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BREAKTHROUGHS AND VIEWS

Do Molecular Chaperones Have to Be Proteins?

R. John Ellis[1]
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

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In recent years the term 'molecular chaperone' has come into general use to describe a range of different proteins that share the common property of assisting the assembly of other proteins. As with all new terms in science, it is important to continually review the precise meaning and usefulness of this term, especially since the literature contains some instances of confusion and misunderstanding. In this article I shall describe the origin of the term 'molecular chaperone', discuss my view of its current meaning and usage, and review the recent proposals that 23S ribosomal RNA (1, 2) and the phospholipid phosphatidylethanolamine (3) should also be regarded as examples of molecular chaperones, and that RNA folding as well as protein folding is a target for chaperone action (4).

ORIGIN

The term molecular chaperone was used first by Ron Laskey and colleagues in 1978 to describe the properties of nucleoplasmin. This protein is abundant in the nucleus of eggs and oocytes from the amphibian *Xenopus laevis,* where it functions to assist the assembly of nucleosome cores from histones and DNA (5). Nucleoplasmin is not a component of the nucleosome nor does it possess any of the steric information required for nucleosome assembly; this information resides in the histones. Instead, nucleoplasmin binds transiently and non-covalently via acidic sidechains to the basic residues on the surface of particular histones, and so reduces their high positive charge density. This reduction prevents the rapid and non-specific aggregation of the histones with DNA that occurs under physiological conditions of ionic strength in the absence of nucleoplasmin. This transient inhibitory role of nucleoplasmin allows the self-assembly properties of histones with DNA to predominate over the incorrect interactions generated by their high densities of opposite charge. Since nucleoplasmin acts both transiently and negatively, its action can be thought of as a molecular mimic of the traditional role of human chaperones in preventing incorrect interactions between people.

The term molecular chaperone was not applied to any other protein until seven years later, when I was struck by the appropriateness of this term to describe the properties of a chloroplast protein implicated as a transient intermediate in the assembly of the oligomeric enzyme ribulose bisphosphate carboxylase-oxygenase (rubisco) in the leaves of higher plants. Plant rubisco oligomers share with nucleosomes the property of failing to self-assemble correctly from a mixture of their separated components, due in this case to the strong tendency of one of the two types of subunit comprising the oligomer (the large subunit) to form insoluble aggregates with itself. The problem in the case of rubisco is not high charge density but the high hydrophobicity of the unassembled large subunits. These large subunits are synthesized inside the chloroplast, but require for their assembly into rubisco oligomers small subunits that are synthesized in the cytosol and subsequently imported across the chloroplast envelope. The assembly protein binds transiently to the hydrophobic surfaces of the newly synthesized large subunits in an ATP-reversible reaction, and so prevents their aggregation until small subunits become available (6,7). The suggestion that the large subunit binding protein could be regarded as a second example of a molecular chaperone was made at a Royal Society discussion meeting about rubisco that I organized in 1985, and was subsequently published in the proceedings (8).

The prevailing paradigm about protein assembly in the early 1980s was that it is essentially a self-assembly process requiring neither energy or additional macromolecules. This view arose from the pioneer work of Anfinsen and others, showing that many pure denatured proteins will fold correctly into their functional conformations *in vitro* when the denaturing agent is removed (9), and from the demonstration that some

[1] Fax: 1203 523568. E-mail: je@dna.bio.warwick.ac.uk.

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oligomeric structures such as tobacco mosaic virus will reconstitute correctly when the separated components of the assembled structure are mixed together (10). So I initially thought that nucleoplasmin and the rubisco large subunit binding protein were special cases that had evolved to overcome assembly problems posed by the strong tendency of nucleosomal subunits and rubisco subunits to aggregate incorrectly. It was a speculative paper by Hugh Pelham (11) that prompted me to extend the chaperone idea further.

This paper does not discuss either nucleoplasmin or the chloroplast binding protein, but suggests that the heat shock proteins (hsps) of the 70 and 90 families function not just under stress conditions to ameloriate the effects of heat on protein structure, but also influence protein assembly and disassembly processes under non-stress conditions. It occurred to me that these ideas could be fused with those prompted by the examples of nucleoplasmin and the chloroplast binding protein to suggest a more far-reaching proposal which implies that protein self-assembly as envisaged at that time is an insufficient explanation to describe how proteins assemble inside cells. Perhaps many molecular chaperones exist, in which case the problem of incorrect interactions inside cells is not confined to a few proteins but is more widespread than appears from the success of the *in vitro* protein refolding and reconstitution experiments. The conditions prevailing in such experiments are, after all, very different from those found inside cells - especially with respect to the concentrations of partially folded intermediate states that many proteins are believed to pass through on the way to their final conformations.

The proposal that many proteins may exist that could be regarded as molecular chaperones was made at a NATO Advanced Study Institute meeting on plant molecular biology organized by Diter von Wettstein in Copenhagen in June 1987. A representative from the journal *Nature* was at that meeting and encouraged me to write a *News and Views* article describing this more general concept. This article appeared in July 1987 with the following opening sentence 'At a recent meeting I proposed the term 'molecular chaperone' to describe a class of cellular proteins whose function is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures occur correctly' (12). The term soon came into widespread use as more examples of molecular chaperone were discovered, and there are now several regular conferences that include this term in their titles, as well as a journal called *Cell Stress and Chaperones*. As with other novel general concepts however, there has been both some refinement of the initial idea to reflect new discoveries, as well as some confusion and misunderstanding as to what the term implies. In the following section I present my current view as to the meaning of the general concept of molecular chaperones.

DEFINITIONS

My current definition of molecular chaperones is that they are a large and diverse group of unrelated proteins that all share the functional property of assisting the non-covalent assembly and/or disassembly of protein-containing structures *in vivo*, but are not permanent components of these structures when they are performing their normal biological functions (13). Assembly is used in this definition in a broad sense, and includes several basic cellular processes such as the folding of polypeptide chains during and after synthesis, the refolding of proteins after their transport across membranes, and the association of folded polypeptides with one another and with other macromolecules to form oligomeric complexes. Molecular chaperones are also involved in protein disassembly processes, such as the partial unfolding and dissociation of subunits that occurs when some proteins carry out their functions, and the repair or degradation of proteins partially denatured by mutation or exposure to environmental stresses such as high temperature or anoxia. Some, but not all, molecular chaperones, are also stress proteins because the cellular requirement for chaperone functions increases under conditions that cause proteins to unfold and aggregate. Conversely some, but not all, stress proteins are molecular chaperones. Thus the stress response can be viewed in many respects as an amplification of a basic chaperone function that all cells require under normal conditions, rather than as a unique function required only under stress conditions.

The above definition is functional and not structural in nature, but it contains no constraints on the mechanisms by which molecular chaperones function to qualify for inclusion; this is the reason for the use of the imprecise term 'assisting'. Thus only two criteria need to be satisfied in order to call a protein a molecular chaperone; it must in some sense assist the non-covalent assembly or disassembly of other protein-containing structures, the mechanism by which it does this being irrelevant, but it must not be a component of these structures when they are carrying out their normal biological functions.

The term non-covalent is used in this definition to exclude those proteins that carry out co- or post-translational covalent modifications. These are often important for protein assembly but are not the proteins being considered here. Protein disulphide isomerase appears to be an exception because it is both a covalent modification enzyme and a molecular chaperone (14), but these activities lie in different parts of the molecule and can be functionally separated by mutation. There is no reason in principle why molecular chaperones should not possess additional functional activities; indeed the combination may be advantageous. An example of this is an oligomeric complex in the inner mitochondrial membrane of yeast cells that is required for

TABLE 1

Proteins Regarded as Molecular Chaperones

Family	Proposed roles	Reference
1. Nucleoplasmins	Nucleosome assembly & disassembly in eggs	5
2. Chaperonins (hsp60)	Protein folding	20-22
3. Hsp27/28	Prevention of aggregation due to stress	25, 26
4. Hsp40 (DnaJ)	Protein folding, transport & oligomer disassembly	20
5. Hsp47	Procollagen folding in ER	27
6. Hsp70 (DnaK)	Protein folding, transport & oligomer disassembly	20
7. Hsp90	Protein kinase & hormone receptor activation	28
8. Hsp100/104	Dissolution of insoluble aggregates, repair of heat-inactivated mRNA splicing	29, 30
9. Protein disulfide isomerase	Folding of disulfide proteins	14
10. Calnexin/calreticulin	Protein folding in the ER	31
11. SecB protein	Protein transport	32
12. PapD protein	Bacterial pilus assembly	33
13. Lim protein	Folding of bacterial lipase	34
14. Prosequences	Folding of subtilisin & α-lytic protease	16, 35
15. Syc proteins	Secretion of YOP proteins by bacteria	36
16. ExbB protein	Folding of TonB protein	37
17. Ubiquitinated ribosomal proteins	Eukaryote ribosome assembly	17
18. NAC complex	Folding of nascent chains	38
19. Signal recognition particle	Folding of presequences	39
20. PrtM/PrsA proteins	Extracellular protein processing in bacteria	40, 41
21. YTA10-12 complex	Assembly of mitochondrial membrane complexes	15
22. NAP-1 protein	Nucleosome assembly in somatic cells	42

both the assembly of ATP synthase and for the degradation of unassembled subunits (15).

Molecular chaperones are found in all types of cell and the list of proteins that meet my definition is growing steadily longer (Table 1). It must be emphasised however that in some cases the evidence that a protein acts as a molecular chaperone *in vivo* is based only on *in vitro* evidence, and physiological confirmation of this conclusion is not yet available. Table 1 is constructed on the basis that members within each family of chaperone are related by high sequence similarity, but members in different families are not. A common error is to use the term chaperonin as synonomous with the term chaperone, but it should be noted that the chaperonins are just one particular family of molecular chaperone. The continuing use of the nonsense term 'molecular chaperonin' in some respectable journals is one illustration of the prevailing confusion.

Another common misconception is that molecular chaperones are necessarily promiscuous i.e. that each can assist the assembly of many different polypeptide chains. This is true for the hsp70, DnaJ and the GroE chaperonin families but not for PapD, hsp47, Lim, Syc, ExbB, PrtM/PrsA and prosequences, which are specific for their protein substrates. Similarly, it is not a universal property of molecular chaperones that they hydrolyse ATP. It is also not necessary that chaperone functions reside in molecules separate from their substrates. Thus some prosequences are required for the correct folding of the remainder of the molecule, but are then removed (16). Another example of such intramolecular chaperones are the terminal ubiquitin residues of three ribosomal proteins in yeast; these residues promote the assembly of these proteins into the ribosome but are then removed (17).

WHY DO MOLECULAR CHAPERONES EXIST?

Given that protein self-assembly is demonstrable under *in vitro* conditions, the most plausible explanation for the existence of molecular chaperones is not that they provide essential steric information required for some proteins to fold and associate correctly, but that they prevent and/or reverse aggregation processes that result from the high concentrations of macromolecules inside cells. In other words, the existence of molecular chaperones does not cast doubt on the *in vivo* validity of the idea that the steric information for proteins to fold and associate correctly resides solely in their primary structures, only that self-assembly needs assistance to operate efficiently under the condition of macromolecular crowding that characterises the intracellular *milieu*.

Aggregation results because some proteins fold via compact intermediates that expose hydrophobic residues on their surfaces. The thermodynamic activity of these compact intermediates is enhanced by two to three orders of magnitude by the macromolecular crowding effect created by the high concentrations of proteins and nucleic acids found inside cells - 340 mg/ml in the case of *Escherichia coli* (18). The binding of molecular chaperones to these surfaces reduces this

aggregation, and genetic evidence shows that this reduction is essential for cell viability in *E. coli* (19). The binding is then reversed under circumstances that favour correct interactions i.e. those that avoid aggregation and result in the functional conformation. In the case of the chaperonin family, these circumstances include the sequestration of individual partially folded chains inside large oligomeric complexes termed Anfinsen cages; inside these cages folding continues to a point where aggregation is no longer a problem 20-22). In the case of the DnaK (hsp 70) and DnaJ families, exposed hydrophobic surfaces on growing polypeptide chains are shielded from one another until enough primary structure has appeared to fold into a domain.

An additional role of molecular chaperones has been suggested - to cause some unfolding of misfolded polypeptide chains to allow fresh attempts at correct folding (23). The extent of protein misfolding into kinetically stable states that are unable to proceed further to the correct conformation *in vivo* is unclear; the general success of protein refolding experiments in regenerating the correct conformations *in vitro* suggests that it may be of little significance, unless some unknown factors operating inside cells increase the probability of misfolding. The GroE chaperonins can unfold partially folded states to some extent at least *in vitro*, but it is possible that this action targets these chains for degradation rather than further folding (20).

Current research on molecular chaperones emphasises the importance of controlling hydrophobic interactions, but it should be remembered that the function of the first protein to be called a molecular chaperone i.e. nucleoplasmin, is to prevent incorrect electrostatic interactions between folded histones and DNA. Thus it is incorrect to think that molecular chaperones are concerned solely with hydrophobic interactions and protein folding, although this is certainly a major function. A more unifying view is to think that cells require a molecular chaperone function to prevent and reverse incorrect interactions that may occur between a variety of interactive surfaces that are transiently exposed to the intracellular environment. Such a generalised view prompts two questions; do macromolecules other than proteins function as molecular chaperones, and do macromolecules other than proteins act as substrates for molecular chaperones?

RIBOSOMAL RNA AND PHOSPHOLIPID AS MOLECULAR CHAPERONES

The generalised view stated above emphasises that the basic feature of chaperone function is the ability to bind transiently to non-native states of macromolecules in such a way as to promote progression towards their biologically active conformations. It follows from this exclusively functional view that any type of molecule that possesses this activity can be called a molecular chaperone.

Two laboratories have independently reported that ribosomes from prokaryotic and eukaryotic sources improve the yield of several enzymes refolding from the denatured state *in vitro* (1,2). This activity was found to reside in the deproteinized 23S RNA component of the ribosomes from *E. coli*, but not in the 16S RNA component, nor in transfer RNA. The 23S RNA is contained in the 50S ribosomal subunit which carries out the synthesis of the polypeptide chain. It is known that the peptidyl transferase activity resides in the 23S RNA itself, so it is significant that the refolding of lactate dehydrogenase by 23S RNA is inhibited by chloramphenicol and by oligonucleotides that hybridize with segments of the RNA implicated in the transferase activity (43). Refolding of denatured rhodanese leaves the ribosomes in an inactive state for further rounds of refolding, from which they can be reactivated by addition of elongation factor G and GTP (2). All these *in vitro* observations suggest that ribosomes may have an innate protein folding chaperone function. However, unlike the case for the DnaK and chaperonin families, this idea has not as yet been supported by any data showing that this property is important *in vivo*. The folding of denatured rhodanese after import into isolated intact yeast mitochondria is absolutely dependent upon the presence of functional chaperonin 60 (24), so in this situation mitochondrial ribosomes do not promote the folding of already synthesized chains; whether they promote the folding of chains they are making is unknown. It has been proposed that most proteins in *E. coli* fold post-translationally (44), so any chaperone activity by ribosomes for these proteins would presumably function after translation is completed, as it does in the *in vitro* renaturation experiments (1,2).

Proteins inserted into membranes fold in an amphipathic environment, and a recent study utilising mutants of *E. coli* unable to make the phospholipid phosphatidylethanolamine (PE) concludes that PE acts as a molecular chaperone required for lactose permease to fold into the conformation required for H^+-coupled lactose accumulation, but not into the conformation required for energy-independent downhill translocation of lactose (3). The crucial observation uses a so-called 'eastern-western' blotting procedure in which phospholipids are applied to the surface of nitrocellulose sheets before transfer of membrane proteins from SDS-polyacrylamide gels. Cells lacking PE make lactose permease chains that are not recognised by a conformationally-sensitive monoclonal antibody directed against a periplasmic loop of the lactose permease chains made by wild type cells. However recognition by this antibody is regained if PE, but not other phospholipids, is applied to the blotting sheet before transfer of the pro-

teins. The antibody also recognises the same epitope on western blots of wild type membranes.

The authors interpret these findings to mean that PE is required to form the epitope in wild type cells, but that once this epitope is formed, PE is no longer required for it to be maintained. The weakness in this argument is that it is not clear where the PE migrates on the SDS gel, so it is possible that some is still associated with the polypeptide. The authors try to rule this out by citing unpresented data from ^{32}P-labelled cells, but it is not certain whether this would have detected residual amounts of PE bound to the permease. Even if this is the case, it still does not rule out the possibility that in the intact membrane PE is necessary as a permanent structural component to maintain the conformation of the epitope required for the active transport of lactose. Thus the present evidence shows that PE has a specific role in determining membrane protein conformation, but the evidence that it acts as a molecular chaperone is suggestive rather than compelling.

RNA CHAPERONES

The term RNA chaperone appeared in the literature in 1993 to describe proteins that act *in vitro* to increase the activity and specificity of ribozymes (45) and to enhance the rate of formation and stability of dimers of HIV-1 genomic RNA (46). However in both these cases the proteins are bound to the functioning RNA, so they fail to meet the second criterion proposed for a molecular chaperone. Herschlag (4) has presented interesting theoretical arguments for the existence of RNA chaperones, based on three considerations: the high thermodynamic stability of RNA duplexes, their low information content relative to proteins due to their paucity of primary structure diversity, and the sequestering of the basepairing faces in the interior of the molecule that renders difficult the specification of unique tertiary structures. There are many examples of RNA molecules misfolding *in vitro* to produce alternative conformers that are kinetically trapped in stable configurations. Herschlag defines RNA chaperones as proteins that aid RNA folding either by preventing misfolding or by resolving misfolded species, and suggests that some RNA-binding proteins may perform this role *in vivo* (4). The evidence that such proteins can act in this way, at least in the test tube, goes back to 1974, when it was reported that protein UP1, a fragment of the hnRNP A1 protein, can renature 5S and tRNAs that are trapped in alternative conformations (47). A good recent example is the S12 protein of the *E. coli* ribosome, which facilitates the correct folding of Group I introns and then can be removed without reducing the stimulated self-splicing activity (48). These *in vitro* properties of the S12 protein thus meet the two criteria for a molecular chaperone. However Herschlag is careful to point out there is as yet no evidence that any RNA-binding protein acts as a RNA chaperone *in vivo*. Nevertheless, the existence of true RNA chaperones appears highly likely, and their appearance early in the evolution of life might have been a vital step in the transition from an RNA to a protein/RNA world by rescuing primitive RNA from kinetic traps (4).

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