BIOforum INTERNATIONAL
BIORESEARCH PLUS BIOTECHNOLOGY

BIOforum international
1 (1997) 20–24

DIPL.-CHEM. SABINE E. NIEBA-AXMANN
PROF. DR. ANDREAS PLÜCKTHUN

Chaperone-Mediated Protein Folding

Chaperone-Mediated Protein Folding

THE AUTHORS

Dipl. Chem. Sabine E. Nieba-Axmann

Prof. Dr. Andreas Plückthun

Institute of Biochemistry
University Zürich
Winterthurerstr. 190
CH-8057 Zürich
Switzerland

Molecular chaperones of the chaperonin (Hsp60) and Hsp70 families are basic constituents of the cellular machinery that mediates protein folding. The *Escherichia coli* chaperonin system GroEL/GroES is well characterized in terms of structure and function. GroEL prevents the aggregation of partially folded or misfolded proteins by complexing them in a form competent for subsequent folding to the native state. Although many detailed biochemical studies have been performed in order to elucidate the mechanism of GroEL function, very little is known about the conformation of GroEL-bound substrate proteins. Here, we summarize amide proton exchange studies in combination with nuclear magnetic resonance spectroscopy or mass spectrometry as a powerful tool to investigate the conformational properties of proteins bound to the chaperonin. From different studies and modelproteins a wide range of folding states has been proposed.

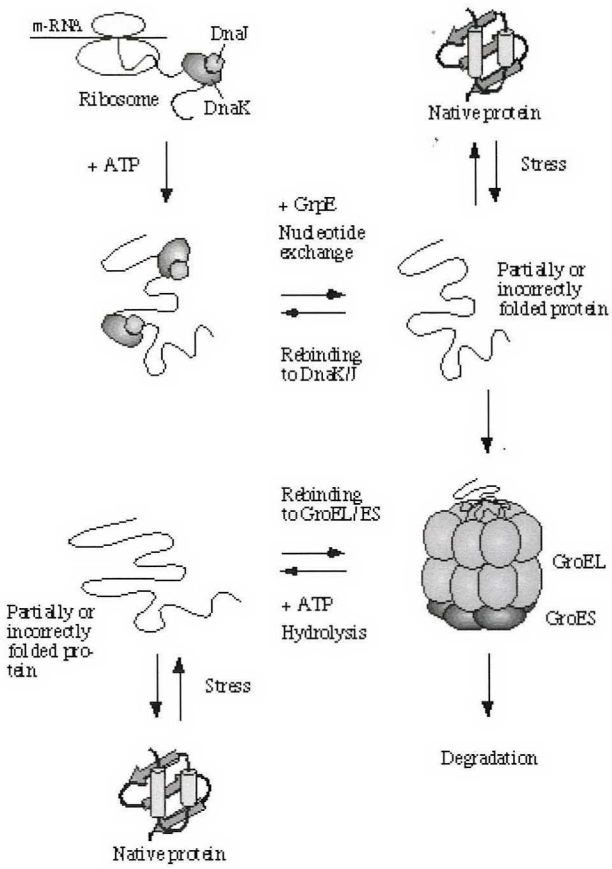

Fig. 1.:
Possible pathways of chaperone mediated protein folding in the cytosol of *E. coli*. Although alternative routes of folding are likely to exist, the pathways depicted here are thought to be involved in the folding of a subset of aggregation-sensitive polypeptides.

Chaperone Assisted Protein Folding

Molecular chaperones were originally defined as proteins that mediate correct folding and assembly of other proteins, but are not themselves components of the final structure [1]. They occur in several unrelated classes and all forms of life, and many of them have also been identified as stress proteins or heat shock proteins (Hsps), because of their specific induction during the cellular response to stress conditions, such as heat. Nevertheless, the majority of them was found to be essential for cell viability under normal growth conditions. More recently, an alternative definition has been proposed: a molecular chaperone is a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate *in vivo*, be it folding, oligomeric assembly, transport to another subcellular compartment or controlled switching between active and inactive conformations. In this review we will focus on the best characterized families, the Hsp60s and the Hsp70s. Both the Hsp70 (DnaK/DnaJ/GrpE) machinery and the Hsp60 (GroEL/GroES) machinery are expressed in the *E. coli* cytosol to prevent the aggregation of a large fraction of newly synthesized polypeptides, even under non-stress conditions. Various proteins can interact with both systems, consistent with the proposed functional cooperation of the Hsp60s and Hsp70s [1]. DnaK and DnaJ bind to unstructured, extended regions of newly synthesized polypeptides with a preference for sequences containing a high proportion of hydrophobic residues (Fig. 1). This mask-

KEYWORDS

**molecular chaperones,
protein folding,
GroEL,
NMR,
amide proton exchange**

ing of exposed hydrophobic surfaces, which in the folded state would be buried in the core of the protein, prevents aggregation of unfolded chains during translation and translocation. The ATP-dependent binding and release of peptides uses the third component of the machinery, GrpE, to stimulate nucleotide exchange.

Aggregation-sensitive intermediates are subsequently transferred from the DnaK-system to the chaperonin GroEL in a GrpE-dependent process, whereas other proteins can fold independently of GroEL (Fig. 1). Folding of these proteins may, however, become GroEL-dependent at higher temperature. GroEL promotes polypeptide folding in an ATP-dependent reaction cycle, which is regulated by its co-chaperonin GroES. It is still unclear what fraction of cytosolic polypeptides interact with chaperones of either the Hsp60 or Hsp70 class during and after translation.

The Chaperonin System

The architecture of the *E. coli* chaperonin system has been studied in great detail by electron microscopy [2], and the solution of the crystal structure of GroEL provides a basis for detailed mechanistic analysis [3]. GroEL is a large oligomeric complex of 14 identical 57 kD subunits that form a double toroid with a central cavity. Each subunit is divided into three distinct domains: The large equatorial domain forms the central core of the ring structure and contains the nucleotide binding site (Fig. 2). The equatorial domain provides most of the contacts between subunits within one heptameric ring and all contacts between the two rings. A small intermediate domain serves as hinge and connects the equatorial domain to the apical domain, which contains the binding sites for non-native proteins and GroES (Fig. 2). The surface residues of the apical domain facing the central cavity are mostly hydrophobic, and mutations of these residues affect the binding of polypeptides and GroES, confirming the assignment of the binding site to the central cavity [3].

GroES, a dome-like heptameric ring with 10 kD subunits [4], binds to one end of the GroEL cylinder in the presence of MgADP or MgATP, forming an asymmetric complex, which contains two distinct substrate binding sites. Binding of GroES causes dramatic conformational changes in the interacting GroEL ring [2]. The apical domains move upwards and outwards relative to the intermediate hinge domains, increasing the size of the central cavity to a dome shaped space (Fig. 2). The opening of the opposite toroid widens to a lesser extent. In the presence of ATP, complex formation and dissociation is very rapid, whereas in the presence of ADP, association is slow and dissociation even slower such that the affinity becomes very high [5]. Thus, GroES first binds to the ring with ATP bound, ATP hydrolysis then evokes stable binding. Turnover of ATP on the opposite ring drives ADP and GroES dissociation once every ATPase cycle, communicated from one ring to the other.

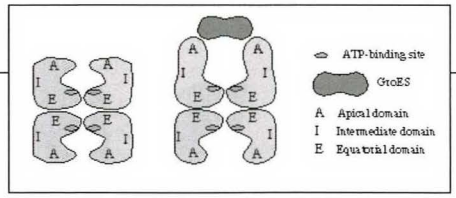

Fig. 2.: Vertical sections of the GroEL double toroid. The domain organization of the GroEL subunits is visualized (A, I, E). Binding of GroES causes dramatic conformational changes in the GroEL cylinder (see text). The apical domains form contacts with GroES with the same regions as the substrate binding site. The GroEL cylinder is greatly elongated.

Initially, non-native proteins bind to the *trans* side opposite from GroES, but they can be enclosed by subsequent binding of GroES to the same side (Fig. 3). By binding to unfolded proteins, mainly by hydrophobic interactions, GroEL creates an environment that prevents aggregation of the bound polypeptide, and protein folding my occur at least partially in the complex. Several mechanisms of GroES action may co-exist (Fig. 3): Polypeptide binding to the asymmetric GroEL-GroES complex accelerates the dissociation of GroES from the opposite ring by stimulating the ATPase activity of GroEL, and by causing rearrangements in the GroEL-GroES complex that facilitates dissociation of tightly bound ADP and GroES. Upon ATP-binding, the liberated GroES may now encapsulate the substrate protein by binding to the same GroEL toroid. Alternatively, GroES can stimulate release of the substrate from the opposite ring via an effect communicated by negative cooperativity [5]. In all proposed models, GroES and ATP cause dissociation of stable GroEL-polypeptide complexes, presumably by removing the apical domains with their hydrophobic binding sites from the bound protein, and ATP turnover is observed in concert with repeated cycles of substrate binding and release (Fig. 3).

Conformation of GroEL-bound Polypeptides: H/D-exchange Measured by Mass Spectrometry and NMR

GroEL has a very high affinity for unfolded proteins. Dissociation constants in the range of 10^{-7}–10^{-9} M have been determined using different methods [5]. Although detailed biochemical studies about nucleotide and GroES binding have been performed, and many studies observing the folding of proteins *in vitro* in the presence of chaperonins have been reported, the conformational properties of the non-native proteins bound to the chaperonin have been very difficult to determine. A powerful tool regarding the investigation of the GroEL-bound state are amide proton exchange studies in combination with two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy or electrospray ionization mass spectrometry (ESI–MS).

The analysis of protein folding intermediates by pulsed H/D-exchange is a relatively new field. During the past decade, the methodology has been continuously refined, and now gives not only highly detailed information about the folding process, but can also be applied on large protein complexes to evaluate the structure of the components. Analysis has mostly been performed using 2D-NMR, but recently it has been shown that the mass differences associated with deuterium incorporation are measurable by MS [6]. H/D-exchange of individual amide protons takes place, when there are structural fluctuations causing an opening of the structure that exposes these protons. Therefore, the exchange rates are a measure of the local stability of the protein during the labeling pulse (Fig. 4). Hydrogen exchange requires that the amide proton is accessible, and one may formulate a reaction scheme, in which one state is accessible to exchange (U) and the other is not (N). All exchange would then occur via the following mechanism, where k_{open} and k_{close} are the local unfolding and folding rates, respectively.

$$N_D \underset{k_{close}}{\overset{k_{open}}{\rightleftharpoons}} U_D \xrightarrow{k_{exchange}} U_H \underset{k_{open}}{\overset{k_{close}}{\rightleftharpoons}} N_H$$

In a two-state model, in which folding is completely cooperative, k_{open} and k_{close} correspond to the global unfolding and folding rates. An important characteristic of such a system is the rate of folding relative to the rate of hydrogen exchange with solvent. Two limiting situations, which both have been found in proteins, are conceivable: one in which the intrinsic exchange rate $k_{exchange}$ is much faster than k_{close} and vice versa. The protection factor PF = $k_{exchange}/k_{obs}$, where kobs is the measured exchange rate, is a measure of the retardation of exchange caused by the local stability of secondary or tertiary structure. A significant limitation of the pulsed H/D-exchange method is that only those peptide amide protons that are stable to

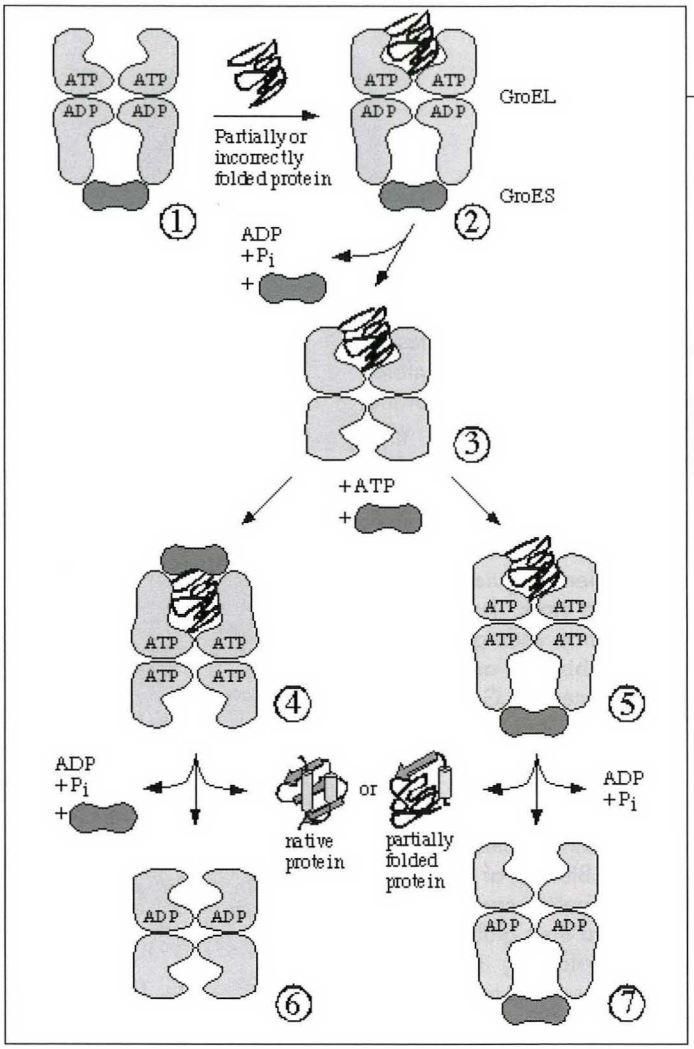

Fig. 3.: Scheme for the GroEL/GroES reaction cycle in protein folding. The denatured or incorrectly folded protein binds to the asymmetrical GroEL-GroES complex (1) in the *trans* ring. GroES dissociates from the complex upon ATP-hydrolysis on the opposite ring (2→3). GroES, together with ATP can now rebind to either ring of GroEL and enclose the bound substrate protein (4) or bind to the opposite ring (5). Rounds of ATP-hydrolysis lead to polypeptide release (4→6, 5→7). Folded proteins leave the GroE complex rapidly, whereas partially folded proteins can rebind to the chaperonin and participate in another reaction cycle. Thus, protein folding is mediated by repeated cycles of polypeptide binding and release.

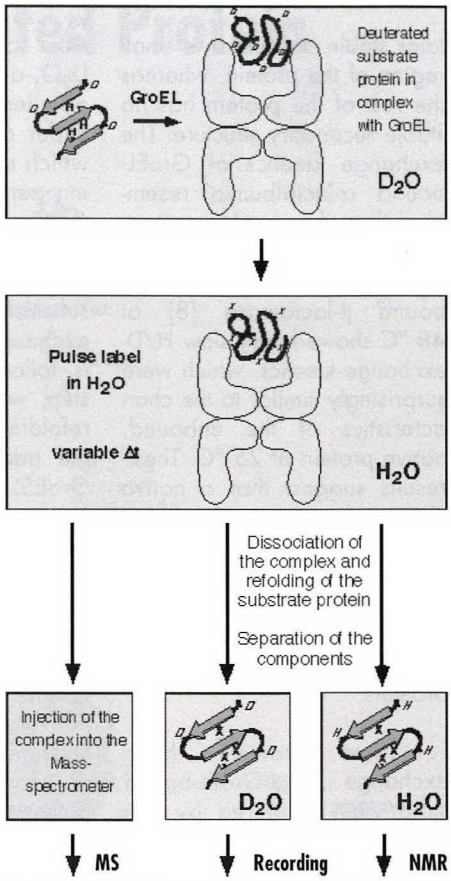

Fig. 4.: Schematic diagram of an experiment designed to measure hydrogen exchange in GroEL-bound substrates by 2D-NMR or MS. First, a complex of deuterated polypeptide and GroEL in D_2O is formed. Then, a labeling pulse in H_2O is applied to this complex. This complex can be injected directly into the mass spectrometer without previous separation of the components. For NMR measurements, the complex is dissociated once in H_2O, once in D_2O. After refolding of the substrate protein, 2D-NMR spectra are recorded. In the polypeptide representation, arrows, gray lines and bold and italic letters represent regular β-sheet secondary structure, hydrogen bonds and slowly and rapidly exchanging amide protons respectively. Protonation at the positions labeled with X can vary between 0 % and 100 %.

Table 1: 2D NMR versus MS to measure amide proton exchange

	NMR spectroscopy	Mass spectrometry
Information	sequence specific information about the formation of hydrogen bonds with high resolution exact assignment of each proton	detection of transient intermediates and different populations occurring at the same time no information about the location of a proton
Size of the protein	up to about 40 kD	not limited
Labeling	^{15}N-labeling of the protein for detection of peptide N-H exchange	no label necessary
Quantities	mg-quantities	µg-quantities
Purity of the sample	highly concentrated (mM) solution of pure protein	complex mixtures possible e.g. polypeptide-chaperone complexes
Conformation	native protein	native or denatured protein
Buffers	no restrictions	no salts or detergents, ultrapure reagents

exchange in the native protein can be used as probes, because only these protons do not further exchange during sample preparation.

There is a fundamental difference in the information that can be obtained by the two methods for following deuterium incorporation, NMR and MS, summarized in Table 1. Like spectroscopic methods (circular dichroism or fluorescence) and calorimetry, NMR measures the average properties of a sample, however, with enormous spatial resolution by observing single amide protons. Using MS, mixtures of different conformers of the same protein in which mass differences arise as a result of H/D-exchange can be analyzed. Even though MS does not average the properties of different populations, it averages, like optical spectroscopy, over the whole protein molecules of a given population. Thus, the combination of NMR spectroscopy providing sequence specific information and MS to detect multiple coexisting species, allows detailed studies on the structural properties of folding intermediates, both isolated and in complex with molecular chaperones (Fig. 4).

A key question in the mechanism of chaperonin assisted protein folding concerns the conformation of the substrate protein bound to the chaperonin during the reaction cycle. In recent studies, the H/D-exchange properties of five different proteins interacting with the *E. coli* chaperonin GroEL have been analyzed. As measured by MS, the exchange in a GroEL-bound destabilized derivative of α-lactalbumin [6] was complete within about 2 h, whereas some 20 deuterons remained in GroEL-bound DHFR [7] after this time, demonstrating that they are sites that are highly protected from hydrogen exchange. For DHFR, the protection factors of positions in the core of the protein were assumed to be at least 10^3. These high protection factors can be explained by a distinct partially folded species or by the existence of two distinct populations, one being highly protected and the other being unstructured. From detailed analysis of the peak width in the mass spectra it was assumed that GroEL-bound DHFR con-

tains stable structure in a small region of the protein, whereas the rest of the protein has no stable secondary structure. The exchange kinetics of GroEL-bound α-lactalbumin resembled that of a weakly protected molten globule state in free solution. MS studies on GroEL-bound β-lactamase [8] at 48 °C showed very slow H/D-exchange kinetics, which were surprisingly similar to the characteristics of the unbound, native protein at 25 °C. These results suggest that a native like conformation of β-lactamase, which was shown to be enzymatically inactive, is bound. Thus, a wide range of folding states has been proposed for different substrate proteins.

For two proteins, H/D-exchange in the GroEL-bound state was monitored by 2D-NMR. Barnase binds transiently to GroEL in a fully unfolded state, since the exchange of those amide protons, which can only exchange from the globally unfolded state, is accelerated up to 25-fold in the presence of catalytic amounts of GroEL [9]. Earlier H/D-exchange studies on GroEL-bound CypA [10] showed that all amide protons had quantitatively exchanged after three association-dissociation cycles. Using 2D-NMR, high spatial resolution is achieved. To improve the temporal resolution, pulse labeling experiments can be performed with kinetically stable GroEL-substrate protein complexes. After complete exchange in D_2O, a labeling pulse of variable length is applied in H_2O under association conditions, which defines a limited labeling period of the substrate in the GroEL-bound state (Fig. 4). These experiments require that refolding of the dissociating substrate is faster than H/D-exchange. The labeling pulse is followed by a quenching step, where dissociation and refolding are accelerated by the addition of MgATP and GroES. Using these experiments, protection factors can be calculated for single protons of a substrate protein that forms a stable complex with GroEL. These protection factors have values around 10^3–10^4 in GroEL-bound CypA and do not vary significantly along the sequence (Nieba-Axmann et al., manuscript submitted). The native protein unfolds locally at some parts in a reversible manner, while the GroEL-bound protein unfolds all at once. Since the H/D-exchange rates are slower than those in a random coil, a dynamic model for the GroEL-CypA-complex can be postulated. The dynamic equilibrium between native-like and unfolded states of CypA is partially shifted towards the unfolded state by the interaction of hydrophobic amino acids with the hydrophobic lining of the GroEL-cavity. Since native CypA is very stable against global unfolding, giving rise to protection factors of up to 10^7 in the native state, the 10^3-fold destabilization in the complex still leaves the equilibrium favoring the native state. The main effect of GroEL seems to be an acceleration of global unfolding and the stabilization of globally unfolded states in the complex. For this unfolding, the binding energy of the hydrophobic interaction with the chaperonin might be used.

For many proteins a single binding and release event is not sufficient to guide them to the native state. Rather, proteins seem to undergo multiple cycles of binding and release in the process of GroEL-assisted folding. Thus, fast folding molecules would no longer be recognized, whereas slow folding or kinetically trapped folding intermediates would be rebound. Binding of these folding-incompetent conformers to GroEL and their global unfolding and refolding in the complex allows these molecules to find a productive pathway after release. GroEL exists in two states, characterized by a conformational change, one with high affinity for substrates and the other with low affinity.

If the association energy between GroEL and the substrate protein is low, the protein may spontaneously leave the GroEL cavity. Proteins with high interaction energy require GroES and ATP, which coordinate conversion of GroEL from a high affinity state to a low affinity state, for fast release.

References

[1] HARTL, F. U.: Molecular chaperones in cellular protein folding. Nature, **381**, 571–580 (1996).

[2] ROSEMAN, A. M., S. CHEN, H. WHITE, K. BRAIG, H. R. SAIBIL: The chaperonin ATPase cycle: Mechanism of allosteric switching and movements of substrate-binding domains in GroEL. Cell, **87**, 241–251 (1996)

[3] BRAIG, K., Z. OTWINOWSI, R. HEGDE, D. C. BOISVERT, A. JOACHIMIAK, A. L. HORWICH, P. B. SIGLER: The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. Nature, **371**, 578–586 (1994).

[4] HUNT, J. F., A. J. WEAVER, S. LANDRY, L. GIERASCH, J. DEISENHOFER: The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. Nature, 379, 37–45 (1996).

[5] CLARKE, A. R.: Molecular chaperones in protein folding and translocation. Curr. Opin. Struct. Biol. **6**, 43–50 (1996).

[6] ROBINSON, C. V., M. GROSS, S. J. EYLES, J. J. EWBANK, M. MAYHEW, F. U. HARTL, C. M. DOBSON, S. E. RADFORD: Conformation of GroEL-bound α-lactalbumin probed by mass spectrometry. Nature, **372**, 646–651 (1994).

[7] GROSS, M., C. V. ROBINSON, M. MAYHEW, F. U. HARTL, S. E. RADFORD: Significant hydrogen exchange protection in GroEL-bound DHFR is maintained during iterative rounds of substrate cycling. Protein Sci. **5**, 2506–2513 (1996).

[8] GERVASONI, P., W. STAUDENMANN, P. JAMES, P. GEHRIG, A. PLÜCKTHUN: β-lactamase binds to GroEL in a conformation highly protected against hydrogen/deuterium exchange. Proc. Natl. Acad. Sci. USA, **93**, 12189–12194 (1996).

[9] ZAHN, R., S. PERRETT, G. STENBERG, A. R. FERSHT: Catalysis of amide proton exchange by the molecular chaperones GroEL and SecB. Science, **271**, 642–645 (1996).

[10] ZAHN, R., C. SPITZFADEN, M. OTTIGER, K. WÜTHRICH, A. PLÜCKTHUN: Destabilization of the complete secondary structure on binding to the chaperone GroEL. Nature, **368**, 261–265 (1994).