



## Folding while bound to chaperones

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Chaperones are important in preventing protein aggregation and aiding protein folding. How chaperones aid protein folding remains a key question in understanding their mechanism. The possibility of proteins folding while bound to chaperones was reintroduced recently with the chaperone Spy, many years after the phenomenon was first reported with the chaperones GroEL and SecB. In this review, we discuss the salient features of folding while bound in the cases for which it has been observed and speculate about its biological importance and possible occurrence in other chaperones.

### Addresses

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Current Opinion in Structural Biology 2018, 48:1–5

This review comes from a themed issue on **Folding and binding in silico, in vitro and in cellula**

Edited by **Stefano Gianni** and **Amedeo Caflisch**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 19th July 2017

<http://dx.doi.org/10.1016/j.sbi.2017.06.009>

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### Introduction

Molecular chaperones are responsible for keeping proteins in their folded form and preventing protein aggregation in the cell [1]. Defects in protein folding can lead to myriad diseases, including Alzheimer's [2], Parkinson's [3], and Huntington's disease [4]. Although much has been learned in recent years, our current understanding of how chaperones aid protein folding remains incomplete. Chaperones have traditionally been divided into two categories: first, ATP-dependent chaperones, which use ATP hydrolysis to drive large conformational changes in the chaperone and promote correct protein folding, and second, ATP-independent chaperones, which bind to proteins that are at risk of aggregating and later pass them to ATP-dependent

chaperones for folding [5]. However, these two categories belie a third possibility—chaperones that aid protein folding without the assistance of ATP hydrolysis. In this scenario, the client protein still folds to its native state, but the chaperone continuously interacts with the client in a passive manner during folding. Three prominent examples of chaperones that exhibit this phenomenon are GroEL (in this case, without its co-chaperone GroES), SecB, and the periplasmic chaperone Spy. Here, we review what is known about this mechanism, speculate on the potential for other chaperones to utilize similar mechanisms, and evaluate its possible physiological role.

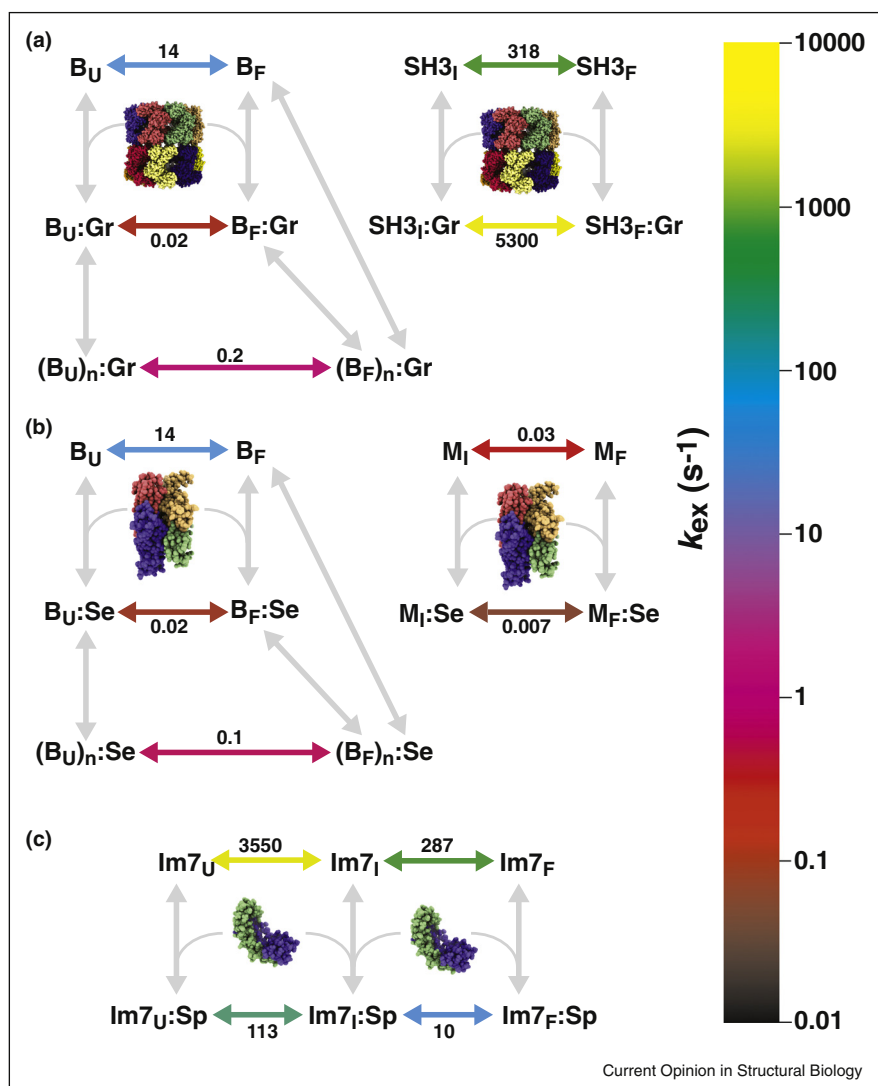
### Folding while bound to GroEL and SecB

The bacterial chaperonin GroEL is the most extensively studied chaperone to date. An essential protein, it aids the folding of approximately 10% of the bacterial proteome [1]. GroEL binds to and hydrolyzes ATP to drive conformational changes that regulate client binding and release [1]. One often overlooked aspect of GroEL's function, however, is that it can help proteins fold without its co-chaperone GroES or ATP [6–8].

As early as 1993, Fersht and colleagues observed that barnase, a model protein for folding studies, could fold while continuously interacting with GroEL [7,8]. The authors performed refolding experiments in the presence and absence of the chaperone, and analyzed its effect on barnase refolding rates. Intriguingly, they found that the asymptote of the refolding rate constant at increasing GroEL concentrations did not go to zero. This result indicated that a significant portion of the refolding that they observed occurred while barnase was continuously bound by the chaperone (Figure 1a) [8]. Although the authors noted that multiple other groups had found conflicting results with different client proteins, a then-recent paper by the Buchner lab suggested that a similar mechanism could be employed by a Fab fragment of a monoclonal antibody [9]. Gray *et al.*'s interpretation, which still rings true many years after these initial studies, was that the interactions between chaperones and their clients are likely different for different clients.

For the past two decades, studies on how GroEL functions as a chaperone have focused on the ATP-dependent aspects. However, a series of recent reports have revived the question as to whether folding while bound to GroEL may be an important feature of its mechanism of action. In two papers, Libich *et al.* use thermodynamically unstable variants of the Fyn SH3 domain to analyze SH3

Figure 1



Currently known mechanisms of folding while bound to a chaperone. The sums of the forward and reverse rate constants for each conformational change step,  $k_{ex}$ , are depicted by different colors (see strip gradient on right) and numbers above the double arrows in s<sup>-1</sup>. Binding and release rate constants are not shown (gray arrows). Subscripts U, I, and F denote unfolded, intermediate, and folded states, respectively. The subscript  $n$  denotes multiples bound to the chaperone simultaneously. Note that the proteins are not scaled to size, and that in the case of SecB and GroEL, the clients are considerably smaller than the chaperones. **(a)** Folding of barnase (B) (left) and SH3 (right) while bound to GroEL (Gr) [6,10<sup>\*\*</sup>]. **(b)** Folding of barnase (left) and MBP (right) while bound to SecB (Se) [18,20]. Note that GroEL concentrations were not saturating, and so the folding while bound rate constants could be overestimated [18]; also, the release and binding of folded MBP to SecB was not modeled [20]. **(c)** Folding of Im7 while bound to Spy [26<sup>\*</sup>].

folding in the presence of GroEL [10<sup>\*\*</sup>,11<sup>\*</sup>]. Using a combination of relaxation-based NMR methods, relaxation-dispersion, and dark-state saturation transfer experiments, they discovered that GroEL preferentially binds the intermediate state of the protein. However, they also found that the transition between the native and intermediate states of SH3 occurs while bound to GroEL and is even catalyzed by the chaperone, leading to rate increases of 2–3 orders of magnitude (Figure 1a) [10<sup>\*\*</sup>]. In a subsequent report using a different variant of SH3, the

authors observed that GroEL can interact with folded SH3 in at least two distinct modes, one of which tumbles at the same rate as GroEL, while the other is bound more loosely [11<sup>\*</sup>]. Although the populations of the unfolded states were too low to observe directly, the authors postulated that interconversion with unfolded states while bound occurs at very low rates [11<sup>\*</sup>]. Even more unexpectedly, recent reports have suggested that rhodanase, a client not thought to engage in folding while bound to GroEL [12], can indeed fold to near

completion while attached to the chaperone surface [13<sup>\*</sup>], with rhodanase's folding potentially regulated through competition between folding interactions and interactions with the chaperone surface [14].

Shortly after discovering that barnase could fold while bound to GroEL, Fersht and colleagues began studying the *Escherichia coli* chaperone SecB. In addition to its well-known roles in membrane translocation [15], SecB can serve as a general chaperone in the cytosol [16,17]. These investigators analyzed barnase folding in the presence of SecB and found that, like with GroEL, barnase could fold while bound, albeit at a slower rate (Figure 1b) [18]. Subsequent studies showed that maltose binding protein (MBP) can also fold while bound to SecB (Figure 1b), although the immature form of the protein (preMBP) cannot [19,20]. Like GroEL, the contrasting behavior of different clients with SecB suggests that the mechanism of folding while bound to the chaperone is not the same with every protein.

### Folding while bound to Spy

Spy is a periplasmic chaperone recently discovered to aid folding in the *E. coli* periplasm [21]. The permeable nature of the outer membrane of the periplasm has severe consequences for the protein-folding environment, as small molecules are free to enter and leave the periplasm at will. As a result, periplasmic proteins are regularly confronted with an array of chemical folding stresses. Furthermore, ATP is not found in the periplasm, meaning that any periplasmic chaperones necessarily operate without ATP. Spy has been shown to protect cells from tannins by acting as a molecular chaperone [21] and is able to help proteins fold independent of ATP [21,22].

Recently, we investigated how Spy facilitates protein folding without employing a regulated client release mechanism, such as ATP-driven conformational changes. In these studies, we examined the effects of Spy on the folding pathway of the protein-folding model Im7 [23–25]. Our goal was to determine if Im7 release is necessary for complete folding or if Spy can allow proteins to fold while bound, as observed previously for GroEL and SecB. To learn how Spy binding affects the folding pathway of Im7, we first determined the binding affinity and kinetic rate constants for Spy binding to the individual folding states of Im7, namely, the unfolded, intermediate, and native states. For these experiments, we used mutants of Im7 that trap the protein either in the unfolded or intermediate state but are still soluble, thereby avoiding protein aggregation that would interfere with our measurements. Using isothermal titration calorimetry (ITC), we found that Spy binds to all three folding states of Im7 in a 1:1 stoichiometric complex with micromolar affinities, with the native state binding with the weakest affinity [26<sup>\*</sup>]. This observation was surprising given that chaperones are generally thought to exhibit low affinity to

natively folded proteins due to the lack of exposed hydrophobic surface [27]. We then investigated the kinetics of complex formation between Spy and the three folding states by monitoring the tryptophan fluorescence of Im7. A fluorescence change was observed for all three variants of Im7 when mixed with Spy. These results confirmed our equilibrium ITC titrations demonstrating that Spy binds to all three states and allowed us to calculate the binding and release rate constants for all three states of Im7 [26<sup>\*</sup>]. Spy binding to all three folding states suggested that Im7 folding may occur while continuously bound to Spy.

To test this hypothesis, we measured the refolding rate constants of urea-denatured wild-type Im7 in the presence and absence of Spy. Although Im7 folding slowed down when mixed with increasing concentrations of Spy, it was not completely inhibited. A global fit analysis to determine the simplest kinetic model revealed that Spy does indeed allow Im7 to fully fold into its native state while remaining bound to the surface of the chaperone (Figure 1c) [26<sup>\*</sup>]. The kinetic model also suggested that Spy binding does not alter the folding pathway of Im7; as in the absence of Spy, Im7 folds through the obligatory intermediate state while bound to Spy. Folding in the protective environment of the chaperone, however, occurs with a kinetic penalty, as folding is slowed down roughly 30-fold [26<sup>\*</sup>]. Spy assists in the folding of client proteins without the need for an external factor that regulates client release because the lower affinity to the native state stems from an increased off rate that triggers preferential release from the native state [28].

### Could folding while bound be a common chaperone mechanism?

The finding that Spy allows Im7 to fold while bound echoes the observations with GroEL and SecB. The discovery of a third chaperone to use this mechanism suggests that it could be more widely used than previously thought. It also raises related questions: Why might a chaperone employ this strategy, what properties allow proteins to fold while bound to chaperones, and how many chaperones use this mechanism?

In our analysis of the Spy mechanism, we proposed that the reason folding while bound could work in the absence of ATP is that the client was able to fold while loosely bound [29,30]. Given that the periplasm lacks ATP, such a mechanism could serve a primitive folding function. Notably, the physiological argument for Spy and the cytosolic chaperones shares many features. Folding while bound to GroEL and SecB was observed for proteins that can thermodynamically drive their own folding. Although these proteins may not require chaperones to fold under normal conditions, the introduction of stress changes the situation. Under at least oxidative protein folding stress, the cytosolic level of ATP drops considerably [31,32],

which presumably prevents ATP-dependent chaperones from actively helping proteins refold via ATP-dependent mechanisms [32]. It is under these conditions that the ATP-independent chaperones are traditionally thought to hold aggregation-prone folding intermediates in partially folded states to be transferred to the ATP-dependent chaperones when stress ceases. The examples cited here, however, suggest an additional component: the folding of the proteins ATP-independently while bound to the chaperone. In the same fashion that Spy can allow proteins to fold during stress in the periplasm, the GroEL cavity could provide a semi-isolated environment for proteins to fold during stress without the threat of aggregation. Smaller proteins could conceivably take advantage of this mechanism, remaining mostly folded and bound until the stress ends. With the cessation of stress, ATP binding can help to release clients, as previously demonstrated [1]. Such a mechanism could prime the cell for fast protein reactivation after stress.

It was commonly thought that proteins must be released from chaperones in order to fold, raising the question as to how folding can occur if the protein is continuously bound. We and others have been able to derive principles for how this process works for Spy based on follow-up studies on Spy and Im7. Crystallography, NMR spectroscopy, and biochemical experiments revealed that Im7 binds to Spy in a highly dynamic fashion [33–35]. Spy binds to the unfolded ensemble of Im7 primarily through hydrophobic interactions, but as Im7 folds, the hydrophobic interactions are replaced with electrostatic contacts elsewhere on Spy's surface [33–35]. These electrostatic contacts enable Im7 to fold while staying in contact with Spy, with only a small decrease in folding speed. Conceivably, a similar mechanism could apply for barnase folding while bound to GroEL or SecB, as at neutral pH, barnase is oppositely charged to the GroEL cavity and to SecB and similarly exhibits a decreased folding rate. However, the mechanism of SH3 folding while bound to GroEL is probably different, as GroEL catalyzes the conformational change in SH3 as opposed to slowing it down. The nature of these interactions therefore remains an interesting area of future study.

The observation of protein folding while bound to Spy, SecB, and GroEL leads one to wonder why this phenomenon has not been observed with other chaperones. Notably, the studies described here all use folding models that do not aggregate, allowing in-depth bulk equilibrium and kinetics measurements. Similar analyses using comparable clients have only been performed on a few chaperones. One such study on trigger factor, using alpha-lactalbumin as the protein client, stopped short of employing high enough protein concentrations to test whether folding occurs while bound to the chaperone [36]. New single molecule techniques have recently opened up the possibility of measuring this property with other chaperones and clients [37].

Optical tweezers experiments found that trigger factor and DnaK bind to multiple different protein folding states, the prerequisite for folding while bound [38,39]. It has even been proposed that folding while bound could occur on Hsp70, given the large number of dynamic conformations assumed by SH3 while bound [40]. How many chaperones and clients exhibit this mechanism remains to be determined. Even chaperones such as CCT/Tric and Hsp90 feature surface heterogeneity reminiscent of Spy [41,42], and thus could potentially also use a similar mechanism.

## Conclusions

Recent investigations have reopened the possibility that folding while bound to chaperones is a physiologically important mechanism. Spy, GroEL, and SecB provide three such examples, but other chaperones could conceivably use a similar mechanism. This simple mechanism could be evolutionarily ancient, with regulation added later by ATP hydrolysis and cofactors. Based on studies with GroEL, the ability to fold while bound likely depends on the properties of the client as well as the chaperone. With Spy, the properties of the chaperone-client complex that favor this mechanism include favorable electrostatic surfaces to allow flexible binding to folding intermediates and the native state, and hydrophobic surfaces to recognize unfolded substrates.

## Conflicts of interest

None.

## Acknowledgements

This work was supported by the National Institutes of Health (R01-GM102829 to J.C.A.B. and K99-GM120388 to S.H.). J.C.A.B. is a Howard Hughes Medical Investigator.

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