Preparing chaperone—client protein complexes for biophysical and structural studies

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Many proteins are prone to misfolding or unfolding and aggregation in the aqueous environment of the cell, either due to their complex ternary structure, cellular localization, molecular crowding, cellular stress, or their intrinsic hydrophobic nature (Díaz-Villanueva et al., 2015; Ellis & Minton, 2006). Molecular chaperones are involved in biogenesis of such proteins, and in ensuring their intactness throughout their life cycle (Hartl et al., 2011; Kim et al., 2013; Sinnige et al., 2020). While there are many different types of chaperones with various cellular roles, essentially all of them are characterized by a basic "holdase" function, i.e. the ability to bind to proteins that are generally in some non-native conformation – often these are called "client" proteins. Chaperones may have more "active" roles in addition to the basic function: "foldases" assist their client protein in folding to its native state (reviewed in (Balchin et al., 2020)), and "disaggregases" help in dissociating protein aggregates (reviewed in (Mattoo & Goloubinoff, 2014)). Understanding how these complexes form, what (residual) structure the client proteins have when bound, and how the client protein may eventually be released again from the chaperone is essential for resolving questions in biogenesis of cells and organelles.

Detailed study of chaperone—client complexes on the molecular level is challenging for several reasons. The interactions involved in the complex formation are often highly dynamic, i.e. binding often relies on avidity of multiple interactions (see reviews (Burmann & Hiller, 2015; L. He & Hiller, 2019; Hiller, 2019; Macošek et al., 2021; Sučec et al., 2021)). Furthermore, chaperones themselves are often large, up to the megadalton range, and can adopt multiple states (Bracher & Hartl, 2013; Skjærven et al., 2015). All these factors make their experimental characterization difficult. Moreover – and this is the focus of the present chapter – chaperone—client complex formation for any experimental study turns out to be challenging. The proteins to be bound to the chaperone are in many cases aggregation-prone. This is particularly the case for membrane proteins in aqueous solutions (Heyden et al., 2012; Schiffrin et al., 2017), but it applies also to many soluble proteins in the state in which they interact with chaperones, i.e. in partially unfolded states (Capaldi et al., 2002; Espargaró et al., 2008; Tsirigotaki et al., 2018). An experimental way to keep aggregation-prone proteins in solution is to add denaturant, but under such conditions many chaperones are also destabilized, which impairs complex formation. Moreover, the shielding of the client by the denaturant also hampers complex formation. Any experimental work that aims to characterize chaperone—client complexes *in vitro* needs to find a set of conditions that enable the formation of the complex. In this chapter, we review the possibilities for such complex formation, starting from some general considerations, and highlighting the different possibilities with recent examples from structural and biophysical studies. The cases we present are to be considered as example cases, with the aim to show the range of methods that have been proposed, rather than an exhaustive review of all chaperone-client complexes that have been reported.

1. Client-chaperone complex formation

1.1. General considerations

In vivo, client—chaperone complexes form either at specific locations where a client protein emerges – for example at the exit of the ribosome tunnel, or the exit of translocation pores, or they form in a situation where the chaperone is rather abundant compared to the client. Examples for the former are the trigger factor chaperone, associated to the ribosome (Ferbitz et al., 2004); Trigger factor's association

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with the ribosome provides a shielded environment for the newly synthesized peptides where a co-translational folding of client domains could occur, with possibly several chaperone molecules bound to the client polypeptide chain (Saio et al., 2014). Another example are the TIM chaperones that localize at the membrane translocation pore TOM in mitochondria, presumably in a position ready to bind membrane-protein precursor polypeptides, as they emerge in the intermembrane space (Shiota et al., 2015).

Some chaperones are abundant in the cell; for example, the family of 70-kDa heat-shock proteins (Hsp70) on its own is estimated to correspond to up to 3% of the total protein mass in eukaryotic cells under non-stress conditions (Finka et al., 2011; Finka & Goloubinoff, 2013). Under stress conditions, the cellular chaperone concentration or activity can be up-regulated, such as in the case of many of the heat-shock proteins (Bakthisaran et al., 2015; Young et al., 2004) and the Spy chaperone (Quan et al., 2011). In cases other than binding nascent polypeptide chains, stress response chaperones are upregulated as a response to misfolding or aggregation of client proteins that become structurally unstable or denatured due to stress conditions like heat, pH changes, osmotic- and oxidative stress, and nutrient starvation (Alam et al., 2021), leading to increased chaperone cellular levels or their activation.

These cellular conditions and environments facilitate the encounter of chaperones and client proteins, and the formation of complexes with affinities that range all the way from low-affinity transient interactions to tight binding with nanomolar affinity.

For an *in vitro* preparation procedure, the complex formation starts from a very different point, namely, in general, from purified proteins (chaperone, client protein). The question is, thus, how one can find suitable conditions for the encounter of the two proteins and complex formation under native conditions.

Formation of a complex between a chaperone (C) and a client protein (P) requires that, under the chosen conditions, the P-C complex is thermodynamically the most stable state, or that it is kinetically trapped state. The desired outcome, namely formation of the P-C complex, is challenged by other reactions that the protein P may undergo with itself (intramolecular processes such as folding and unfolding), with other copies of itself (such as oligomerization or, quite commonly, aggregation) or with other components in the system (e.g. other protein binding partners, a lipid bilayer, small molecules). In order to understand how the chaperone—client-protein complex may form, it is instructive to understand these possible reactions. A simplified scheme of this set of reactions is shown in Figure 1. The rate of these reactions differs from one chaperone—client pair to another. Therefore, understanding this set of reactions provides a way of seeing the possibilities that the experimentalist has to prepare P-C complexes.

Any protein P exists in aqueous solution in an equilibrium between folded conformations (P_{folded}) and possibly a multitude of different partially unfolded intermediate states (P_{int}) and an ensemble of unfolded states ($P_{unfolded}$; Fig. 1). The distinction we make here between folded, partially unfolded and fully unfolded is somewhat arbitrary, as this is rather a continuum of states, and even the "folded state" is to be seen as a dynamic ensemble of conformations. For the sake of the discussion, we keep this simplified scheme here. The kinetics and thermodynamics of this equilibrium on the continuum from folded to unfolded is protein-dependent. Many proteins are able to fold only in a proper environment or in the presence of proper binding partners – this is particularly true for the vast majority of membrane proteins, which are unable to fold in aqueous solution.

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It is primarily the partially or fully unfolded states that are prone to aggregation: by the encounter of several copies of the protein ($(P)_n$) an aggregated state, $P_{aggregated}$, may form. The (partially or fully) unfolded states are generally more prone to aggregation than the folded states. The reason for this finding is that in the folded states of globular proteins, hydrophobic parts are mostly buried within the core, and they may become exposed upon unfolding. Likewise, the polypeptides that finally get inserted into a lipid bilayer as membrane proteins are most often highly hydrophobic. The aggregated state that brings together the hydrophobic parts is often highly stable.

Molecular chaperones enter this picture of protein folding, unfolding, aggregation, and possibly insertion of the protein (into a membrane or a complex) because they can interact with one or several of these states and protect them from aggregation. They may also play an active role by lowering energy barriers in folding processes (Chakraborty et al., 2010; Hartl et al., 2011). From the practical standpoint of this manuscript, namely for the experimental preparation of chaperone—client complexes, it is important to understand where the chaperones intervene, and what the relevant kinetic rate constants are, compared to the competing processes, and how the chaperone—client complexes can be thermodynamically favored. Figure 1 schematizes the possible reactions generally, and the following sections then discuss the situation for several chaperone—client complexes, highlighting which practical experimental approach was successful for obtaining complexes.

Most chaperones bind to parts of proteins that are (partly) unfolded and expose hydrophobic residues (Rüdiger et al., 1997); this realization is linked to reports of chaperones that bind to locally "frustrated" sites in proteins (L. He et al., 2016; L. He & Hiller, 2018), which are prone to local unfolding. P-C complex formation, thus, tends to be favored when the P_{folded} state is disfavored over the (partially) unfolded ones. For the preparation of P-C complexes that start with purified soluble protein P, it is, thus, often helpful to increase the population of (partially) unfolded states. *In vitro*, destabilization of P_{folded} can be achieved by different means. One way is by acting on the chemical properties of the protein sequence itself, namely through destabilizing mutations or by truncation of the sequence. Such changes disrupt interactions that form in the folded state. Similarly, the disruption of intramolecular disulfide bonds can equally favor the unfolded states. Other factors that can be utilized to act on the $P_{folded}/P_{int}/P_{unfolded}$ equilibrium are the addition of chemical denaturant or application of high or low temperature and pH or high hydrostatic pressure.

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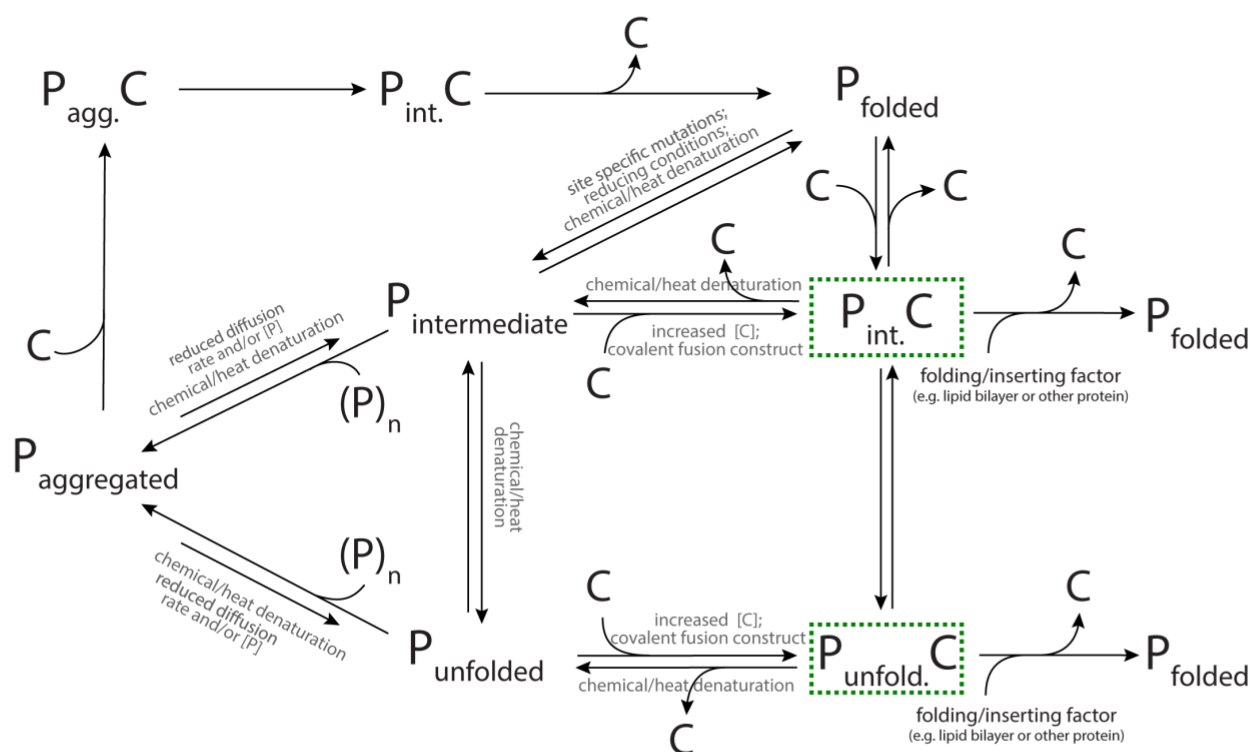

Figure 1. *Schematic overview of the reactions that may occur in a system composed of a chaperone (C) and a client protein (P). The preparation of complexes of chaperones and client proteins may proceed by direct binding of a chaperone to a folded state or to (partially or fully) unfolded states. In the formed complexes the protein P differs generally from the folded state, and the complexes are depicted by green dashed boxes. The distinction between an "unfolded" client and a partially folded one ("intermediate" or "int.") are arbitrary, and shown here to denote that there is a range of possible residual structures in chaperone-bound clients. Several other reactions involving P can occur and compete with P-C complex formation. These are the folding of P to a state that is not folding-competent, the aggregation of P or, if available, the interaction of P with binding partners or a membrane (right in this scheme). Note that also the apo protein in its folded or partially unfolded state may bind to such an additional binding partner (not shown). The various reaction equilibria and their kinetics can be modulated by changing the experimental conditions or by modifying the client protein. The effect of some of these conditions on the reaction equilibria is indicated near the arrow. The condition shifts the equilibrium in the direction of the arrow. For example, the rate of aggregate formation can be reduced by immobilizing P (section 2.5.) or reducing the concentration of P; the folding/unfolding equilibrium can be shifted towards unfolded states by e.g. denaturing conditions, reducing conditions or mutations of P (section 2.3.). From one P/C system to another, the relative importance of these reactions, i.e. the relative rate constants and thermodynamic constants, can vary largely.*

These different approaches – either the mutation/truncation of P or the change in buffer conditions, temperature or pressure – differ in an important aspect: all the changes in solvent, temperature or pressure can also affect the chaperone (e.g. unfold it) and its ability to bind client proteins. Thus, while such changes in solvent conditions may achieve the desired increase in [$P_{unfolded}$] or [P_{int}], the formation of the complex (P_{int}-C, $P_{unfolded}$-C) may be disfavored, i.e. the reaction $P_{unfolded}$ + C → $P_{unfolded}$-C or P_{int} + C → P_{int}-C either has a very low reaction rate or its equilibrium is on the left-hand side of these equations. Forming

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the P-C complex, thus, requires the removal of the conditions that denature/destabilize the chaperone (such as denaturant or high temperature).

A practically very important reaction is the one that converts soluble protein into insoluble aggregates. For the formation of soluble P-C complex, this reaction is a major threat. The essential question for *in vitro* complex formation is whether the formation of aggregates is thermodynamically and/or kinetically favored over the formation of the P-C complex. The coupled equilibrium between aggregated, free and chaperone-bound protein, thus has a few important parameters: the kinetics and thermodynamics of the aggregation of soluble P, and the kinetics and thermodynamics of P-C complex formation. The thermodynamic stabilization of the soluble form over the aggregated form can be achieved by the addition of denaturant or high hydrostatic pressure. However, as discussed in the last paragraph, if such conditions are incompatible with keeping the chaperone intact, this strategy does not lead to productive formation of P-C complex. Therefore, in most cases, one can rather act upon the kinetics of aggregate formation vs. the kinetics of complex formation, as discussed in the following.

As aggregation is an intermolecular process involving many copies of P, the kinetics of formation of aggregates is concentration dependent. It can, therefore, be slowed down when the concentration, [P], is reduced, or the rate of diffusion of P is reduced, such that encounter of copies of P is slowed down. As the kinetics of P-C formation is also concentration dependent (namely on both [P] and [C]), a rather high [C] and low [P] helps favoring the reaction $P + C \rightarrow P\text{-}C$ over the aggregation reaction $P + P_n \rightarrow P_{n+1}$. The experimentalist's options in this sense include drip-diluting the protein from a denatured state (e.g. from a solution containing urea or other chaotropes) to a buffer that contains the chaperone and sustains the chaperone's integrity. Another possibility is to have the protein synthesized in the presence of chaperone, such that a nascent protein would encounter chaperones before having the chance to encounter other copies of P and aggregate. This strategy is exploited in *in vitro* protein synthesis systems containing chaperones (Rampelt et al., 2020; Schneider et al., 2010; Weinhäupl et al., 2018).

The other parameter that the experimentalist can influence in order to slow down the aggregation process is by limiting its diffusion coefficient and thus limit the probability of encounter of copies of P. A useful approach that exploits this principle is based on immobilizing the protein to e.g. a resin via an affinity tag. Doing so, the encounter of proteins is strongly reduced (the diffusion coefficient is essentially infinitely small), and added chaperone C can bind with P, without having the competing process of aggregation of P.

The P-C complexes have often a substantial degree of flexibility, as revealed by numerous atomic-level studies reviewed in (Hiller & Burmann, 2018; Sučec et al., 2021). The client protein can exist in a range of conformational states when bound to C, from fully unfolded to largely folded (see the equilibrium of P_{int}-C and $P_{unfolded}$-C in Figure 1).

The release of P from the P-C complex is an important parameter that determines if biochemical, biophysical and structural studies of P-C can be performed. The different states of P have different affinity to C. Although not strictly necessary, it can be assumed that less native-like states of P have a higher propensity to bind C. Accordingly, the off-rate, $P\text{-}C \rightarrow P_{int} + C$ or $P\text{-}C \rightarrow P_{unfolded} + C$, depends on the conformation of the client protein. Rapid off-rate and slow on rate is obviously counter-productive for obtaining large quantities of stable P-C complex. As discussed above, the off-rate is enhanced and the on-rate reduced in the presence of conditions that destabilize the chaperone (e.g. with denaturants).

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One way of increasing the rate of P-C complex formation (P + C → P-C) is by covalently binding/fusing the client protein to the chaperone; this is best done by introducing a flexible linker between the sequences of P and C, such that the interactions between chaperone and client protein are minimally perturbed. In such fusion constructs, the off-rate is not necessarily altered as compared to a non-covalent protein complex. However, as the distance between C and P is short, the probability of encounter is enhanced. As an additional effect, the covalent fusion can hamper aggregation of P, even in its detached state, due to steric hindrance.

For completeness, Figure 1 (right) also sketches an additional reaction that can occur, particularly in the cell. The protein may become inserted into a complex with a cognate binding partner, or inserted into a membrane, and this reaction may strongly stabilize its folded state, and thus essentially remove it from the equilibrium. For example, a membrane-protein precursor that gets inserted into the membrane is generally not bound by soluble chaperones any more. While this may be an important reaction in the cell, experimentally it is, of course, simply avoided by not having the respective binding partner or lipid bilayer present.

1.2. Overview of the possibilities to prepare chaperone-client complexes

Following these basic considerations, we can enumerate several strategies which, in principle, can be successful for generating P-C complexes. In the simplest case, mixing of a purified chaperone sample with a purified client protein may lead to spontaneous formation of a complex; this approach may need destabilizing mutations in the client protein, and/or covalently linking the client to its chaperone. Reducing conditions may also be considered as a similar strategy that impacts the client protein by disrupting disulfide bonds, but not the chaperone (unless it contains disulfide bonds). Alternatively, heating the mixed sample or applying pressure may allow the formation of complex, provided that the chaperone remains intact at the elevated temperature/pressure. Another possibility is to choose the solvent conditions to either destabilize the (soluble) protein, or to extract it from the aggregate state in which it may have been produced. The denatured protein can then be mixed with the chaperone, and simultaneous or subsequent removal of the denaturing conditions may allow the complex formation. This removal can be achieved in different ways (e.g. sudden dilution, dialysis; discussed below). Lastly, complex formation may be achieved directly in the host organism in which the protein is produced, and the intact chaperone—client complex may be extracted. Somewhat similarly, the client protein may be captured upon production, using a cell-free (*in vitro*) protein synthesis system.

Rationally designing the most appropriate complex-formation strategy may, in principle, work, based on the knowledge of the kinetics and thermodynamics of the reactions outlined in Figure 1. However, it is not often the case that these details are known, and trying a few different schemes is common. The following sections discuss these different strategies with reported examples from the literature.

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2. Different complex-formation approaches in practice

2.1. Forming complexes in solution by mixing chaperones and their soluble client proteins

2.1.1. Hsp70 (DnaK) complexes

In some cases, the preparation of P-C complexes can be achieved in a straightforward manner by mixing chaperone and client. An example for such a case is the complex formed by DnaK (bacterial Hsp70) and the human telomere repeat binding factor 1 (hTRF1) (Sekhar et al., 2015). In solution, hTRF1 exists in an equilibrium of folded and unfolded states, but the folded state largely dominates; at 35 °C, ca. 4% are unfolded (Gianni et al., 2003) and at 25 °C only ca. 0.3% of the population is unfolded (Sekhar et al., 2015). There are two equilibria present in solution under the conditions chosen by the authors (25 °C, concentrations of ca. 0.5-0.6 mM): (i) folding/unfolding is heavily skewed towards the folded state with a population of 99.7%, and rate constants of ca. $1 s^{-1}$ for the unfolding process and $288\ s^{-1}$ for the folding process; (ii) binding/release to the DnaK is with a dissociation constant in the low micromolar range (K_d ~18 µM, $k_{on} = (1.1 \pm 0.2) \times 10^6\ M^{-1}\cdot s^{-1}$, $k_{off} = 20.4 \pm 0.2\ s^{-1}$). The binding is proposed to proceed from the native unbound state (N) via the unfolded free state (U), to the bound state (Sekhar et al., 2015). In the complex hTRF1 has some residual secondary structure, and it seems plausible that DnaK can bind to hTRF1 when the latter has some residual helicity. In this particular case, the sample preparation involved mixing of hTRF1 with an excess of DnaK (for the experiments in which hTRF1 was isotope-labeled for NMR) or DnaK with an excess of hTRF1 (for NMR studies of DnaK).

Sekhar, Kay and co-workers used similar sample-preparation strategies – namely mixing of chaperone and client – to resolve an important mechanistic question: does complex formation proceed via selection of the unfolded state that pre-exist in solution, or does the chaperone rather bind to the folded state, and then induce unfolding of the client in the bound state? The two schemes are sketched in Figure 2, following the general scheme of Figure 1. (Hereby, we considered that the aggregation-propensity can be ignored, i.e. the client is largely soluble.) Sekhar *et al.* used two different proteins, one with a native α-helical fold and one with a native β-sheet fold, to resolve this question for both classes of proteins. They used marginally stable and slowly folding proteins, which allows dissecting the folding/unfolding equilibrium and the binding/release process. In an elegant combination of NMR dynamics experiment, such as zz-exchange and CEST, they demonstrated that conformational selection (left in Figure 2) is dominant (Sekhar et al., 2018). Thus, the chaperone preferentially binds to pre-existing unfolded state(s).

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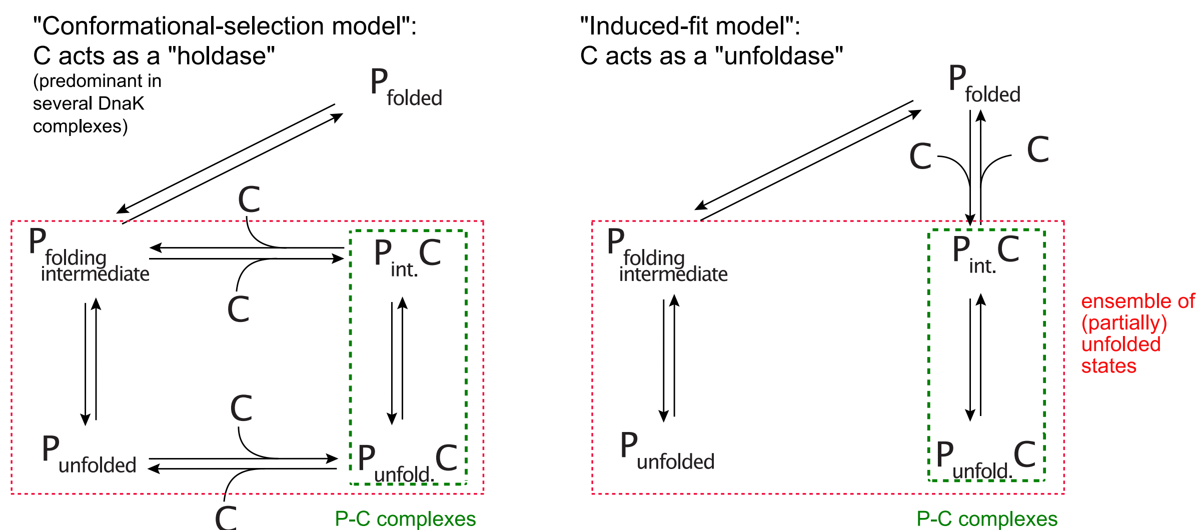

Figure 2. *Schematic overview of the relevant reactions that can lead to the formation of a complex of a chaperone (C) with a soluble client protein (P) that has negligible aggregation propensity. P and C are mixed and the inherent property of P to expose (partially unfolded) parts, and the ability of C to recognize and bind these parts, leads to complex formation. For the case of DnaK, discussed in section 2.1., the left mechanism, namely selection of (partially or fully) unfolded states by the chaperone, is dominant* (Sekhar et al., 2018).

Similarly to working with full-length clients, another option is to use fragments thereof, which has been done by the same group (Rosenzweig et al., 2017): using soluble peptides of the hTRF1 client and several NMR labeling schemes, multiple chaperone binding sites on the client were detected. Comparison of DnaK-bound state populations of these different binding sites (peptides) indicated that multiple DnaK chaperones bind to the hTRF1 client. Conceptually, what happens when working with fragments of clients is that the folding/unfolding equilibrium is heavily skewed towards the unfolded state – the fragment is often unable to fold – and hence the equilibrium N←→U is eliminated from the reaction scheme.

2.1.2. Complexes of the chaperone Spy with Im7

The client-chaperone recognition and interaction of the stress-induced Spy foldase (Quan et al., 2011) has been studied with the small soluble protein Im7. Im7 functions by binding to its cognate partner colicin E7, but in isolation Im7 tends to (partially) unfold, due to a number of locally frustrated sites. For this reason, Im7 has been used as a model protein in a number of protein-folding studies. Conditions and mutants of Im7 have been reported in which Im7 remains soluble, either in partially folded or unfolded states (Capaldi et al., 2001; Friel et al., 2009; Whittaker et al., 2007). Complexes with Spy can be formed by mixing the protein with the chaperone (L. He et al., 2016; Horowitz et al., 2016; Stull et al., 2016). Wild-type Im7 is in a partially unfolded state when it interacts with Spy, where the residues experiencing local frustrations are driving the sequence-unspecific binding to Spy (L. He et al., 2016; L. He & Hiller, 2018). In addition to the wild-type Im7 client, site specific mutations were introduced that were shown to prevent

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Im7 folding (Pashley et al., 2012), while preserving its solubility during the production and purification. Mixing of this soluble but folding-incompetent Im7 variant with the chaperone was successful to prepare samples suitable for crystallography (Horowitz et al., 2016) and for NMR studies (L. He et al., 2016). Although crystallography is challenging to interpret in this case of a highly disordered client (Wang, 2018), NMR showed that a combination of hydrophobic and charged residues appears to be required for binding to the unfolded client. The ITC and stopped-flow experiments showed that Spy binds to both folded and partially folded clients with similar affinities (3-10 µM) (Stull et al., 2016). In these examples, the concentration of the complex was high enough for NMR (several hundred µM) and ITC studies.

In kinetic measurements, chemically denatured wild-type Im7 was used and its chaperone-bound folding was followed upon dilution into different concentrations of the Spy chaperone (Stull et al., 2016). In other assays, chemically denatured client malate-dehydrogenase (MDH) and DTT-reduced α-lactalbumin (α-LA) were used as fast-aggregating clients to probe the anti-aggregating activity of wild-type Spy (Stull et al., 2016) and its variants (W. He et al., 2020). The aggregation was followed by light scattering upon diluting denatured clients in the presence or absence of wild-type Spy or Spy variants.

2.1.3. Hsp90 and Hsp110 complexes

The 90-kDa heat-shock protein (Hsp90) is another well-studied system of which complexes could be formed by mixing with clients. Street *et al*. probed binding and complex formation of the protein Δ131Δ (131-residue fragment of staphylococcal nuclease; globally unfolded protein fragment) to Hsp90. Fluorescently labeled Δ131Δ was mixed with Hsp90, and the binding was followed by the fluorescence polarization anisotropy titration experiments, with the increasing concentrations of the binding partners (up to 50 µM). Such straightforward titration experiments are possible due to the solubility of both the client and the chaperone. The dissociation constant was found to be in the low µM range (comparable to Spy-Im7 above), with a 1:1 stoichiometry; binding involves a partially structured region within the unfolded client (Street et al., 2011). Rüdiger and co-workers studied the complex formed by Hsp90 and the intrinsically disordered protein Tau (Karagöz et al., 2014) using NMR methods and electron paramagnetic resonance (EPR) (Weickert et al., 2020). Hsp90 and Tau were mixed at concentrations of tens to hundreds of micromolar, which is possible due to the relatively high solubility and comparably low aggregation-propensity of Tau (as compared to other client proteins described below).

A recent example investigated how the Hsp100 chaperone ClpB, a disaggregase, interacts with client protein. A 20-fold excess of α-casein, an intrinsically disordered client protein, was mixed and incubated with an ATPase-deficient variant of ClpB (0.7 mg/mL) and structurally investigated by cryo-EM (Deville et al., 2017). Deville *et al.* prepared different mutants of ClpB at concentrations in the range 1.6 mg/mL to 8 mg/mL and incubated them with casein (in excess). By comparing the structural features of the mutant complexes with client protein, they proposed a mechanism of ATPase initiation and client binding by the disaggregase (Deville et al., 2019). Recent work by Yin *et al.* investigated the interactions of ClpB, DnaK and the client casein, i.e., a bi-chaperoning system, using cryo-EM (Yin et al., 2021). For preparing the complexes, a simple incubation of the components proved successful.

Taken together, for many soluble client proteins, generating complexes with chaperones is achieved by mixing solutions of the two binding partners. The possibility of obtaining complexes spontaneously provides opportunities for measuring binding affinity. The complex formation is facilitated by an inherent propensity of the client protein to unfold without aggregate formation (in the client's wild-type form or

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by mutations). At least in some documented cases (e.g. DnaK discussed above), the unfolded state is selected by the chaperone. If the binding affinity for the unfolded client is sufficiently high, then even a small proportion of unfolded client suffices to generate large quantities of P-C complex in the coupled set of reactions of unfolding and binding.

2.2. Making a client protein bind by adjusting sample conditions

2.2.1. Hsp60 (thermosome) complexes formed at high temperature

If the population of unfolded client is very low under native conditions, then it may be feasible to change the sample conditions to promote unfolding. For example, if the chaperone has a high tolerance to heat, then it may be straightforward to identify client proteins that become unfolded – and prone to chaperone-binding – at temperatures where the chaperone is still functional. This strategy has been chosen for making complexes of the Hsp60 chaperone from the hyperthermophilic archaeon *P. horikoshii* and two mesophilic client proteins (lysozyme and malate synthase G). *P. horikoshii*, an organism that is found in deep-ocean hydrothermal vents, has a growth optimum beyond 90 °C. Its Hsp60, also called thermosome, forms a ca. 1 MDa large assembly from 16 copies of the 60 kDa large subunits, which assemble into two large barrel-like chambers. *Mas et al.* have studied binding of lysozyme to thermosome (Mas et al., 2018). In the absence of chaperone, heat unfolds lysozyme with a midpoint unfolding temperature of ca. 70 °C. Folding and unfolding occurs on a time scale of milliseconds in this temperature range. The unfolding is reversible: cooling the sample restored the folded state, without significant accumulation of aggregates. *Mas et al.* then mixed thermosome with lysozyme in an NMR tube at concentrations in the tens-of-micromolar range. At ambient temperature the interaction is negligible whereas at high temperature, lysozyme binds to the chaperone, evidenced by e.g. translational diffusion measurements that probe the size of the client protein. This finding shows that the chaperone binds preferentially (or exclusively) to the unfolded state. Interestingly, NMR showed, that the midpoint temperature of unfolding decreases by ca. 5 °C in presence of chaperone. This suggests that the presence of the chaperone shifts the equilibrium ($P_{folded}/P_{unfolded}$) towards the unfolded state. The Hsp60-lysozyme$_{unfolded}$ interaction at high temperature is dynamic: NMR data show that binding and release are fast on the NMR time scale, i.e. in the sub-millisecond range. Cooling down the sample shifts the equilibrium of lysozyme to its folded state, which does not interact with the chaperone any more. Repeating the experiment at different ratios of lysozyme and thermosome (i.e., titration) allows measuring the proportion of lysozyme bound to thermosome, through NMR chemical shifts. This allowed the authors to determine the dissociation constant (K_d ~1.6 μM); NMR measurements of isotope-labeled thermosome allowed mapping the interaction site. The short lifetime of this dynamic complex strongly contrasts with other cases discussed in sections 2.4. and 2.5., whose lifetimes are more than six orders of magnitude longer.

When applied to a different client protein, the 83-kDa large malate synthase G (MSG), the same strategy with the same chaperone resulted in a somewhat different behavior (Mas et al., 2018). First, when MSG alone is heated, it aggregates at elevated temperature. When a mixture of MSG and *P. horikoshii* Hsp60 is heated, the chaperone protects MSG from aggregation (at least in part). The complex, once formed at high temperature can be brought to low temperature without dissociation, i.e. the complex formation is irreversible, or, more precisely, the off-rate of the client is very low. This might at least in part be explained by the size of the client (83 kDa), which essentially fills the cavity of the chaperone cage. A further difference between these two cases is that MSG is much more aggregation-prone than lysozyme.

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2.2.2. Trigger factor complexes obtained via temperature or disulfide-bond breakage

Another example of applying heat for client-selective denaturing is the study of trigger factors (TF) holdase function where a temperature sensitive variant of maltose binding protein (MBP) showed reduced aggregation at high temperatures (50°C) in the presence of TF (Saio et al., 2014). The TF chaperone does not bind to the folded client (MBP variant at lower temperatures); however, by increasing the temperature partially unfolded states of the client protein become more populated, leading to complex formation. In the same study, with a different client protein, reducing conditions were used to keep the client unfolded and chaperone-binding prone: the client protein, wild-type PhoA protein, contains four cysteine residues and, *in vivo,* folds in oxidizing periplasmic environment. NMR was used to study the state of PhoA under reducing conditions. It was shown that several regions had only low (20-60%) secondary structure propensities (Saio et al., 2014). Isotopically (^{15}N) labeled full-length PhoA, as well as the PhoA fragments, were titrated in reducing conditions with the unlabeled TF. This approach allowed identifying the binding site(s) of the chaperone (Saio et al., 2014). These studies were successful because the TF chaperone is insensitive to the reducing agent because it lacks cysteine residues. This approach has a drawback, however, as the resulting sample may not be homogeneously reduced (see discussion in section 2.3). In addition to NMR experiments, isothermal titration calorimetry (ITC) with the fragments of the client protein and multiangle light scattering (MALS) experiments of the complex were performed to determine the stoichiometry of the complex and the binding affinity. The chaperone appeared monomeric upon interaction with the client and the dissociation constant was between 25 and 200 µM (Saio et al., 2014). The same two client proteins (PhoA and MBP) were used for characterization of client-protein binding by the chaperone SecB in similar conditions (reducing buffer or introducing mutations to make client soluble and un-foldable). The resulting complexes were studied by NMR, surface plasmon resonance (SPR) and bio-layer interferometry (BLI) (Huang et al., 2016).

Taken together, in cases where the client does not spontaneously bind (unlike those presented in section 2.1.), the use of harsher conditions for the client protein (high temperature, reducing conditions). By choosing chaperones from hyperthermophilic organisms or chaperones that do not rely on disulfide bonds, such conditions may be found.

2.3. Mutating client proteins to make them chaperone-binding prone (or reduce their aggregation propensity)

As already mentioned above in some of the examples (Im7 in section 2.1. and MBP in section 2.2.), site specific mutations are used to stabilize unfolded conformations of the client in order to increase the population of chaperone-binding competent states. The phosphatase A (PhoA) protein, encountered in section 2.2.2 contains native disulfides which are important for folding. The binding-competent unfolded state can be enhanced under reducing conditions (Huang et al., 2016; Saio et al., 2014), or by mutating the cysteines to, e.g. serines. The cysteine-less variant of full-length PhoA (proPhoAS4) shows no indication of residual structure based on its NMR spectrum, in contrast to the reduced form of a wild-type protein, for which some residual secondary structure propensity has been observed (Clerico et al., 2021). The reduction of the cysteines in a wild-type PhoA may not be complete, and it may change over time as the reducing agent gets consumed (oxidized). Consequently, a mixture of unfolded and partially folded species of the client protein are present in solution, and the unfolded portion of the client has a tendency decrease over time (Clerico et al., 2021). The cysteine-less variant has been used for studies of binding to

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the substrate-binding domain (SBD) of DnaK (Clerico et al., 2021). The practical drawbacks of a cysteine-less variant, however, can be that (i) its production requires solubilization and purification from inclusion bodies and (ii) it may allow only for short time storing at low concentrations, because aggregation from the unfolded state is more rapid than if the protein was folded during storage (Clerico et al., 2021).

Mutating cysteines may also be of use in a different context: the formation of intermolecular disulfide bonds may enhance aggregation of client proteins. For forming complexes of membrane proteins, i.e. highly aggregation-prone polypeptides, Weinhäupl *et al.* developed a pull-down approach with the chaperone TIM9·10 described in section 2.5. The chaperone contains essential disulfide bonds while the two client proteins chosen by the authors (ADP/ATP carrier; GDP/GDP carrier) contain four and one cysteines, respectively (Weinhäupl et al., 2018). The authors found that the presence of the native cysteines enhances the propensity of the client protein to aggregate. Employing reducing conditions is not an option because the chaperone unfolds when its disulfides are broken. Hence, in this case, mutating the native cysteines in the client proteins to Ala or Ser was a successful solution for complex formation without accumulating aggregates (see section 2.5.).

2.4. Complex formation upon removal of a denaturant

Some client proteins may not be kept in aqueous solution at all, i.e. the aggregation reaction may be predominant. For such proteins one needs to find conditions in which they can encounter (in solution) the chaperone proteins; of course, these conditions shall not be denaturing, so as to maintain the chaperone in a folded and active state. Membrane-protein (MP) precursors on the way to their membranes (e.g. bacterial outer-membrane proteins or MPs of organelles) are generally such highly-insoluble polypeptides. Bacterial outer membrane proteins (OMPs) with a native β-barrel fold require purification in chemically denaturing conditions from inclusion bodies. Chemical denaturation at high concentrations of chaotropic agents such as 8 M urea or 6 M guanidine-hydrochloride implies denaturing of all proteins present in solution and shielding of all (hydrophobic) intra- and intermolecular interactions. Consequently, the denaturing agent needs to be removed for client-chaperone complex formation. It has been shown that by drop-wise dilution (drip dilution) of denatured Omp into a solution containing a native chaperone of OMPs, Skp, leads to successful complex formation (Burmann et al., 2013). Drip dilution is a very fast dilution of the denaturant by dropwise addition of denatured sample into a typically 100 times bigger volume of the refolding buffer over a period of 1 h, keeping unfolded protein at minimal concentration during this procedure (Burgess, 2009). In the denaturing buffer, the OMP is unfolded, but as soon as the OMP is rapidly dissolved in chaperone-containing buffer at very low denaturant concentration, the OMP may bind to the chaperone, but it may also aggregate. (The folding reaction is negligible, as membrane proteins generally do not fold to a defined structure outside a membrane.) The relative rate constants of aggregation vs. binding are, thus, determining whether a complex forms. The aggregation rate constant is temperature-dependent, and aggregation is slowed at low temperature (Xie & Wetlaufer, 1996). The aggregation furthermore depends on the concentration of the client protein; diluting into sufficiently large volume and good mixing during dilution is, therefore, helpful to suppress aggregation. The rate constant of P-C complex formation is dependent on the concentrations of both the client protein and the chaperone. The drip dilution method implies high dilution of the client protein concentration too, causing additional decrease of aggregation rate due to reduced diffusion-collision probability of the unfolded client molecules. However, to ensure that most of the chaperone is in the client bound state, excess of

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denatured client needs to be added in a drop-wise manner until precipitation due to lack of free chaperone is observed (Burmann et al., 2013). In the preparation of Skp-OMP complexes, the Hiller group has added an excess of OMP (solubilized in 6 M guanidine) in a drop-wise manner to Skp in native buffer (such as 25 mM MES, 150 mM NaCl, pH 6.5), under stirring, until precipitation appeared (Burmann et al., 2013; Callon et al., 2014). The solution was then centrifuged before analysis by structural and biophysical methods. In some special cases, the assembly buffer in which client is drip-diluted can contain reducing agent such as DTT (see section 2.2. for keeping client binding-prone in chaperone compatible conditions) if working with disulphide bond-free chaperone. Once the chaperone-client complex is formed, the assembly buffer can be exchanged for the analysis compatible buffer without chemical reductant (Thoma et al., 2015).

Essentially the same method was applied for forming complexes of trigger factor (TF) with OmpA. Here, the purification of the client protein was performed in denaturing conditions and complexes were formed by diluting the client sample 20-fold in the presence of chaperone in reducing conditions to keep the OmpA unfolded and TF-binding prone (Saio et al., 2014).

It is noteworthy that the kinetics and thermodynamics of complex formation between chaperones and highly aggregation-prone polypeptides are very different from those of the soluble clients (described in sections 2.1., 2.2. and 2.3.). For Skp complexes with several β-barrel client proteins of the OMP family, the dissociation constant was consistently in the one- to two-digit nanomolar range (Qu et al., 2007). The life time of the Skp-OmpA complex is ca. 2.6 hours (Burmann et al., 2013), which is more than six orders of magnitude longer than complexes formed by e.g. thermosome and lysozyme, and more than three orders of magnitude longer than the DnaK complexes (section 2.1.).

In a similar way, a GroEL/ES-client complex was obtained for cryo-EM studies capturing chaperone 'in action' with an encapsulated client. The clients used in this study were denatured in acidic urea buffer and mixed with an open-state GroEL/ES chaperone solution in reducing conditions, by drip-diluting the denaturant 50-fold, after which ATP was added to ensure the encapsulation of the client (D. H. Chen et al., 2013).

Complex formation by the drip dilution method requires that the binding is faster than aggregation and that the complexes are stable. The method works for forming complexes of bacterial outer-membrane proteins with different chaperones (Skp, SurA, trigger factor). We found that in another case of membrane-protein precursors as client proteins, the drip dilution method fails: the case of mitochondrial-carrier membrane proteins and their native chaperones, the hetero-hexameric TIM9·10 chaperone. Drip dilution of different guanidine-solubilized mitochondrial carriers into a solution of TIM9·10 (testing a range of optimization parameters), has not produced complex; rather, the client protein precipitated (not published). A reason for this failure may be that mitochondrial carrier proteins (α-helical in their native state) are more hydrophobic than OMPs. This higher hydrophobicity may lead to faster aggregation compared to OMPs. Additionally, TIM chaperones disassemble above approx. 100 mM guanidine-hydrochloride (unpublished data). The guanidine-sensitivity of the chaperone might result in destabilization of the chaperone at the locally high denaturant concentration as the drop gets diluted into chaperone-containing buffer.

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In a Förster resonance energy transfer (FRET) study, DnaK, introduced in section 2.1., has been studied in complex with rhodanese (Kellner et al., 2014). Of interest is that the rhodanese–DnaK complex is strictly dependent on the presence of the J-domain protein DnaJ (which is essential for ATP hydrolysis of Hsp70 chaperones) and ATP. Kellner *et al.* prepared samples from rhodanese that was denatured in 4 M guanidinium buffer, and then diluted the protein into a buffer without denaturant, but with DnaK, DnaJ, GrpE (a nucleotide exchange factor) and ATP. The particularity of single-molecule FRET is its ability to work at very low concentration (25 to 75 pM in this study).

2.4.1. Complex formation by dialysis

Another method towards formation of complexes from a denaturant solution is dialysis. In such a procedure, one would mix the client protein and the chaperone in the presence of denaturant, and dialyze out the denaturant. At the initially high denaturant concentration, not only the client protein is solubilized but, most often, the chaperone is also perturbed; moreover, the shielding by the chaotropic agent likely hampers the interaction between client and chaperone. Dialysis may, thus, work if during the time-dependent reduction of denaturant concentration, the chaperone's integrity and binding capacity are restored before the aggregation of the client protein becomes dominant. This approach has been reported to be successful e.g., for the DegQ chaperone/protease, (Malet et al., 2012; Mauldin & Sauer, 2013). In the case of the above-described TIM9·10 chaperone with mitochondrial carriers, dialysis approaches proved unsuccessful: after dialysis, we could only obtain aggregated client protein and free chaperone (unpublished). It is likely that this is due to the fact that the hetero-hexameric chaperone is disassembled at a concentration of ca. 100 mM guanidine, likely much less than what is needed to keep mitochondrial carriers in solution.

2.5. Hampering aggregation by client immobilization: complex formation with a pull-down approach

As outlined above, drip dilution and dialysis were unsuccessful for generating complexes of TIM9·10 and mitochondrial carriers (such as the ADP/ATP carrier Aac1 and the GDP/GTP carrier Ggc1). Weinhäupl *et al.* have developed an approach for complex formation that strongly reduces aggregation and allows obtaining homogeneous and long-lived complexes, stable over days to weeks (Weinhäupl et al., 2018; Sučec et al., 2020) (Figure 3). The method comprises first the binding of unfolded client protein in denaturant (such as 6 M guanidine) on an affinity column, via an affinity tag on the client-protein construct (His_6-tag). As the individual molecules are attached to the beads at fixed location, they are unable to diffuse and encounter each other and thus to form multimeric aggregates. Then, the buffer is exchanged to a more native buffer without denaturant ("binding buffer"). The concentration of denatured client loaded on the column is kept low: on a 5 ml affinity resin approximately 2.5 mg of unfolded client is loaded. This helps to reduce the aggregation rate. Immediately after removal of denaturant, an excess of chaperone, typically 2 times higher compared to the client protein, is passed through the column. The non-bound chaperone is washed off the column, and the complex then eluted (e.g. with imidazole). In a slightly modified version of this protocol, the denaturant was not removed before addition of the chaperone. Instead, a 2-fold excess of chaperone (compared to loaded client protein) was added in one fifth of the column volume and the denaturant concentration gradually decreased by diluting the flow-

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through solution 1:1 with binding buffer before passing it onto the column again. This process was repeated until denaturant concentration was below 0.05 M (roughly in a total volume of 10 times the column volume). In this latter approach, the chaperone is presumably disassembled in the beginning and when the denaturant concentration is below a critical level (for TIM9·10 this is of the order of 100 mM), the chaperone assembles and is able to bind the client protein.

For TIM chaperones and mitochondrial carriers, both variants work, and the former (namely removing the denaturant from the column before adding the chaperone) is experimentally somewhat simpler. After desalting and concentrating the eluted complex, the final concentration of chaperone complex was of the order of 150 µM, well suited for structural studies. The sample was stable for days of measurement at up to 35 °C. The lifetime of the complex was ca. 4 hours, i.e. when apo-chaperone was added to P-C complex, it took 4 hours to transfer the client from one chaperone to another. No significant aggregation of the client was observed under these conditions. Interestingly, however, gel filtration experiments of the complex were unsuccessful: the TIM9·10 complex with mitochondrial carriers dissociates during passage on a size exclusion column (unpublished data). In contrast, with another client protein, Tim23, the P-C complexes of both TIM9·10 and a homologous chaperone, TIM8·13, could be analyzed by gel filtration and coupled SEC-MALS (see supplemental figures S12 and S13 in (Sučec et al., 2020)). Characterization of these complexes has also been done by other biophysical/biochemical methods, such as analytical ultra-centrifugation, small-angle X-ray scattering, NMR-detected translational diffusion experiments or SDS-PAGE (Weinhäupl et al., 2018; Sučec et al., 2020). Note that with the drip-dilution method one can determine dissociation constants K_d, by following e.g. fluorescence spectra upon titrating the client protein into a chaperone solution (Qu et al., 2007). In the pulldown method describe here, determining affinities or stoichiometries is not possible.

It is worth noting that this approach to complex formation was not successful for the TIM chaperones with a precursor of the outer membrane β-barrels. None of the above methods (drip dilution, dialysis, pull-down) resulted in complex formation. The β-barrel client protein was found to aggregate in all these conditions (reported in (Weinhäupl et al., 2018)). The authors were able to generate complexes of weak affinity (K_d in the low mM range) only with a β-turn fragment of the β-barrel client, but only if the β-turn was stabilized by cyclization of the peptide. A likely explanation for this observation is that in a cyclic β-turn element the side chains that are hydrophobic and hydrophilic point to the two faces of the β-turn element. In other words, such an element creates one hydrophilic and one hydrophobic face – and the latter presumably binds. In a totally disordered polypeptide, the hydrophilic/hydrophobic side chains point randomly in different directions, which likely impedes the chaperone binding.

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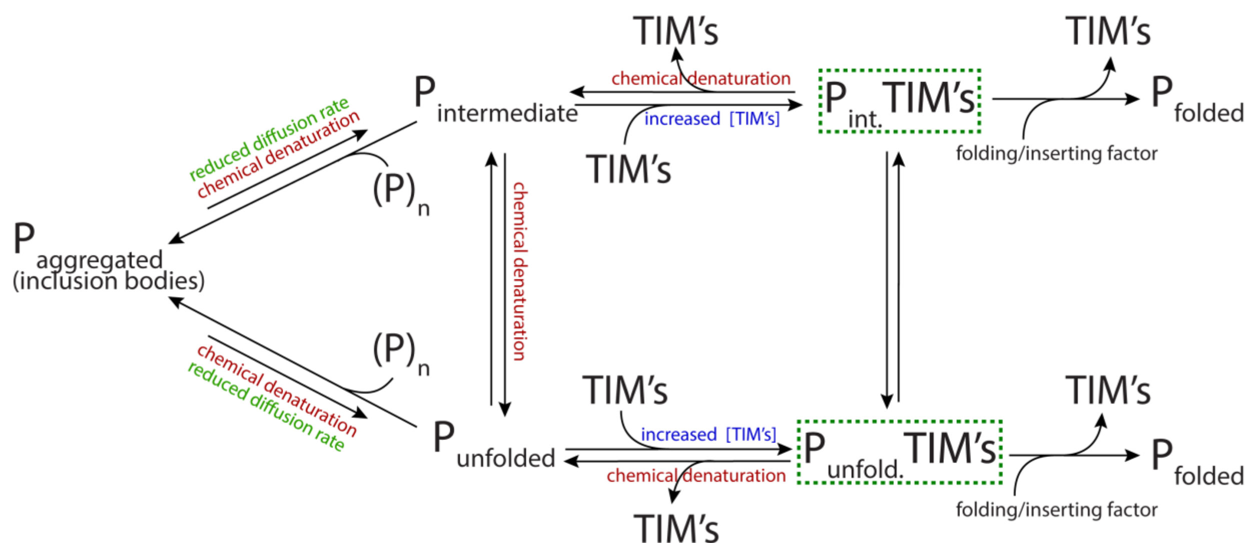

Figure 3. Scheme of different approaches for the mitochondrial intermembrane space chaperone complex formation. *Method developed for complex formation with mitochondrial membrane proteins* (Weinhäupl et al., 2018) *is comprised of three steps. In the first step (red) hydrophobic client protein is unfolded from the inclusion bodies using strong chemical denaturant that prevents formation of protein aggregates and keeps the client in an unfolded state. However, strong denaturant is preventing any possible interaction with the client due to chaperones instability under such conditions. In the second step (green) client is bound to the affinity column, which reduces the local concentration of the client and its diffusion rate and the rate of clients aggregation is reduced once the denaturant is removed. In the third step denaturant is removed and saturating concentration of the chaperone is added (blue) to the bound client increasing the rate of complex formation. In a second method, the client is produced by a cell-free (in vitro) expression system which contains the chaperone, in addition to the usual components required for in vitro translation. In a second method, complex formation in a cell-free system, client in produced in in vitro expression system while providing increased concentration of purified chaperone. In this approach there are no aggregation—prone client species (i.e. unfolded and free client) as long as there are free chaperones in the cell-free reaction solution.*

2.6. Purifying P-C complexes from the cell

Direct purification of the P—C complex from the host organism would be biologically most relevant complex for biophysical studies and characterization. In order for this approach to be successful, the stability of the P-C complex must be sufficiently high such that the complex survives all steps from cell disruption to purification. This approach was shown to be successful in the case of the Hsp60 chaperone GroEL/ES (Fei et al., 2014) and the bacterial periplasmic chaperone/protease DegP with outer-membrane proteins (Krojer et al., 2008). The majority of DegP purified from *E. coli* is co-purified as 12-mer and 24-mer with bound OMPs (OmpA, OmpC, OmpF and LamB). These complexes could be separated from the apo-chaperone by ion exchange chromatography. The yield of purified complex was sufficient for electron microscopy structural studies to determine the active chaperone state (Krojer et al., 2008). However, this approach is often discarded for client-chaperone sample preparation due to the heterogeneity of the sample, where chaperone with different client proteins can be present. In a study by *Fei et al*. a subsequent refolding of GroEL was applied after chaperones purification to reduce unwanted client-bound particles (Fei et al., 2014). However, co-purification of chaperone-client particles may in fact be

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highly desirable, in particular if coupled to cryo-EM, where the separation into free and client-loaded classes can be done post acquisition.

Co-expression of the client and substrate protein from the same expression plasmid in the bacterial cells was applied to obtain the trigger factor in a complex with the S7 (ribosomal subunit protein) and OmpA client proteins for crystallography studies (Martinez-Hackert & Hendrickson, 2009). This was done after identifying the TF-substrate proteome in *E. coli* by co-purification with TF and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The complexes of trigger factor formed during co-expression could be co-purified on the affinity column with only one binding partner carrying the affinity tag, and its stability is sufficient for characterization by the size-exclusion chromatography and analytical ultracentrifugation with a yield high enough for crystallographic studies (Martinez-Hackert & Hendrickson, 2009).

Baculovirus co-expression of human Hsp90 complexes in the insect cells was used for production of chaperone-cochaperone-client complex for (cryo-) electro-microscopy studies. With addition of a mimetic of the ATP γ-phosphate (molybdate) during the complex co-purification, the closed state of an Hsp90-Cdc37-Cdk4 ternary complex was captured with the client in the semi-folded state (Vaughan et al., 2006; Verba et al., 2016). Interestingly, Verba *et al.* also reported that simple mixing of proteins individually produced and purified did not result in successful complex formation. This may be due to modifications that occur after complex formation or additional factors required for the complex formation (Verba et al., 2016).

2.7. Capturing emerging client proteins in a cell-free system

Another method of chaperone-client complex formation, with a client being highly aggregation prone, is during *in vitro* protein synthesis (cell-free expression) of a client (Schneider et al., 2010), in the presence of purified chaperone (Rampelt et al., 2020; Weinhäupl et al., 2018). *In vitro* protein synthesis using bacterial cell extract in the presence of recombinantly produced and purified TIM9·10 chaperone significantly improved the solubility of mitochondrial carrier precursors (Mpc3, Mpc2, Ggc1) in a chaperone concentration-dependent manner. The majority of the hydrophobic carrier precursor aggregate during the cell-free expression in the absence of detergent or chaperone. From these experiments it was concluded that the mitochondrial pyruvate carriers (Mpc) depend on the TIM9·10 chaperone during their import. In addition, binding to the previously established hydrophobic binding site on the chaperone could be confirmed (Rampelt et al., 2020). High local concentration of chaperone in the cell-free reaction mixture is enabling clients capture on its exit of the ribosome, reducing the probability of client aggregation.

The protein yield in cell-free expression systems is often of the order of 0.5 mg per milliliter of reaction mixture, and can reach up to 5 mg per ml. Constant improvements in the methodology enable the production of 'difficult-to-obtain' proteins (some eukaryotic, post-translationally modified, membrane or 'toxic-for-the-cell' proteins; (Jin & Hong, 2018)), as well as specific labeling schemes for NMR studies, including deuteration (Imbert et al., 2021). In parallel with the reduction of costs, this method is becoming used more often to obtain samples for structural biology studies (Terada et al., 2016). However, it often

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requires extensive optimization of the reaction conditions that is protein dependent, time consuming and often expensive.

2.8. Fusing chaperone and client into a single polypeptide chain

An additional approach to generate complexes is to fuse a client protein (or a fragment) to a chaperone, i.e. to generate a single polypeptide containing both proteins, separated by a suitable linker. Conceptually, this approach shifts the equilibrium (P / P-C) towards the bound form because of the proximity; furthermore, the presence of the chaperone may hamper the aggregation reaction, and may also hamper folding of the client protein. This approach was used, for example, for stabilizing a predominantly unfolded variant of maltose binding protein (MBP) to trigger factor separated by a 25-residue linker for a binding site(s) verification (Saio et al., 2014).

2.9. Chaperones bound to protein aggregates

A particularly interesting process is how chaperones bind to pre-existing protein aggregates. Chaperones have been shown to play key roles in suppressing protein aggregation by interacting with aggregation-prone proteins at different stages, including in oligomeric and fibrillized forms. In one recent example, the interaction of J-domain proteins (Hsp40) with amyloid fibrils formed by the protein Tau has been monitored. From a preparation standpoint such samples are, arguably, simpler than the above examples that aim to generate a soluble sample: the pre-formed amyloid fibers were incubated with the chaperone and monitored by negative-stain EM (Irwin et al., 2021).

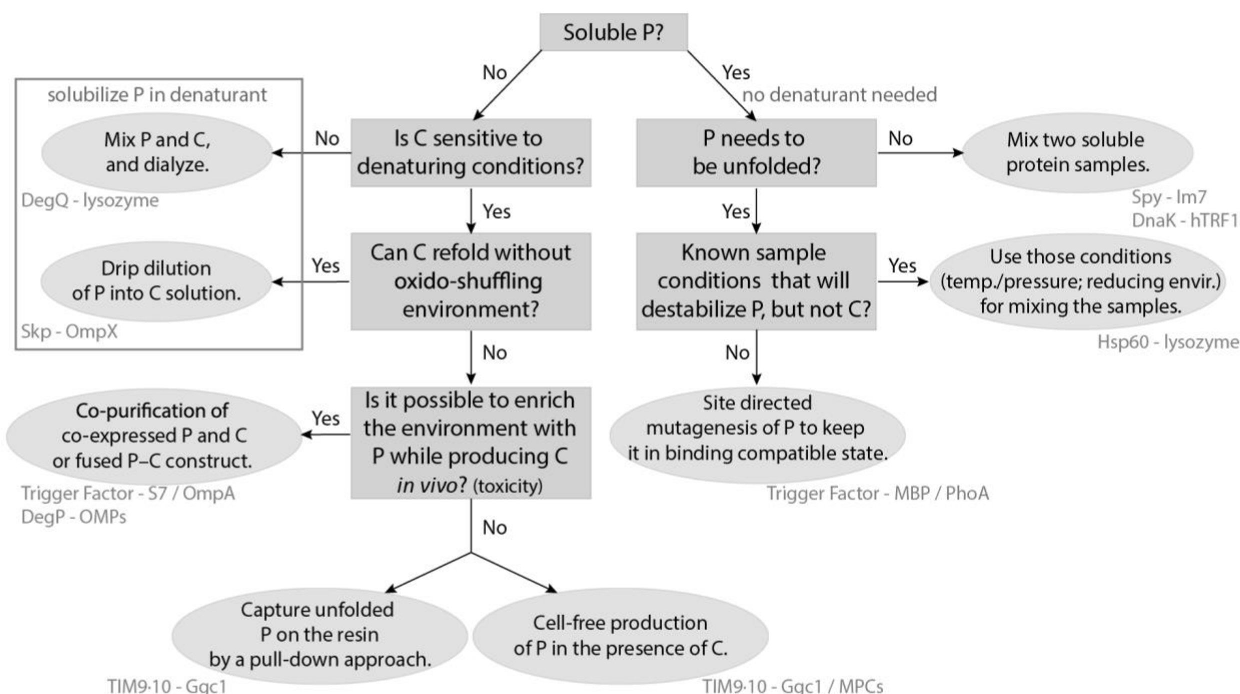

Figure 4. Overview of the main methods used for preparing chaperone—client complexes. *Examples for these different cases are given by the name of the involved proteins. See main text for details.*

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3. Concluding remarks

We have outlined here a number of strategies that can be successful for preparing chaperone complexes with client proteins, with the aim to characterize these complexes structurally and functionally. These strategies are summarized in the scheme of Figure 4. The properties of the various client proteins and chaperones vastly differ, and so do the preparation schemes, affinities and complex life times. In our view, it is not possible to make precise predictions which of the protocols will be successful, but general trends nonetheless emerge. If the client protein is largely soluble, then not aggregation of P, but rather the stability of the folded state of P may hamper successful P-C complex formation. In such cases, destabilizing P by mutations or appropriate sample conditions (denaturing, heat, possibly also pressure) is a promising route, provided the chaperone resists. The studied cases suggest that the binding and release reaction in such complexes are fast, often on millisecond time scales.

If the client protein is highly aggregation-prone, then protocols that start with denatured state are the way to go. Although the number of reported cases is small, we propose that the most aggregation-prone client proteins may not be amenable to the drip-dilution approach, and they may rather need e.g. a pull-down approach (Weinhäupl et al., 2018) or a cell-free production or isolation from the native environment.

The steady improvement of methods for the structural and dynamical study of chaperone complexes, including cryo-EM, solution- and solid-state NMR and optical spectroscopies such as single-molecule FRET comes jointly with a better understanding of the sample preparation protocols. Biochemistry, biophysics and structural-biology tools as well as *in vivo* experiments have allowed understanding the fascinating mechanisms of chaperone function at the atomic level, which is not only of fundamental interest but also of biomedical importance.

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