provides an environment permissive for folding. This mechanism has been demonstrated convincingly for the E. coli GroEL/GroES system and is thought to prevent protein aggregation during folding. Group II chaperonins are apparently independent of a GroES-like co-chaperone and it is not clear to what extent sequestration of non-native substrate plays a role in their mechanism. Recent evidence suggests that group II chaperonins in eukaryotes and archaea cooperate functionally with an oligomeric cofactor, termed prefoldin or GimC. Prefoldin is structurally unrelated to GroES and, in contrast to GroES, has a chaperone activity on its own. We have obtained the high-resolution crystal structure of archaeal prefoldin. The structure and its functional implications will be discussed.

Mechanisms of protein folding and misfolding *in vitro* and *in vivo*

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Our understanding of how a protein folds has increased enormously recently, principally through the combination of experimental and theoretical methods. Views of folding ranging from simple two-state transitions to more complex mechanisms involving parallel pathways and partially folded states have emerged. An important question that remains is the role of intermediates in folding- do they guide folding molecules towards the native state or are they misfolded species that might lead to aggregation or be substrates for chaperones that assist folding *in vivo*. In this presentation this issue will be addressed by drawing on our recent data of protein folding of a variety of different protein systems. In particular, recent data using fast mixing will be used to illustrate the role of an intermediate in the kinetic folding pathway of a small all helical protein and the conformational properties of an intermediate in the folding of the all-beta sheet protein of β_2-microglobulin that is involved in a human amyloid disease will be described. Finally, the potential influence of chaperones on protein folding and misfolding will be discussed using our knowledge of the conformational properties of intermediates and the nature of substrate recognition by chaperones.

Twelve years later: re-questioning the biochemical definition of chaperone activity *in vitro*

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The biochemical activity of a molecular chaperone was first demonstrated *in vitro* in the case of GroELS from *Escherichia coli*, which specifically bound and prevented the aggregation of a denatured protein, and assisted it's correct refolding in a strict ATP and GroES-dependent manner (Goloubinoff et al., 1989). Although chaperone activity was initially defined as assisted-correct refolding of a denatured protein, the ability to bind and prevent protein aggregation was since often used instead. Yet, recent work describe some powerful chaperones that cannot prevent aggregation, others that can but need not to prevent aggregation in order to assist protein refolding. Therefore, chaperones have two distinct biochemical activities that do not necessarily overlap: prevention of aggregation and assisted-refolding. Prevention of aggregation is more important during than after stress. It is commonly measured by light scattering, but results are variable and often ignore small soluble aggregates. Chaperone refolding activity is more important after than during stress. It is commonly expressed as a fraction (%) of recovered protein at the end of the refolding reaction (yields), as compared to a native control before denaturation. But this expression lacks essential information about chaperone's specific activity. When folding activity is correctly expressed, in terms of numbers of refolded substrate proteins, per time unit, per chaperone molecule (rates), astoundingly low efficiencies are revealed that may question the biological relevance of *in vitro* chaperone assays.

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Allostery in chaperonin function

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The GroE chaperonin system comprises GroEL and its cofactor GroES. It assists protein folding in an ATP-regulated manner. Steady-state studies carried out during the past several years suggested a nested model for cooperativity in GroEL function with positive intra-ring cooperativity and negative inter-ring cooperativity (1). According to this model, each GroEL ring is in equilibrium between a **T** state, with low affinity for ATP and high affinity for non-folded proteins, and an **R** state with high affinity for ATP and low affinity for non-folded proteins. The GroEL double-ring structure is thus in equilibrium between the **TT**, **TR** and **RR** states. Steady-state studies carried out very recently (2) on CCT from bovine testis suggest that it also has positive intra-ring cooperativity and negative inter-ring cooperativity in ATP hydrolysis, with respect to ATP, as previously observed in the case of GroEL. It is shown that the relatively weak positive intra-ring cooperativity found in the case of CCT may be due to heterogeneity in its subunit composition. Cooperativity in GroEL with respect to ATP has also been analysed using transient kinetics. Rates of the **T** Æ **R** transition were measured at different ATP concentrations. The results were found to be consistent with the nested model. The steady-state and transient kinetic data for wild-type GroEL and mutants were combined in order to obtain information regarding the pathway of the allosteric transition (3). The data suggest that in the transition state of the reaction, there is some concerted twisting of the apical domains of each subunit which precedes vertical opening of the rings. The data also suggest that the allosteric switch is concerted (4) in agreement with simulations (5). Finally, more recent data regarding the role of allostery in the folding kinetics of model substrates in vitro (6) and in vivo (7) will be presented.

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Session 2
Chair: Keith R. Willison

What does kinetics tell us about chaperonins?

Anthony R. Clarke

There are two major models to describe the basic mechanism of action of the GroE system. The 'unfoldase' mechanism asserts that any protein molecule which misfolds will bind to GroEL and be unfolded by interactions with hydrophobic sites on the protein-binding surface. Subsequent nucleotide/GroES driven dissociation will then release the unfolded molecule to allow it the chance to refold productively. Alternatively, adherents of the 'encapsulation' mechanism argue that misfolding is a multi-molecular process involving aggregation of non-native chains, so that if a protein molecule were allowed to fold in an isolated capsule, then folding would be a highly efficient process. This mechanism requires that the unfolded molecule is first bound, then released into a closed cavity and finally let loose from the cavity once folded. Both mechanisms are plausible and they are not mutually exclusive.

Experiments in our laboratory have concentrated on attempting to distinguish between potential mechanisms by defining (1) the ligand-binding properties and reaction kinetics of the system with respect to the dynamics of nucleotide binding and hydrolysis and (2) the influence of different conformational states on the ability of GroE to bind to unfolded protein substrates and to its co-protein GroES. The kinetics of binding, encapsulation and ejection of a real protein substrate will be described and the unusual reciprocating mechanism of the two GroE rings will be defined in terms of the ability of ADP to act as a non-competitive inhibitor and the ability of the rings to act as cooperative units during the hydrolytic cycle.

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GroEL-GroES-mediated folding

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While structural states of the GroEL-GroES machine itself are becoming increasingly well-defined, the fate of the polypeptide substrate remains less understood, at least in part because non-native states are not stable and likely to be ensembles of individual species. Because it seems desirable to understand the full range of GroEL-GroES actions on substrate proteins, we have been studying the binding and folding of stringent substrate proteins like Rubisco from *R. rubrum* and mitochondrial MDH from pig heart, which require the full GroEL-GroES-ATP system for productive folding. Studies of several sorts seek to address the occurrence and importance of ATP/GroES-driven unfolding and the influence of the *cis* cavity on folding.

Structure and allosteric movements in wild type and mutant chaperonins

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A.M. Roseman, MRC Laboratory of Molecular Biology, Cambridge
A.L. Horwich, H.S. Rye, G.W. Farr, Yale University, New Haven
B. T. Sewell, EM unit, University of Cape Town
S. van der Vies, Vrije Universiteit Amsterdam

We have been using cryo-electron microscopy and 3D reconstruction to examine the conformational changes and allosteric interactions in wild-type and mutant chaperonins. In *E. coli*, chaperonin-assisted protein folding proceeds through cycles of ATPase action by the large chaperonin GroEL, and involves concerted, rigid body movements of the 3 domains in each subunit. Transient binding of the small chaperonin GroES forms an enclosed chamber, inside which folding of a protein subunit takes place. Access to the chamber and to the binding sites is regulated by the nucleotide cycle. Binding, encapsulation and release states alternate between the two back-to-back 7-membered rings of GroEL, which communicate through the inter-ring interface. Combining the cryo EM maps with atomic structures of the domains provides a detailed structural basis for understanding the allosteric mechanism of chaperonins and control of their interactions with folding protein substrates.

Binding of ligands or mutations lead to dramatic and often unexpected conformational changes in GroEL complexes. GroEL subunits contain two flexible hinge regions, and a wide range of different movements is observed. The GroEL folding cycle is initiated by the cooperative binding of ATP in one of the heptameric rings. The cryo EM structure of a mutant GroEL with a slowed ATPase rate (D389A) at 11 Å resolution, combined with an accurate docking of the subunit domains into the EM density, reveals changes in the inter-ring interface and in the orientation of domains in one of the rings. Examination of an interface mutant, E461K, reveals a surprising reorganisation of the inter-ring interface, and provides a structural basis for the temperature-sensitive behaviour of this mutant. Comparisons of ADP and ATP forms of GroEL-GroES complexes suggest that there are also changes in the GroES oligomer.

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Mechanisms of GroE-assisted protein folding

Johannes Buchner

Protein folding is known to be a spontaneous reaction *in vitro*. In the cell, however, a sophisticated machinery of molecular chaperones exists which supports the folding process of newly synthesised or stress-damaged proteins. The GroE chaperone complex of *E. coli* exhibits a complicated double ring structure which is required for assisting protein folding under nonpermissive conditions. Using *in vitro* protein folding assays we have analysed the interaction of GroE with maltose binding protein (MBP) and citrate synthase (CS) in their nonnative states. In the case of CS, GroEL is able to bind several distinct nonnative conformations. Processing of a chaperone-bound protein requires ATP-induced conformational changes and, in many cases, the association with cofactors. For the GroE system, binding of ATP and the cofactor GroES leads to the formation of a cavity in which a single protein molecule can fold. Our experimental systems allow to define the requirements of the GroE system for catalysed folding and for the folding of subunits of oligomers. Defining further the influences of GroE on the folding trajectories of proteins will be important to understand both molecular chaperones and protein folding.

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Entirely concerted domain movements of GroEL

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The domains of GroEL undergo large rigid-body movements in response to the binding of various ligands. The binding of ATP to the equatorial domain induces the allosteric T->R transition, a clockwise twisting motion of the apical domain relative to the equatorial domain. The subsequent binding of GroES to the R state GroEL•ATP complex induces the R->R' transition, the huge upward displacement and further twisting of the apical domain to form the asymmetric GroEL•ATP•GroES complex. The theory of nested cooperativity (Horovitz & Yifrach, 2000) as well as molecular dynamic simulations (Ma et al., 2000) indicate that these movements are concerted in nature. Here we provide experimental evidence for the entirely concerted nature of these domain movements.

Two inter-domain salt bridges, K327-D83 and R197-E386 are among the interactions that stabilize the T state. K327-D83 links the apical and equatorial domains of the same sub-unit while R197-E386 links the intermediate domain of one sub-unit with the apical domain of the adjacent sub-unit. Murai et al. (1996) created the double cysteine mutant K327C-D83C and showed that the R->R' transition was prevented when all seven sub-units were oxidized. We show here that the introduction of a *single* inter-domain disulfide bond in the ensemble of seven sub-units is sufficient to prevent GroES binding to that ring. We have also created the double cysteine mutant R197C-E386C and show that the introduction of a *single*, covalent, inter-domain cross-link is likewise sufficient to prevent GroES binding.

Release of the ligands from the *cis*-ring of the asymmetric GroEL•ADP•GroES complex is triggered by the binding of ATP to the *trans*-ring. We show here that this ring-to-ring communication does not require the breakage of K327-D83 salt bridge, or, by inference, the T->R transition in the *trans*-ring.

The T->R transition can be prevented by restraining the Cα to Cα distance of K327-D83 to no more than about 12.8Å. This can be accomplished by oxidizing the K327C-D83C mutant or by reacting the two cysteines with *o*-phenylene dimaleimide. The introduction of a *single*, covalent, inter-domain bond between K327 and D83 into *each* ring of GroEL is sufficient to prevent the TT->TR->RR transitions. This is consistent with an entirely concerted domain movement, during the T->R transition, as well as with the basic premises of nested cooperativity. Cross-linkers, such as *m*- and *p*- phenylene dimaleimide, that permit the Cα to Cα distance of K327C-D83C to exceed about 12.8Å fail to prevent the T->R transition although they do prevent the subsequent R->R' transition accompanying GroES binding.

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Session 3
Chair: George H. Lorimer

A dynamic model for the allosteric mechanism of GroEL

Martin Karplus

GroEL-assisted protein folding is regulated by a cycle of large coordinated domain movements in the 14-subunit double-ring assembly. The transition path between the closed (unliganded) and the open (liganded) states, calculated with a targeted molecular dynamics simulation, shows the highly complex subunit displacements required for the allosteric transition. The early downward motion of the small intermediate domain induced by nucleotide binding emerges as the trigger for the larger movements of the apical and equatorial domains. The combined twisting and upward displacement of the apical domain determined for a single subunit is accommodated easily in the heptamer ring only if its opening is concerted. This is the source of cooperative ligand binding within a ring. It suggests that GroEL has evolved so that the motion required for heptamer cooperativity is encoded in the individual subunits. A calculated model for a di-cis 14-subunit assembly is destabilized by strong steric repulsion between the equatorial domains of the two rings, the source of negative cooperativity. The simulation results, which demonstrate that transient interactions along the transition path are essential for GroEL function, provide the first detailed structural description of the motions that are involved in the GroEL allosteric cycle.

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From minichaperones to GroEL

Alan Fersht, Cambridge

We have successfully used a fragment of the apical binding domain of GroEL as a system for obtaining detailed information on GroEL-substrate interactions and also for practical purposes, harnessing its residual chaperoning activity for refolding recombinant proteins. A major goal is to build up the mechanism of GroEL by detailed studies on the structure and activity of the "minichaperone" and the effects of adding more features of the structure of GroEL. We have now extended our structural analyses and have mapped out more of the binding site of GroEL using crystallographic studies on the minichaperone. We have investigated the activity of constructs of minichaperones in artificial seven-membered rings. We have extended these studies one step further by detailed investigation of single ring versions of GroEL. These studies have encompassed measuring activity *in vivo* and *in vitro*. From these, we can see how the complex machine of GroEL has evolved from a simple chaperoning activity to its current highly efficient state.

Gene duplication and the evolution of CCT/TriC

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Gene duplication has been an important factor in the evolution of the eukaryotic chaperonin CCT (TriC). Eight CCT subunit families (paralogs) have been characterized. We have sequenced five CCT genes from Trichomonas vaginalis and seven from Giardia lamblia, representatives of amitochondriate eukaryotic lineages suggested to have diverged early from other eukaryotes. Our data show that (1) the duplications producing the paralogs predate the divergence of Trichomonas and Giardia from other eukaryotes and (2) the duplications producing CCTdelta and epsilon, as well as alpha, beta and eta, appear to be the most recent in the evolution of the CCT gene family. Our analyses show significant differences in the rates of evolution of archaeal chaperonins compared to the CCTs, as well as among the different CCT subunits themselves. To examine the pattern and degree of conservation in the different CCT subunits, we estimated the rate of sequence evolution at amino acid sites for each subunit. All the subunits were found to possess unique "signatures" that are invariant or nearly so with respect to the other CCTs. When mapped onto the crystal structure of the Thermoplasma chaperonin, these signatures were present in the apical, intermediate and equatorial domains. Our results have implications for the evolution of subunit-specific function(s) in CCT.

The Chaperone Complement of *Thermoplasma acidophilum*

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The archaeon *Thermoplasma acidophilum* has become an important model organism for studying the components of the cellular pathways of protein folding and degradation, most notably amongst them the thermosome –the archetype of the group II chaperonins- and the 20S proteasome (for recent reviews see 1,2).

The genome of *Thermoplasma acidophilum*, which is the smallest of all known free-living organisms (1 564 905 base pairs or 1 509 ORFs) has recently been sequenced, providing us with the full complement of proteins involved in folding and degradation (3).An ongoing analysis of the proteome of *Thermoplasma acidophilum* indicates that the small heat-shock protein (hsp20),the thermosome and VAT-ATPase,a homologue of Cdc 48 with foldase and unfoldase activity (4),are amongst the most abundant cellular proteins (W.Graml et al.,in preparation).Average *Thermoplasma* cells contain between 500 and 1000 copies of them.

Following a description of the structures of the thermosome and of VAT, ,a report will be given on recent advances towards the long term goal of depicting protein complexes such as the thermosome in an undisturbed cellular context. Cryo-electron tomography of whole prokaryotic cells has entered the realm of molecular resolution (3-4 nm), which allows to detect and identify molecules by virtue of their structural signature (5).This opens up entirely new possibilities to map the territorial distribution of molecules and their interactions *in situ*.

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Crystal structure of a thermosome

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The crystal structure of the thermosome from the archaeon *Thermoplasma acidophilum* at 2.6A (Ditzel et al., 1998) resolution reveals two features unique to group II chaperonins: a different type of inter-ring contact compared to group I chaperonins and a mechanism of particle closure that explains why group II chaperonins can function without a GroES-like co-chaperonin.

The thermosome consists of two types of highly homologous subunits (α and β) which are arranged in an alternating fashion within each ring. Two of these rings are arranged in a head to head fashion by contacts between the equatorial domains. The subunits are eclipsed generating pairs of αα and ββ subunits across the double ring. In contrast, in group I chaperonins the subunits are in a staggered arrangement (Sigler et al., 1998). This may reflect a different type of allosteric signalling between both rings.

Although the protein has been crystallised in the absence of nucleotide or in the presence of MgADP a closed conformation is found in both cases. A lid domain that closes the particle in analogy to GroES in group I chaperonins is formed by the lid segments of the apical domains that has also been implicated in substrate binding (Klumpp et al., 1997). The enclosed cavity is large enough to accommodate a protein of about 50 kDa. The arrangement of the equatorial, intermediate and apical domains is very similar to the GroES-bound ring of the asymmetric GroEL-GroES complex. Binding of MgADP-AlF3 that mimics the transition state of ATP hydrolysis suggests this conformation as transition state of ATP hydrolysis. Therefore, the closed conformation will probably have to be adopted during ATP hydrolysis.

The folding pathway of tubulins include the interaction with a set of cofactors that bind and stabilise essentially folded tubulins after interaction with CCT. These cofactors form a super-complex containing both α- and β-tubulin from which the native αβ-tubulin heterodimer is released in a GTP-dependent reaction (Tian et al., 1999). The crystal structure of Rbl2p, the yeast homologue of cofactor A, that specifically binds to β-tubulin is presented (Steinbacher, 1999).

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Crystal structure of the human p14/cofactor A protein and localization of the β-tubulin interaction sites

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Alpha and β-tubulin fold via a series of chaperone-assisted steps, and at least five different protein cofactors are implicated in the post-chaperonin tubulin folding pathway. Recently, some of these cofactors have been implicated in microtubule dynamics[1,2]. Although in most cases folding can be ended after polypeptides release from the chaperonin, tubulin folding is completed after a second GTP-hydrolysis dependent step[3]. P14 or cofactor A was the first tubulin cofactor discovered. Cofactor A was purified from bovine testis and identified as a cochaperonin for CCT/TriC [4]. P14 was purified from pig testis and defined as a molecular chaperone for β-tubulin folding. This protein was identified as a β-tubulin monomer release factor from C_{300} multimolecular complexes, intermediates in the post-chaperonin folding step[5]. The human p14 encoding cDNA was cloned and the fully renatured active protein purified from *E.coli* was shown to bind noncovalently to β-tubulin *in vitro* in the presence of an uncharacterized protein named cofactor Z and purified tubulin heterodimers[6]. Homologs of p14 were identified in yeasts. The p14 homolog of budding yeast, Rbl2p, is required for normal meiosis and has about 30% identity to p14/cofactor A proteins[7]. P14 is more abundantly expressed in mouse testis than in other tisues. It is upregulated from the onset of mitosis through spermiogenesis. Additional experiments have shown that p14 plays a double role enhancing the dimerization rate of β-tubulin as well as a reservoir capturing excess of β-tubulin[8].Recently, the crystal structure of Rbl2p at a resolution of 2.2 A has been obtained. The protein crystallizes as an antiparalel homodimer through one of the few hydrophobic patches present on the surface of each monomer[9]. In this study we present the structure of the human p14 protein at a resolution of 1.8 Å. Although both monomeric proteins have the same overall structure, H2 in p14 is broken by proline 65. We also present data suggesting that human p14 is a protein that interacts as a monomer with β-tubulin via three different regions along the p14 molecule. One region is located in the amino-terminal region. The second which gave the strongest signal, is located inside the most conserved central region. The third region is located inside the smallest C-terminal helix. Additional in vitro competition experiments with peptides derived from the conserved central region confirmed the participation of this region in the interaction of p14 with β-tubulin.

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Session 4
Chair: F. Ulrich Hartl

Structural studies by cryo-electron microscopy of the cytosolic chaperonin CCT

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Despite their diverse origin, chaperonins share homologies, both at the level of their sequence [1], as well as in their general morphology [2]. While Type I chaperonins (comprising bacterial GroEL, mitochondrial Hsp60 and chloroplast RBP), form a quite uniform subgroup, type II chaperonins (cytosolic CCT, archaebacterial thermosome and thermophilic TF55) are more diverse, both in sequence and structure. Type I and II share a general shape and size based on two stacked rings, assembled back to back and built by multiple subunits arranged following radial symmetry. Each ring encloses a cavity that undergoes drastic changes during the functional cycle of the chaperonins, changes that are most probably related to the assistance of the substrate folding, as this cavity is the place where the substrate is enclosed [3]. Besides this overall similarity, the resolution of the structure of the GroEL monomer [4] and that of the thermosome [5] revealed a common organization in three domains, the main difference being the presence of one additional apical protrusion in the thermosome subunit. These basic similarities strongly support the idea of a common ancestor that evolved to accomodate to different environments and needs.

The main structural differences among both chaperonin groups are that while type I chaperonins are homo-oligomers, type II are oligomeric complexes built by different albeit homologous proteins. Another striking difference between type I and II chaperonins is the presence of a co-chaperonin in the type I that controls the opening and closing of the cavity. The type II counterpart is a built-in subunit domain that is placed as an apical protrusion in the rings capable of extensive movements, allowing the closure and opening of the cavity.

The activity of chaperonins is supported by a number of structural transitions during the folding cycle. The best known example is GroEL, which depending on the binding of nucleotide adopts different conformations that have characteristic affinity for the substrate. The concerted change between these states involves a complex set of signals within each ring and between both rings, signals that are also probably required for the proper activity of type II chaperonins. The most important functional difference between GroEL and the cytosolic CCT is that while the former is involved in the productive folding of many substrates [3], the latter is much more selective, and plays a fundamental role in the proper folding of tubulin and actin [6]. In an attempt to analyze the structural basis of this different behavior, we have studied the three-dimensional structure of CCT isolated from mouse testis by cryo-electron microscopy of frozen samples [7]. The comparison of the apo-CCT with the structure obtained from the nucleotide-containing AMP-PNP-CCT reveals that binding of the nucleotide generates a conformational change of the ring that resembles the thermosome

crystal structure [5], and it is consistent with a transition from the open to the closed conformation of the chaperonin.

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Interaction between eukaryotic chaperonin CCT and the cytosolic proteins actin and tubulin

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Folding of many proteins *in vivo* requires interaction with macromolecular complexes known as chaperonins. These proteins are ubiquitous oligomeric assemblies that have been classified into two distinct families that share limited but significant sequence homology: type I, present in eubacteria and endosymbiotic organelles, and of which the bacterial GroEL is the best known representative, and type II, present in archaebacteria and the eukaryotic cytosol, which are represented by the thermosome and CCT (Chaperonin Containing TCP-1), respectively (Bukau and Horwich, 1998; Willison, 1999; Gutsche *et al.*, 1999). All the chaperonins share a common architecture, a cylinder made up of two back-to-back stacked rings, each one enclosing a cavity where folding takes place. The atomic structures of GroEL (Braig *et al.*, 1994) and the type II thermosome (Ditzel *et al.*, 1998) have revealed a common subunit architecture consisting of three domains, apical, intermediate and equatorial. There are, however, numerous differences between type I and type II chaperonins, one of which is the absence in the of cochaperonins for type II family members, whose role in the closure of the cavity during the chaperonin working cycle is fulfilled instead by a helical protrusion in the apical domain (Klumpp *et al.*, 1997; Ditzel *et al.*, 1998; Llorca et al., 1999a).

The most important difference between these two chaperonins is related to their substrate specificity: whereas GroEL interacts with a broad range of substrates (Houry *et al.*, 1999) using a non-specific recognition mechanism based on hydrophobic interactions (Bukau and Horwich, 1998; Chen and Sigler, 1999; Shtilerman *et al.*, 1999), the main *in vivo* substrates of CCT are actins and tubulins (although other proteins that bind to CCT are being found continuously, suggesting a possible broader role of CCT in protein folding; Leroux and Hartl, 2000).

It has already been shown that CCT binds actin through a mechanism that is both geometry dependent and subunit specific (CCTδ, CCTβ and CCTε subunits are involved in actin binding; Llorca *et al.*, 1999b). To gain further insight into the folding mechanism of CCT and to search for a common pattern of interaction with tubulin, the other major *in vivo* substrate of CCT, we have carried out electron microscopy and biochemical analysis of CCT:tubulin complexes. The three-dimensional reconstruction from cryoelectron micrographs of the eukaryotic cytosolic chaperonin CCT complexed to tubulin shows that CCT interacts with tubulin (both α– and β–isoforms) using five specific CCT subunits. CCT-tubulin interaction has a different geometry to the CCT-actin interaction and a mixture of shared and unique CCT subunits is used in binding the two substrates. Docking of the atomic structures of both actin and tubulin to their CCT-bound conformation suggests a common mode of chaperonin-substrate interaction. CCT stabilises quasi-native structures in both proteins which are open through their domain-connecting hinge regions suggesting a novel mechanism and function of CCT in assisted protein folding.

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ATPASE CYCLE OF AN ARCHAEAL CHAPERONIN

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Chaperonins are double-ring protein folding machines fueled by ATP binding and hydrolysis. The exact relationship between the nucleotide-bound state and the conformation of the folding machine has so far only been understood for the group I chaperonins, typified by the *E. coli* GroEL (1). In contrast, it remains elusive for the group II members - thermosome and TRiC/CCT (2). A combination of enzymological studies (3) with small-angle neutron scattering (4) and cryo-electron microscopy (5) enables us to analyze the ATPase cycle of the thermosome. We systematically compare recombinant α-only and native αβ-thermosomes from *Thermoplasma acidophilum*, which also helps to clarify the role of the heterooligomeric composition of the natural chaperonin. Although, at first approximation, all subunits of the α-only thermosome bind nucleotides tightly and independently, the native protein has two different classes of ATP binding sites. Both apo-thermosomes are open in solution, but ATP binding induces their further expansion rather than a closure expected from a GroE-based analogy. At least after half-saturation with ATP, an anticooperativity in hydrolysis is observed for α-only and αβ-thermosomes, which once again highlights that archaeal chaperonins have unique allosteric properties. Furthermore, for the α-only thermosome, the steady-state ATPase rate is determined by the cleavage reaction itself. However, for the αβ-thermosome, the rate-limiting step seems to be associated with a post-hydrolysis isomerisation into a non-covalent ADP-Pi species prior to the release of the γ-phosphate. In solution, this process is associated with a closure of the chaperonin that subsequently re-opens during phosphate release. The same closure can be triggered by the crystallization buffer. At present, we are applying cryo-electron tomography under conditions defined by these studies in order to gain a higher resolution insight into the ATPase cycle of the group II chaperonins. We show that in vitrified ice, as well as in solution, ATPase cycle exerts a strict control over the conformation of the *T. acidophilum* thermosome.

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Facilitated folding of cytoskeletal proteins

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Actins and tubulins are the principal targets for facilitated folding by the chaperonin CCT in eukaryotic cells. While still associated with the ribosome, nascent actin and tubulin chains associate with prefoldin (Hansen et al., 1999), a heterohexameric chaperone protein that functions by escorting these target proteins to CCT. Prefoldin interacts with CCT, and hands off its target protein in a nucleotide-independent manner (Vainberg et al., 1998). During *in vitro* folding of actin and tubulin by CCT, we show that prefoldin acts to direct released target protein back to CCT, but trapping experiments show that it does not prevent CCT from releasing target protein nor does it alter the ATPase activity of CCT.

While native actin is produced *in vitro* by incubation of unfolded actin with CCT and ATP alone, tubulin folding further requires GTP and five tubulin-specific chaperones, cofactors A-E. These chaperones act as a tubulin dimerizing machine, bringing together unstable, quasi-native α and β tubulins (folded by CCT) into a tubulin/chaperone-containing supercomplex (Tian et al., 1997). Native tubulin heterodimer is released from this complex upon hydrolysis of its bound GTP (Tian et al., 1999). A subset of these chaperones also act post-translationally as GTPase-activating proteins for native tubulin. The post-folding effects on tubulin of these chaperones are regulated by a subfamily of G proteins, the ARF-like proteins or Arls (Bhamidipati et al., 2000; A. Bhamidipati, S.A. Lewis and N.J. Cowan, in preparation).

We have expressed the individual subunits of prefoldin in *E. coli* and purified them. Upon co-incubation, the subunits self-assemble into the prefoldin protein, which functions indistinguishably from prefoldin purified from a tissue source. There are pairwise associations of sets of subunits, but the longest prefoldin subunit (pfd3) appears to organize the entire complex. *In vitro* assembly makes possible the identification of those subunits within the prefoldin particle that interact with CCT and with their principal target proteins.

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Mutational analysis of interactions between CCT and cytoplasmic actins *in vitro* and *in vivo*

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The major *in vivo* substrates of the eukaryotic cytosolic chaperonin are members of the actin and tubulin families of cytoskeletal proteins. Actins bind and hydrolyse ATP whereas tubulins are GTP binding proteins. Both proteins form highly regulated polymer systems in the cytosol which are involved in functions as diverse as cell membrane ruffling, chromosome segregation and muscle contraction. There is strong evidence from signal transduction studies that the actin and tubulin systems interact and communicate with each other. CCT is the first machinery to be characterised which interacts with both actins and tubulins.

Chaperonin containing TCP-1, CCT, seems to have co-evolved with the modern actins and tubulins. The gene duplications which gave rise to the 8 separate CCT genes, found in all eukaryotic species examined so far, occurred approximately 2 billion years ago (Kubota, Hynes and Willison 1995). It is around this time that the first eukaryotic cell(s) is believed to have evolved. We have proposed that the evolution of actin and tubulin folding function through CCT activity may have helped drive cells across the prokaryotic-eukaryotic divide (Willison 1999, Willison and Grantham 2000). Many thinkers down the years have considered the evolution of a specialised cytoskeleton to be the major, novel acquisition of the eukaryotes.

We have characterised the CCT 16-mer genetically and biochemically and also structurally with our colleagues in Madrid. The 8 CCT subunits have a single arrangement within the apparently indentical two 8-mer rings. We have shown that the 8 CCT subunits have different functions in substrate recognition and that the recognition mechanism is geometrically specific. Actin binds to CCT in two 1:4 modes using two subunit combinations; CCTβ-CCTδ and CCTε-CCTδ. Tubulin binds to CCT in two 1:5 modes using five subunit combinations; CCTα/CCTη-CCTβ/CCTγ/CCTθ and CCTδ/CCTθ-CCTβ/CCTζ,CCTε.

The chemical nature of the interactions between CCT subunit apical domains and actin and tubulin folding intermediates is poorly understood. We have used solid phase peptide arrays to screen the human β-actin and α-,β- and γ-tubulins for CCT binding sites (Hynes and Willison 2000, Ritco-Vonsovici and Willison 2000).In the case of β-actin the CCT binding sites are found in loop regions located on the surface of native actin. These CCT-binding sites on β-actin are called SitesI, II and III. We have screened the CCT binding properties of more than 60 β-actin mutants by *in vitro* translation assays in reticulocyte lysate. The data are consisent with the cryo-electron microscopical analysis of CCT-actin complexes and the biochemistry. Mutations in both the large and small domains of actin which affect binding and processing have been found. There seems to be co-operativity between the sites during folding.

This accumulating data set points towards CCT being involved in the folding of a restricted set of actin folding intermediates, perhaps one(s) that are kinetically trapped. In this presentation we describe the discovery and characterisation of a stable folding intermediate of β-actin. We suggest that β-actin cannot fold to the native state without CCT function and futhermore that the native state of actin is not the thermodynamically most stable state of the monomer. The native state is proposed to be stablized by nucleotide and divalent metal ion acting in conjunction to form a clasp which holds the two sides of the actin polypeptide across its cleft.

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Conformational changes of the cytosolic chaperonin CCT during the folding of actin and tubulin

Oscar Llorca

Folding of many proteins in vivo requires interaction with macromolecular complexes known as chaperonins. The most important difference between the prokaryotic chaperonins and the cytosolic chaperonin CCT is related to their substrate specificity: whereas GroEL interacts with a broad range of substrate using a non-specific recognition mechanism based on hydrophobic interactions, the mainin vivo substrates of CCT are actin and tubulins. ATP hydrolysis is required for the correct folding by CCT of both substrates, but there is still not a clear view of the effect of ATP binding and hydrolysis on both the chaperonin and the substrate to be folded. Cryo-electron microscopy and 3D reconstruction in the presence of several nucleotides has been carried out to establish the conformational intermediates generated during actin and tubulin CCT-mediated folding.

Session 5
Chair: Alan Fersht

Genetic analysis of the yeast CCT complex

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Function of mutationally altered Cct subunits. The CCT double-ring chaperonin complex of *Saccharomyces cerevisiae* is comprised of 8 essential subunits, Cct1p-Cct8p, and assists the folding of substrates such as actins and tubulins. We have constructed by oligonucleotide-directed mutagenesis a total of 90 single and multiple amino acid replacements of Cct6p, including changes of charged to alanine residues and uncharged to charged residues. The replacements were targeted, in part, to residues corresponding to functionally critical regions identified in the published crystal structure of the *Escherichia coli* chaperonin, GroEL. Lin *et al.* (1997) showed that the critical hydrophobic residues and clusters of hydrophilic residues in regions corresponding to those from the apical domain of GroEL implicated in peptide binding and peptide release, and certain residues in the putative equatorial domain implicated in subunit-to-subunit interaction. In contrast to their homologous counterparts in Cct2p and Cct1p, the highly conserved putative ATP binding motifs of Cct6p were relatively amenable to mutations. These results suggest that the entire Cct6p molecule might be essential for assembly of Cct complex, and might participate in binding substrates. However, a functional hierarchy in ATP binding/hydrolysis among Cct subunits appears to exist, as suggested by the high tolerance of Cct6p to mutations within the putative ATP binding pocket.

Model of cooperative ATP binding. Lin and Sherman (1997) further addressed the structural and functional organization of the CCT complex by genetic analyses of subunit interactions and catalytic cooperativity among five of the eight different essential subunits, Cct1p-Cct8p. The *cct1-1*, *cct2-3*, and *cct3-1* alleles, containing mutations at the conserved putative ATP binding motif, GDGTT, are cold-sensitive, while single and multiple replacements of the corresponding motif in Cct6p are well tolerated by the cell. Lin and Sherman (1997) demonstrated that *cct6-3* (L19S), but not the paralog *cct1-5* (R26I), specifically suppresses the *cct1-1*, *cct2-3*, and *cct3-1* alleles, and that this suppression can be modulated by mutations in a putative phosphorylation motif, RXS, and the putative ATP binding pocket of Cct6p. These results suggest that the CCT ring is comprised of a single hetero-oligomer containing eight subunits of differential functional hierarchy, in which catalytic cooperativity of ATP binding/hydrolysis takes place in a sequential manner different from the concerted cooperativity proposed for GroEL.

Unassembled Cct subunits have physiological effects. Fractionation experiments with strains containing an epitope tagged *CCT1*-Myc and a multicopy His tagged *CCT6*-$(His)_7$ revealed that overproducing *CCT6* resulted high level of the Cct6p subunit, but only the normal level of the assembled CCT complex. Thus, overexpression of a single *CCT* gene causes an increased level of the Cct subunit but not of the Cct complex. Nevertheless, overexpression of certain Cct subunits suppresses a wide range of abnormal phenotypes, including those caused by the following: a conditional mutation, *tor2-21*, defective in the unique *TOR2* function; a conditional mutation, *lst8-2*, defective in the regulatory sorting in

the late secretory pathway; a conditional mutation, *rsp5-9*, defective in a ubiquitin-protein ligase; and the growth inhibition caused by the concomitant overexpression of Sit4p, a protein phosphatase, and Sap155p. Overexpression of *CCT6* suppressed all of the traits, whereas overexpression of four other *CCT* genes weakly suppressed one or at most two traits. The examination of 73 altered forms of Cct6p revealed that the *cct6-24* mutation, containing GDGTT → AAAAA replacements of the conserved ATP-binding/hydrolysis motif, was unable to suppress any of these traits, although *cct6-24* allele was completely functional for growth. These results provide evidence for functional differences among Cct subunits and for physiological properties of unassembled subunits. We suggest that the suppression is due to the competition of specific Cct subunits for activities that normally modify various cellular components. Furthermore, we also suggest the Cct subunits can act as suppressors only in certain states, such as when bound to ATP.

Suppression of defective subunits. Conditional *cct* mutants having defects in chaperonin subunits can be suppressed by overproduction of various normal genes. The temperature-sensitive *cct4-1* mutation, which has a G345D replacement in the apical domain of Ccp4p, was used to systematically isolate multicopy suppressor. The characterization of 38 suppressors revealed a wide range of unrelated genes, including four independent isolates of the expected *CCT4*. Representatives of these suppressors were shown to also act on some other *cct* conditional mutants. Surprisingly, 24 of the 38 suppressors encoded ribosomal proteins, representing 16 different type; and 3 of the 38 suppressors encoded two types of protein phosphatases. Ribosomal proteins are highly charged, nucleic-acid-binding proteins that are detrimental to the cell unless safely assembled within a ribosome. In fact, excess ribosomal proteins are degraded with half-lives of 0.5 to 3.0 minutes. We suggest that excessive ribosomal proteins signal the cell to overproduce the CCT complex, a condition that could compensate for the partial defect. Such a hypothesis is under investigation.

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Role of nucleotide exchange and hydrolysis during chaperonin-assisted folding of actins and tubulins

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Actin and tubulin polypeptide chains acquire their native conformations in the presence of the cytoplasmic chaperonin containing TCP-1 (CCT, also called TRiC) and, in the case of α- and β- tubulin, additional protein cofactors.

We have shown that nucleotide exchange and ATP hydrolysis act as a switch between conformational forms of CCT that interact either strongly or weakly with unfolded substrates (Melki and Cowan, Mol. Cell. Biol., **14**: 2895-2904, 1994).

The specificity of protein binding to CCT was investigated by the use of a range of labeled denatured target proteins as well as total labeled soluble HeLa cell extracts. We demonstrated that CCT recognizes a large number of proteins *in vitro* and binds them with high affinity. By the use of denatured labeled β-actin and β-tubulin as model substrates for binding to CCT, we have found that the stoichiometry of CCT-target protein complexes is two target proteins chains per CCT particle. By electron microscopy, sedimentation velocity and intrinsic fluorescence measurements, we documented the large conformational changes that CCT exhibits in its ATP- and ADP-bound forms as well as upon target protein binding (Melki et al., Biochemistry **36**, 5817-5826, 1997).

The mechanism of nucleotide binding was further dissected. The stoichiometry of nucleotide binding to CCT particles as well as the stereochemistry of the metal ion-nucleotide complex (Cr-ATP) bound to CCT subunits were determined. In addition, the average number of ATP molecules hydrolyzed by CCT per folded actin molecule, was measured spectrophotometrically (Melki, submitted, 2000). Our data allow a better comprehension of the molecular events that occur on CCT during target proteins assisted folding.

A model summarizing our findings will be presented and discussed. This model describes in details the mechanism of nucleotide exchange and hydrolysis on CCT and allows a better comprehension of the conformational changes that affect CCT during target proteins assisted folding.

The use of monoclonal antibodies to study interactions between CCT and its major substrates actin and tubulin

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The eukaryotic chaperonin containing T-complex polypeptide 1 (CCT) is required in vivo for the production of correctly folded actin and tubulin. It is a 900 kDa oligomer formed from two rings, each containing eight different subunits, which surround a central cavity. Unlike the eubacterial chaperonins which seem to have a broad range of folding substrates, it would appear that the actins and tubulins represent the major folding substrates of CCT. The complex arrangement of divergent CCT subunits and its limited number of folding substrates, are highly suggestive of specific, sequence-dependent interactions occurring between CCT and its substrates. Indeed, actin has been shown to interact with CCT in a subunit-specific and geometry-specific manner when viewed by cryoelectron microscopy. Furthermore, actin remains accessible to digestion by trypsin while bound to CCT, suggesting there are differences in the folding mechanisms of CCT and the eubacterial chaperonins. It has previously been demonstrated that the binding of a monoclonal antibody which recognises the C-terminus of CCT a and partially occludes the chaperonin central cavity has no effect on the rates of actin and tubulin folding in rabbit reticulocyte lysate. This study will describe the characterisation of monoclonal antibodies which recognise the apical, substrate binding domains of CCT and their effect on the processing of actin and tubulin in rabbit reticulocyte lysate and in vivo.

Target protein recognition by the class II chaperonin CCT and by prefoldin

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Actins, tubulins and other proteins require the class II cytosolic chaperonin containing TCP1 (CCT) to reach their native state (see Leroux and Hartl, 2000). This diversity of non-homologous proteins interacting transiently with CCT raises the question how this chaperonin accomplishes specific binding to the non-native forms.

In earlier work, using truncation analysis, we showed that actin is recognized by CCT via binding information comprised in rather large, hydrophobic segments, termed recognition determinants. Actin has three such sequences that bind in a co-operative manner (Rommelaere *et al.*, 1999). Similarly, β-tubulin (Dobryzinski *et al.* 1996) and α-tubulin (De Neve *et al.*, unpublished) contain binding information in delineated sites with a hydrophobic nature. Peptides, mimicking the recognition determinants from actin, efficiently compete with the CCT-tubulin interaction suggesting that actin and tubulins are contacted by similar CCT subunits. If this is true then common features of target proteins allowing specific interaction with CCT should be present in these non-homologous proteins.

Therefore we embarked on a project to localise these common recognition features. To maintain the integrity of the target protein as much as possible intact we decided to use alanine scanning mutagenesis. Given the size of the determinants (40-75 amino acids) a window of five consecutive alanines is feasible. So far we performed alanine-scanning in the middle recognition determinant of actin (amino acids 240-289 in β-actin, see Rommelaere *et al.* 1999). We did this in a shortened form of actin as well as in the full length molecule. The short form lacks the first six amino acids (actin7-375), has therefore compromised stability and shows good CCT-binding.

When analyzed in actin(7-375) we show that indeed this portion of actin possesses binding information and that it is confined to smaller subregions or hot spots. Somewhat to our surprise, the majority of these mutants adopted a stable native conformation when analysed in the full length actin. This strongly indicates these mutants were recognized by CCT in a manner compatible with subsequent folding. Comparing the two results suggest that sequences required for interaction with CCT are different than sequences required for maintaining the stable conformation.

The landmark paper of LLorca *et al.* (1999) shows that only two or three CCT-subunits contact actin consistent with our observation on three recognition determinants (Rommelaere *et al.* 1999). This and our observation that actin peptide mimetics compete with tubulins for CCT-binding suggests that not all of the eight related CCT-subunits contact target proteins. Thus these may serve other functions and this may well be interaction with prefoldin since a ternary complex between prefoldin, actin and CCT can be formed (Vainberg *et al.* 1998). This predicts that the interaction sites for prefoldin and for CCT in target proteins are mutually exclusive. Therefore we mapped the prefoldin interaction sites in actin

and in tubulin. Both target proteins contain two prefoldin interaction sites with a common signature sequence despite the fact these proteins are non-homologous. The prefoldin interaction sites are located before and after the most N-terminally located CCT-interaction sites. This finding is consistent with a hypothesis whereby docking of prefoldin subunits on CCT leads to directed presentation of the target protein to CCT.

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Role of the chaperonin TRiC/CCT in the assembly of the VHL-elongin BC tumor suppressor complex

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Disease often arises from the loss of function of key cellular proteins. von Hippel-Lindau (VHL) disease is caused by loss of function of the VHL tumor suppressor protein. We found that the folding of VHL in mammalian cells and its assembly into a functional complex with its partner proteins, elongin B and elongin C (herein elongin BC), is mediated by the chaperonin TRiC/CCT. VHL associates with TRiC and this interaction is required for formation of the VHL-elongin BC complex. A 55 amino acid domain of VHL is both necessary and sufficient for binding to TRiC. Importantly, this domain is frequently mutated or deleted in individuals with VHL disease or sporadic renal clear cell carcinomas. We are currently defining the structural elements within the TRiC-binding domain that are required for chaperonin binding, as well as characterizing tumor-causing mutations that disrupt the normal interaction with TRiC and impair VHL folding. Our results may uncover a class of inactivating mutations that interfere with polypeptide folding by targeting its interaction with molecular chaperones.

POSTERS

Dihydropicolinate synthase (DHDPS), a GroE substrate

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Although GroEL and GroES are abundant proteins in the E. coli cell, not enough is produced to fold all nascent proteins. [FASEB J. (1996)10: 5-9]. In order to help identify obligate substrates of the GroELS chaperonin our laboratory has engineered a strain of E.coli so that the synthesis of the chaperonin GroEL and its cochaperonin GroES is controlled by arabinose. When GroELS synthesis is prevented by removal of arabinose and the remaining chaperonin diluted by growth, broth grown cells continue to grow at a normal rate for five generations. During this period GroELS is depleted and becomes undetectable in cell extracts. Growth rate then slows; and, after a total of approximately seven generations, cell lysis occurs. Lysis is due to a defect in the peptidoglycan cell wall, resulting from a lack of the essential cell-wall precursor, diaminopimelic acid (DAP). Supplying extra DHDPS from a high copy plasmid on which dapA is cloned delays lysis. [Nature (19980 392: 139] suggesting that activity of this enzyme is reduced in lysing cells.

We have now shown that DHDPS activity is reduced to approximately 15% of normal just before lysis. However, the amount of soluble DHDPS protein, measured immunologically on SDS gels, is not reduced, suggesting that the enzyme may be misfolded in these conditions. DHDPS is only active as a homotetramer. We therefore speculated that, in depleted cells, it might, as a consequence of misfolding, be failing to form tetramers. However, analysis using native gels fails to support this idea; DHDPS from both GroELS containing and depleted cells migrates as a tetramer. We therefore suggest that the DHDPS made in GroELS depleted cells is structurally different from wild type enzyme in some more subtle way that compromises its enzymatic activity, while permitting the interactions between subunits that lead to tetramer formation.

Ligand independent assembly of bacterial synthetic CD1 using an artificial folding machinery

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The *in vivo* and *in vitro* assembly of classical and non-classical antigen presenting molecules is thought to be dependent on the presence of antigen, foldases and molecular chaperones. Artificial folding machinery consisting of immobilised chaperone fragments and foldases which catalyse the rate- limiting steps of disulfide formation/reshuffling and Xaa-Pro peptide-bond isomerization was used *in vitro* to generate native, ligand-associated and ligand-free functionally active human CD1 molecules on a fast time scale. The folding of CD1 molecules is consequently likely to be a ligand-independent process. We predict that this method will prove general and enable the efficient *in vitro* refolding of other members of the extensive and biologically important immonoglobulin supergene family of proteins.

Chaperonin-dependent ribosome biogenesis in *E. coli*

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It has been shown that the E. coli chaperone DnaK is necessary for the late stages of 50S and 30S ribosomal subunit assembly in vivo. Here we emphasise a role for other heat-shock proteins, including the chaperonins GroEL/GroES, in ribosome biogenesis at high temperature. 1. E. coli mutants carrying the groEL44-ts and groEL673-ts alleles accumulate ribosomal precursor particles sedimenting at 45S at the non-permissive temperature (45°C), in addition to normal 50S ribosomal subunits. 2. An E. coli mutant carrying the groES619 allele shows the same behavior at 19°C. 3.Overproduction of GroEL/GroES compensate for the ribosome assembly defects of a DdnaK null mutant at 44°C, suggesting that a chaperone network controls ribosome assembly. Thus GroEL/GroES chaperonins are directly or indirectly implicated in the 50S ribosomal subunit biogenesis, at least at high temperature.

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Excluded volume effects on the refolding and assembly of an oligomeric protein: GroEL, a case study

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We have studied the effect of macromolecular crowding reagents, such as polysaccharides and BSA, on the refolding of tetradecameric GroEL from urea-denatured protein monomers. The results show that productive refolding and assembly strongly depends on the presence of nucleotides (ATP or ADP), and background macromolecules. Nucleotides are required to generate and assembly-competent monomeric conformation, suggesting that proper folding of the equatorial domain of the protein subunits into a native-like structure is essential for productive assembly. Crowding modulates GroEL oligomerization in two different ways. First, it increases the tendency of refolded, monomeric GroEL to undergo self-association, at equilibrium. Second, crowding can modify the relative rates of the two competing self-association reactions, namely, productive assembly into a native tetradecameric structure and unproductive aggregation. This kinetic effect is most likely exerted by modifications of the diffussion coefficient of the refolded monomers, which, in turn, determine the conformational properties of the interacting subunits. If they are allowed to become assembly-competent before self-association, productive oligomerization occurs, while if they are not, unproductive aggregation takes place. Our data demonstrate that the spontaneous refolding and asembly of homo-oligomeric proteins, such as GroEL, can occur efficiently (70%) under crowding conditions similar to those expected in vivo.

Limits of protein folding inside GroE complexes

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The molecular chaperones GroEL and GroES facilitate protein folding under conditions where no spontaneous folding occurs. By analysing the influence of the GroE system on the thermal unfolding pathway of citrate synthase (CS), we could show that the GroE system stabilises the dimeric enyzme, by giving monomeric CS unfolding intermediates the chance to fold productively. A prerequist for this folding step is the encapsulation of CS monomers inside GroEL/GroES complexes. The resulting `high energy` CS intermediate is association-competent even under conditions where the native enzyme unfolds (1). Furthermore, we were able to demonstrate that the time of encapsulation under non-permissive conditions in cis-complexes is of critical importance, especially for oligomeric proteins, as long term incubation in stable cis-complexes leads to irreversible misfolding of monomeric CS intermediates. In contrast, incubation of monomeric CS intermediates in the presence of ATP hydrolysing GroE prevents irreversible misfolding and holds CS in a reactivatable state (2). Taken together two distinct folding steps could be identified. The structure of the respective folding intermediates is under investigation.

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STRUCTURE-FUNCTION RELATIONSHIPS OF AN ARCHAEAL CHAPERONIN

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The reaction cycle of the chaperonins is thought to consist of a nucleotide-regulated alternation between an open substrate-acceptor state and a closed folding-active state. The cavity of the ATP-loaded group I chaperonins, typified by *E. coli* GroEL, is sealed off by a co-chaperonin, whereas the group II chaperonins - archaeal thermosome and eukaryotic TRiC/CCT - possess a built-in lid provided by protrusions of the apical domains of the chaperonin itself [1, 2]. However, the means of allosteric regulation of the group II chaperonins by adenine nucleotides are still largely unexplored and the reasons for subunit differentiation are currently unclear. On the one hand, our structural study of the isolated α– and β-apical domains of the *Thermoplasma acidophilum* thermosome reveals an inherent plasticity of the protrusions, which are speculated to play a dual role in substrate binding and controlling access to the central cavity of group II chaperonins [3]. The current stage of the search for native substrates will be presented. On the other hand, a combination of enzymological studies [4] with small-angle neutron scattering [5] enables us to investigate the functional cycle of the thermosome. We provide quantitative information about the strength of nucleotide binding, the rate and cooperativity of hydrolysis, and the conformational rearrangements of the recombinant α-only and the native αβ-chaperonin upon ATPase cycling. Thus, we address the influence of the heterooligomeric composition of the thermosome on its structural and functional properties. Furthermore, we will present our ongoing analysis of the native αβ-thermosome by cryo-electron tomography, which gives a higher resolution insight into the functional cycle of the group II chaperonins.

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Structural and functional properties of *E. coli*-derived nucleoplasmin. A comparative study of recombinant and natural proteins

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Fourier transform infrared spectroscopy, circular dichroism and prediction techniques have been used to investigate the conformational properties of nucleoplasmin isolated from oocytes and eggs of *X. laevis* and overexpressed in *E. coli*. A simple and fast method allows purification of recombinant nucleoplasmin free of truncated and/or aggregated forms, and therefore provides a suitable sample to carry out the structural and functional comparison between these proteins. The secondary structure of the three proteins estimated from both spectroscopic techniques was very similar, and was found to be 31-33% loops, 27-34% β-structure, 22-26% turns and 9-14% α-helix. Prediction studies,in good agreement with experimental data, also suggest that β-structure is the major regular conformation, and that loops and turns are the most abundant conformational features within the secondary structure of nucleoplasmin. Furthermore, the combination of experimental and prediction data indicates that the secondary structure elements of the protein might be segregated into two regions: the N-terminal fragment (comprising residues 1-125) with holds all the putative buried β–strands, and the solvent-exposed C-terminal region, that is suggested to fold into one or two short helices and o long ending loop. The phosphate/protein monomer molar ratios obtained from chemical analysis and mass spectrometry are 0, 3 and 7-10 for recombinant, oocyte and egg nucleoplasmin, respectively. Phosphorylation does not significantly affect the secondary structure of the protein, but clearly modulates its ability to decondense sperm nuclei and to remove basic proteins from DNA.

Class I smHSPs in *Quercus suber*: protection against stress

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In higher plants, smHSPs (15-30 KDa) form a very diversified family of proteins that has been related to cell protection against different stresses, including oxidative stress. Among them, class I smHSPs is a very abundant multigenic subfamily of immunologically related proteins. From a cork oak phellem cells cDNA library, we cloned a 17 KDa smHSP (Qs_HSP17)1. Phellem (cork) cells are subjected to stress, specially oxidative stress associated to suberin synthesis. Using antibodies raised aigainst Qs_HSP17 we showed that class I smHSPs accumulate in phellem and other tissues that like phellem, are subjected to endogenous oxidative stress (xylem and sclerenchyma). We also showed that class I smHSPs are induced in response to thermic, hídric and oxidative (H2O2) stresses2. The immunodetection of these proteins in 2D gels indicates that different protein species are induced depending on tissue and stress. One of these protein species, which appeared in all the studied patterns, was correlated with Qs_HSP17. Other 17 KDa protein species may correspond to post-translational modifications of Qs_HSP17. Interstingly, a group of very small protein species (about 10KDa) accumulates in phellem and xylem tissue and are specifically induced by H2O2. Besides, the heterologous expression of Qs_HSP17 enhances E.coli viability under heat stress. This result supports a possible in vivo chaperone activity for Qs_HSP17.

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The allosteric mechanism of GroEL

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GroEL-assisted protein folding is regulated by a cycle of large coordinated domain movements in the 14-subunit double-ring assembly. The transition path between the closed (unliganded) and the open (liganded) states, calculated with a targeted molecular dynamics simulation, shows the highly complex subunit displacements required for the allosteric transition. The early downward motion of the small intermediate domain induced by nucleotide binding emerges as the trigger for the larger movements of the apical and equatorial domains. The combined twisting and upward displacement of the apical domain determined for a single subunit is accommodated easily in the heptamer ring only if its opening is concerted. This is the source of cooperative ligand binding within a ring. It suggests that GroEL has evolved so that the motion required for heptamer cooperativity is encoded in the individual subunits. A calculated model for a di-cis 14-subunit assembly is destabilized by strong steric repulsion between the equatorial domains of the two rings, the source of negative cooperativity. The simulation results, which demonstrate that transient interactions along the transition path are essential for GroEL function, provide the first detailed structural description of the motions that are involved in the GroEL allosteric cycle.

Identification of CCT-binding sites in human tubulins

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The actins and tubulins are the obligate substrates in vivo of the chaperonin containing TCP-1 (CCT). The precise elements of recognition between the chaperonin and its substrates remain largely unknown. We have used a solid phase peptide binding assay to screen the human alpha-, beta- and gamma-tubulin sequences for CCT recognition. Multiple regions seem to be implicated in interactions between tubulins and CCT. These potential CCT-binding sites are highly dispersed throughout the primary sequences of the human tubulins. Additionally, using site-directed mutagenesis we assessed the contribution of the selected residues in the C-terminal domain of beta-tubulin to CCT binding. Various hot spots have been identified even though, in each case, their replacement by alanine does not reduce dramatically the total affinity of beta-tubulin for CCT. The CCT-binding information in the tubulins is probably confined to multiple specific regions each having weak or moderate affinity for CCT apical domains. The main binding region seems to be located between residues 263 and 384, but there are no single amino acid residues in this substrate, which make large contributions to the binding energy, although we have detected a minor contribution by F377. These biochemical results are understandable in the context of our recent structural analysis of CCT-tubulin complexes by cryo-electron microscopy and image reconstruction, which shows that, in one stage of an *in vitro* binding reaction between CCT and tubulin diluted from guanidinium chloride, 10 major, stable contacts between tubulin and CCT are involved. Thus, specificity is achieved through the co-operation of many specific, albeit weak, interactions.

The cytosolic class II chaperonin CCT and prefoldin recognize delineated sequences in their target proteins actin and tubulin

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The cytoskeletal proteins, actin, alfa- and beta-tubulin need the assistance of several chaperones to reach their correct native state. The newly synthesised polypeptides first interact with prefoldin (1), which subsequently delivers the polypeptides to the cytosolic chaperonin containing TCP-1 (CCT), where folding occurs (2). By analysing truncated actin and alfa-tubulin molecules synthesised in reticulocyte lysate, which contains endogenous CCT and prefoldin, we could demonstrate that recognition of actin (3) and alfa-tubulin by CCT and prefoldin is mediated by delineated sequence stretches. There are three CCT-binding sites in actin and there may be three or four in alfa-tubulin. Internal deletion of the sites in actin or alfa-tubulin favour a model for co-operative binding of target proteins to CCT. In addition we show that actin recognition by class II chaperonins is different from that by class I. An alanine scan in the middle site of actin, shows that amino acids 245-249 and 265-274 (part of the hydrophobic plug) are important for CCT binding. Surprisingly some of the alanine scan mutants fold to the native conformation suggesting that these mutants are recognised by CCT in a conformation compatible with subsequent folding. Actin as well as alfa-tubulin bind prefoldin through two sites, which are in both cases adjacent to the N-terminal CCT binding site. Alanine scans of these sites show that interaction partly occurs through a motif : ER/HGY/I. Peptide mimetics, representing the CCT or prefoldin binding regions, inhibit target polypeptide interaction, suggesting that actin and tubulin contact similar CCT or prefoldin subunits.

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Dynamic relationship between CCT and CCT micro-complexes in Tetrahymena reciliating cells

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The Tetrahymena cytosolic chaperonin CCT (Chaperonin Containing TCP-1) is a hetero-oligomeric complex of 900 kDa composed of eight distinct subunits encoded by a family of related genes (1-5). Sucrose gradient fractionation of Tetrahymena post-mitochondrial extracts analyzed under native conditions revealed, besides the 900 kDa complex, the presence of smaller complexes (CCTmc) composed by sets of CCT-subunits. These CCTmc have apparent molecular masses ranging 120 kDa-360 kDa. In cells recovering their cilia, at different times, the pattern of molecular mass distribution of CCTmc is the same as in control cells. Interestingly, at 15 minutes of cilia recovery we observed a clear decrease in the amounts of the 900 kDa chaperonin contrasting with an increase in the levels of some CCTmc. After this period the levels of the 900 kDa complex starts to increase to levels found in control cells whereas CCTmc are decreasing. These data were confirmed by the results obtained upon purification of CCT/CCTmc using a FPLC Superose 6 column and subsequent analysis by Western blot. Moreover, tubulin was detected in the same elution profile region of CCTmc suggesting an association between CCTmc and tubulin. However, we cannot exclude that other complexes containing tubulin but not CCT-subunits could be present in this region. Our data taken together shows the existence of a dynamic balance between CCTmc and the 900 kDa chaperonin in reciliating cells. Further experiments are in progress to better understand the possible functions associated with CCTmc.

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Characterisation of At-HSP17.6A, a smHSP involved in osmotic stress tolerance in *Arabidopsis thaliana*

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The high abundance and different cellular localization of small heat shock proteins in plants indicates functional divergence among the smHSP classes (Vierling 1991, Waters et al. 1996). We have characterized At-HSP17.6A, encoding a cytoplasmic class II smHSP in Ararbidopsis. Expression of At-HSP17.6A is not detectable in leaves, stems, roots and flowers under normal growth conditions, but highly induced during seed development. Upon stress conditions, At-HSP17.6A transcripts increased during heat and osmotic stress. The expression is transiently enhanced by NaCl or PEG (polyethylene glycol)-induced-osmotic stress and the induction is slower than upon heat shock. At-HSP17.6A was overexpressed in Arabidopsis via Agrobacterium. Our preliminary data showed that At-HSP17.6A overexpressing plants gained better tolerance against osmotic stress but not against heat stress. SmHSPs are believed to function as molecular chaperones (Gething 1997). Such a function for plant smHSPs has been demonstrated in vitro (Heckathorn et al. 1998, Lee et al. 1997), however a direct link between chaperone activity of smHSPs and whole plant stress tolerance has not been established. Does At-HSP17.6A act as a molecular chaperone during osmotic stress? Further experiments are planned to understand the molecular basis of improved osmotolerance upon At-HSP17.6A overexpression.

Cryo-electron microscopy of a mutant GroEL complexed with GroES and ATP

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Chaperonin-assisted protein folding proceeds through cycles of ATP binding and hydrolysis by the large chaperonin GroEL. GroEL mediates protein folding in the central cavity of a ring bound by ATP and GroES (*cis* ring) and alternates its rings as folding-active *cis* complexes[1,2]. Structural changes of GroEL upon ATP binding are central to the mechanism of GroEL-assisted protein folding[3]. The GroEL/GroES/ADP crystal structure has been solved[4], but due to the fast turnover of the reaction cycle ($t_{½}$ ~ 15 s), it has been difficult to capture the ATP-bound state. By using the mutant Asp398Ala (D398A), which binds ATP but hydrolyses it at an extremely slow rate ($t_{½}$ ~ 20 min), we were able to capture the ATP-bound state of the GroEL/GroES bullet complex. Using cryo-electron microscopy, we have obtained three-dimensional reconstructions at 30 Å resolution. In the ATP bullet structure, the *trans* ring apical domains of GroEL twist anticlockwise compared with the ADP bullet crystal structure, viewed from outside the oligomer. Surprisingly, this twist is opposite to that observed for apical domains bound to GroES.The apical domains of GroEL, bearing the substrate binding sites, are its most mobile regions. In the crystal structure of the ADP bullet, the *cis* apical domains are twisted clockwise by 90° from the unliganded conformation but the *trans* ring is not twisted. In our EM structure of the ATP bullet, the *trans* apical domains have a large anticlockwise twist, suggesting that they may undergo a total excursion even larger than 90° during the functional cycle. We previously observed a similar anticlockwise twist in ATP-bound forms of the GroEL mutant R197A[5].

There are also differences in ATP and ADP *cis* rings of our EM maps of the D398A ATP bullet and the wild-type football structure. At low resolution it is not possible to interpret the *cis* apical domain rotations in detail, but one obvious difference is in the shape of GroES. This is unexpected, but consistent with the original report of inter-subunit flexibility in GroES[6].

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