

GroEL provides a folding pathway with lower apparent activation energy compared to spontaneous refolding of human carbonic anhydrase II

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Abstract The kinetics of the refolding of the enzyme, human carbonic anhydrase II (HCA II), at different temperatures, together with the *Escherichia coli* chaperonin GroEL, has been studied. The Arrhenius plots for the spontaneous, GroEL-assisted, and GroEL/ES-assisted refolding of HCA II show that the apparent activation energy (E_a) is lower in the presence of the chaperonin GroEL alone than for the spontaneous reaction, whereas the apparent activation energy for the GroEL/ES-assisted reaction is almost the same as for the spontaneous reaction (85, 46, and 72 kJ/mol, for the spontaneous, GroEL, and GroEL/ES-assisted reactions, respectively).

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Key words: GroEL; Human carbonic anhydrase II; Chaperone; Refolding

1. Introduction

The protein folding problem was discussed already in 1969 by Levinthal [1] in the context of the great number of conformations available to an unfolded protein, and the one or very few that represents the native state. In light of that, the protein folding problem was seen as a seemingly impossible search for the minimum-energy native state among an almost infinite number of conformations. More recently, protein folding has been discussed in the context of a model comprising a 'folding funnel' [2,3]. In this model, the multidimensional energy landscape has the shape of a funnel with increasingly lower energy (higher stability) for conformations that are closer to the native state. The width of the funnel, that represents the entropy, becomes increasingly narrower for conformations closer to the native state. The funnel in the energy landscape provides a mechanism by which a protein can quickly collapse to a small number of conformations, and thereby avoiding the Levinthal paradox [2]. It has been suggested that the sides of the funnel are not smooth, but instead display local energy minima that act as barriers that can be increasingly higher for conformations close to the native state [2]. These energy barriers can be viewed as kinetic traps, where substantial fractions of intermediate conformations can be found. There is some dispute as to whether these intermediates are off-path-

way forms that are permanently misfolded, or if they constitute intermediates on their way to the native state [4].

The concept of a 'folding funnel' in the energy landscape hence provides a model for avoiding the Levinthal paradox, and at the same time the kinetic-trap phenomenon appears. This is a potential problem for all intermediates, including on-pathway forms, because they are not completely folded and may therefore still have unburied hydrophobic patches and consequently be prone to aggregation. In the living cells, the matter of aggregation in the folding of proteins is solved by several classes of proteins known as molecular chaperones [5–7]. One of these is the hsp60 class, which comprises proteins present in prokaryotes as well as eukaryotes [8–10].

Different mechanisms of action of the *E. coli* chaperonin GroEL and its co-chaperonin GroES are discussed today. One mechanism is based on the central cavity in GroEL, which is suggested to give the aided protein the opportunity to fold without interference from other proteins, and thereby avoiding unfavorable protein aggregation [11–13]. Another mechanism proposed [14], implies that GroEL plays a more active role by converting misfolded forms of the substrate protein to more unfolded forms and then let the polypeptide attempt to reach the native state again. Experimental support for this mechanism has been gained by more recent studies [15–19].

A mode of action, in which the folding takes place in contact with the chaperonin, would allow GroEL to possibly lower high-energy barriers between intermediates. Todd et al. [17] have presented evidence for what they call an 'iterative annealing' mechanism, in which GroEL assists proteins out of kinetic traps by unfolding the trapped intermediate. The mode of action of chaperonins in the context of high energy barriers was also recently discussed by Chan and Dill [20]. Based on a theoretical study, these authors argue that the chaperonin may assist in increasing the recovery of the native state by lowering the energy barrier between the intermediate and the native state. Intuitively, this might seem to lead to an increased rate of refolding. However, Chan and Dill show that, in most cases, the overall rate will still be slower due to the on and off rates of the interaction between the chaperonin GroEL and the protein being assisted.

In the present study, we have studied the kinetics of the folding of a C206S mutant of human carbonic anhydrase II (HCA II_{pwt}) in the presence and absence of the *Escherichia coli* chaperonin GroEL and its co-chaperonin GroES. HCA II [21] belongs to the group of proteins, such as barnase [22], maltose-binding protein (MBP) [23] and glucose-6-phosphate dehydrogenase [24] which does not need ATP or ATP/GroES for GroEL to cause an increase in the refolding yield. We have earlier reported that GroEL can rapidly bind to, and inactivate, HCA II_{pwt} at high temperatures [25]. We then concluded that GroEL probably substantially unfolds HCA II_{pwt} in the process

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Abbreviations: HCA II, human carbonic anhydrase II; HCA II_{pwt}, pseudo-wildtype human carbonic anhydrase II; GuHCl, guanidine hydrochloride; ANS, 1-anilino-8-naphthalenesulfonic acid; MBP, maltose binding protein

of binding. Our results in the present study point to an active role for GroEL in the folding process of HCA II_{pwt}. Hence, GroEL does not only unfold this particular protein and leave it to fold spontaneously without contact with GroEL, but a substantial fraction of the folding must take place in contact with the chaperonin, that provides a folding route with a flatter energy landscape than the spontaneous reaction.

2. Material and methods

2.1. Protein preparations

Pseudo wild-type HCA II (HCA II_{pwt}), with the single cysteine residue mutated to a serine (C206S), was expressed in *E. coli*, and prepared by affinity chromatography as described earlier [26]. GroEL was prepared as described earlier [21], with an addition of a step in which GroEL was incubated with GroES and ATP, followed by gel filtration as described by Mitzobata et al. [27]. GroES was prepared from the supernatant of the Polymin P precipitate in the purification scheme of GroEL [28]. GroES was precipitated with (NH₄)₂SO₄, dialyzed and applied to a DEAE-Sephacel column. Thereafter, the GroES fraction was incubated batchwise with Blue Sepharose CL-6B (Pharmacia) in a buffer containing 50 mM Tris-HCl, pH 7.5 over night at 4°C. Several contaminating proteins bind to the dye-affinity column, while GroES does not.

2.2. Denaturation and reactivation of HCA II_{pwt}

HCA II_{pwt} was denatured by incubating for 1 h in 5 M GuHCl in 0.1 M Tris-H₂SO₄, pH 7.5, at room temperature. Reactivation was achieved by dilution to 0.3 M GuHCl and a protein concentration of 0.21, 0.85 or 5.1 μM, respectively; this step was performed by rapidly mixing the denatured enzyme solution (30 μl) into the reactivation-dilution solution (470 μl), already having the desired temperature. The reactivation solution was 0.1 M Tris-H₂SO₄, pH 7.5 and, when indicated, GroEL or GroEL/ES was included, at 2.0-fold molar excesses (as oligomers) over HCA II_{pwt}. When GroEL and GroES were present, the reactivation buffer also contained 10 mM KCl, 10 mM MgSO₄ and 1 mM Mg-ATP. Due to the limited solubility of GroEL, when reactivating at the high protein concentration (5.1 μM), an equimolar concentration (as oligomer) of GroEL over HCA II_{pwt} was used. The reactivation was monitored by the CO₂-hydration assay described elsewhere [29,30].

2.3. Calculations

The calculations were made in TableCurve 2D (Jandel Scientific). The rate constants were calculated from the equation:

$$y = a(1 - e^{-bx}) + c e^{-dx}$$

where a is the maximal value for the potential yield, b is the rate constant for the increasing phase, c is the amplitude for a possible decreasing phase (at temperatures higher than 35°C, HCA II_{pwt} was slowly inactivated with time), d is the rate constant for the decreasing phase. It must be emphasized that the term $c \cdot \exp(-dx)$ only came into play at temperatures higher than 35°C.

3. Results

3.1. Spontaneous reactivation of HCA II_{pwt} at various temperatures

As expected, the rate of the reactivation of the GuHCl-denatured HCA II_{pwt} increased with increasing temperature (Fig. 1). However, there is no marked change in the maximal yield at temperatures between 3–40°C (75–80%) [25]. An Arrhenius plot of the rate constants at temperatures between 5–40°C shows an apparent activation energy (E_a) of 85 kJ/mol (Fig. 2, ○).

3.2. GroEL-assisted reactivation of HCA II_{pwt} at various temperatures

As in the spontaneous reaction, the rate of recovery of the native state increases with increasing temperature (Fig. 3). The

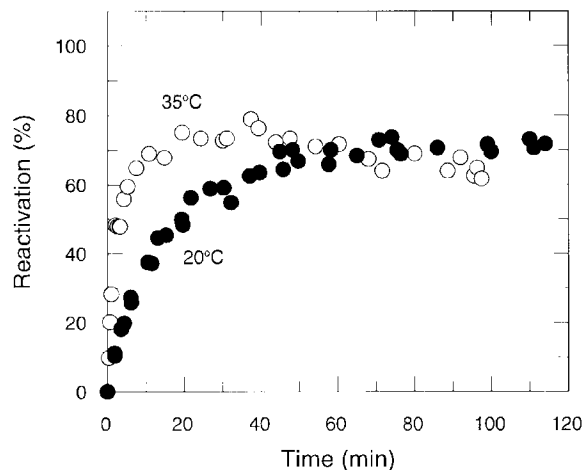


Fig. 1. Spontaneous refolding of HCA II_{pwt} at 20°C (●) and 35°C (○).

Arrhenius plot shown in Fig. 2 (●) indicates an apparent activation energy of 46 kJ/mol for the GroEL-assisted reaction. This value is substantially lower than the corresponding value for the spontaneous reaction. It is noteworthy that the rate of reactivation is, for all temperatures tested, faster in the spontaneous reactivation (Figs. 1 and 3), despite the fact that the apparent activation energy is lower in the presence of GroEL (Fig. 2).

3.3. GroEL/ES-assisted reactivation of HCA II_{pwt} at various temperatures

When GroEL, GroES and ATP is present during the re-folding, the rate, as well as the apparent activation energy are only slightly lower compared to those of the spontaneous reaction (Fig. 2, ▲). Although the apparent activation energy is not much effected by the presence of GroEL/ES during folding, it is evident that the GroEL/ES does interact with the refolding HCA II_{pwt}, since the yield is enhanced compared to that of the spontaneous reaction [21].

3.4. Reactivation at different concentrations of HCA II_{pwt} with and without GroEL

The apparent activation energy is very similar for the re-folding of HCA II_{pwt} at different concentrations of the enzyme, in the absence and presence of GroEL, albeit higher without GroEL. The following apparent activation energies were determined at 0.21 and 0.85 μM HCA II_{pwt}: 40 and 46 kJ/mol, respectively for the GroEL-assisted reaction and 86 and 85 kJ/mol, respectively for the spontaneous reaction (Figs. 2 and 4). The yield is the same at the investigated concentrations of HCA II_{pwt}, 70–75% for the spontaneous reaction, and 90–100% when GroEL is present. Identical yields for the spontaneous reaction at rather different concentrations of protein indicate that the folding intermediates do not have a strong tendency to aggregate. In this respect it is of interest to note that the C206S mutant used in this study differs from the wild-type HCA II_{pwt}. The yield of the latter has earlier been noted to decrease at increasing concentrations of refolding protein [31]. The rate of reactivation, for the spontaneous and GroEL-assisted reaction, decreases with increasing protein concentration (Figs. 5 and 6, Table 1).

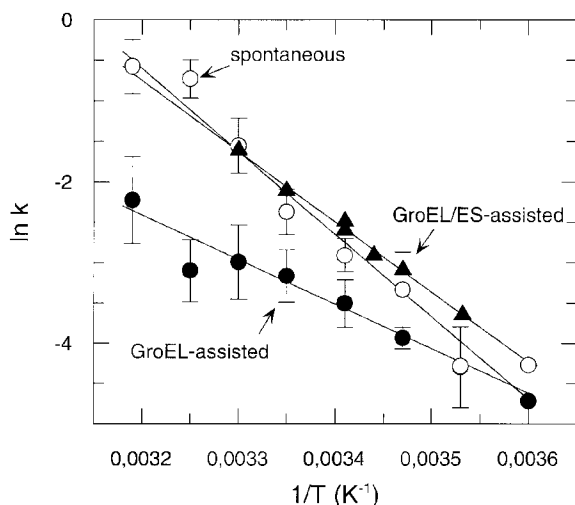


Fig. 2. Arrhenius plot. Rate constants from spontaneous (○), GroEL-assisted (●) and GroEL/ES-assisted (▲) refolding of HCA II_{pwt} at 0.85 μM.

4. Discussion

Several mechanisms have been suggested for the action of molecular chaperonins. According to the Anfinsen's cage model [11–13], the chaperoned protein is hindered to aggregate by folding without contact with potential aggregation-prone intermediates. The chaperonins have also been suggested to unfold misfolded proteins, and hence play a more active role in the folding [14]. Evidences for a more active role of the chaperonin have been gathered from different experimental studies and can explain the folding assistance that occurs when misfolding, rather than aggregation, is the cause of a low yield in the spontaneous reaction. By unfolding misfolded intermediates, and thereby letting these partition once, or if need be, several times, between the native and misfolded states, the chaperonin can help the protein to reach the native state at a high yield [15–19,32].

Recently Chan and Dill suggested that chaperonins ought to lower the kinetic barriers between intermediate states [20]. This is an appealing mechanism, not only because it predicts that the chaperonin unfolds misfolded intermediates, and lets them repartition, but also because it allows the assisted protein to take the most efficient route to the native state.

We have studied the refolding of HCA II_{pwt}, and mutants thereof, for several years [31,33]. Interestingly, when comparing the spontaneous refolding reactions at 0.21, 0.85, and 5.1 μM HCA II_{pwt}, the yield is 70% over the range of protein concentrations (Fig. 5, Table 1). Hence, HCA II_{pwt} has a low tendency to aggregate during the refolding conditions used,

Table 1
Comparison of rate constants, when reactivating, at different concentrations of HCA II_{pwt}, with and without GroEL

[HCA II _{pwt}] (M)	Spontaneous (min ⁻¹)	GroEL-assisted (min ⁻¹) ^a
0.21	0.132	0.041
0.85	0.099	0.029
5.1	0.048	0.010

^a0.43 μM GroEL was used at 0.21 μM HCA II_{pwt}, 1.7 μM of GroEL was used at 0.85 μM HCA II_{pwt} and 5.1 μM of GroEL at 5.1 μM HCA II_{pwt}.

and the 30% that does not reactivate must partition to inactive misfolded state(s).

Although HCA II_{pwt} shows a low tendency to aggregate during refolding from GuHCl, it is still efficiently chaperoned by GroEL. Hence, it seems unlikely that GroEL only provides a cavity in which HCA II_{pwt} is allowed to refold without contact with other proteins. One noteworthy feature of the interaction between this protein and GroEL is the fact that HCA II_{pwt} is efficiently chaperoned by GroEL even in the absence of GroES and ATP. The rate of the GroEL assisted refolding of HCA II_{pwt} is slower than that of the spontaneous reaction. The same yield as in the GroEL-assisted reaction is obtained by the complete GroEL/ES system, but with an increase in the overall rate to close to that of the spontaneous reaction [21]. The GroEL-assisted reactivation of HCA II_{pwt} is sufficiently rapid to facilitate studies of the reaction without the addition of ATP or GroES. Hence, the interaction between the chaperonin and HCA II_{pwt} can be investigated without the complications of simultaneous conformational changes due to ATP hydrolysis or GroES binding.

We have previously found that there is an equilibrium between inactive (probably GroEL-bound) and active HCA II_{pwt} at temperatures above 35°C [25]. The same equilibrium was reached whether denatured HCA II_{pwt} was refolded from GuHCl in the presence of GroEL, or native HCA II_{pwt} was incubated with GroEL. At 50°C HCA II_{pwt} was completely inactivated if GroEL was present, but virtually all enzymatic activity was recovered when the temperature was lowered. Interestingly, when native HCA II_{pwt} was incubated at elevated temperatures, the presence of GroEL caused a more rapid inactivation of HCA II_{pwt}. From these results, we concluded [25] that the mode of action of GroEL when assisting the refolding of HCA II_{pwt}, is to unfold intermediates, and to let them refold spontaneously in solution, as has been suggested in other cases [14]. Hence, GroEL seems not only to provide sequestered refolding of HCA II_{pwt}, but to interact with the refolding HCA II_{pwt}. To further analyze the involvement of the chaperonin in the refolding reaction of HCA II_{pwt}, we have investigated the kinetics of the refolding reaction at different temperatures, and concentrations of proteins.

Although the yield of spontaneously refolded HCA II_{pwt} is 70% over a rather broad range of protein concentrations, the

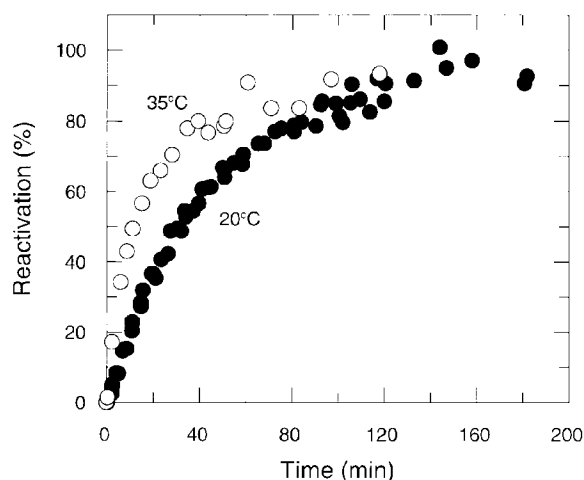


Fig. 3. GroEL-assisted refolding of HCA II_{pwt} at 20°C (●) and 35°C (○).

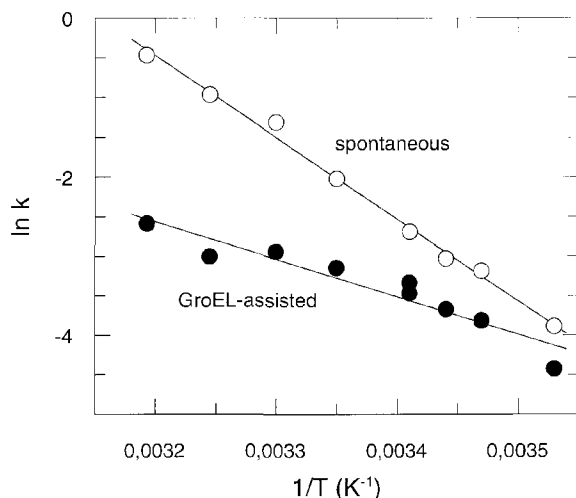


Fig. 4. Arrhenius plot. Rate constants from spontaneous (○) and GroEL-assisted (●) refolding of HCA II_{pwt} at 0.21 μM.

rate of reactivation decreases with increasing protein concentration by a factor of 2.8 when comparing the reactivations at 0.21 and 5.1 μM (Fig. 5, Table 1). Evidently, folding intermediates interact reversibly to slow down the folding process ($I_1 \leftrightarrow (I_1)_n$; Fig. 7), without resulting in off-pathway irreversible aggregation reactions. This indicates that the spontaneously refolding HCA II_{pwt} does not refold in a unimolecular reaction at the investigated concentrations, but that even the spontaneous reaction is quite complex.

If GroEL is present during the refolding reaction, the rate of reactivation is also concentration dependent, and the rate at the highest protein concentration (5.1 μM GroEL, and 5.1 μM HCA II) is 4.1 times slower than the rate at the lowest concentrations (0.21 μM HCA II, and 0.42 μM GroEL) (Fig. 6, Table 1).

The observed lower rate, in the presence of GroEL than in the absence, can be explained by the chaperonin interacting with folding intermediates, and temporarily withholding these from reaching the native state (Fig. 7). In the indicated mechanism, the HCA II_{pwt} undergoing spontaneous refolding is suggested to partition between the native state (*N*), and a misfolded, inactive state (*I_{mis}*). The nature of this misfolded state is not known, but is unlikely to be an aggregated form since the yield of refolding is the same over a rather broad range of protein concentrations. In

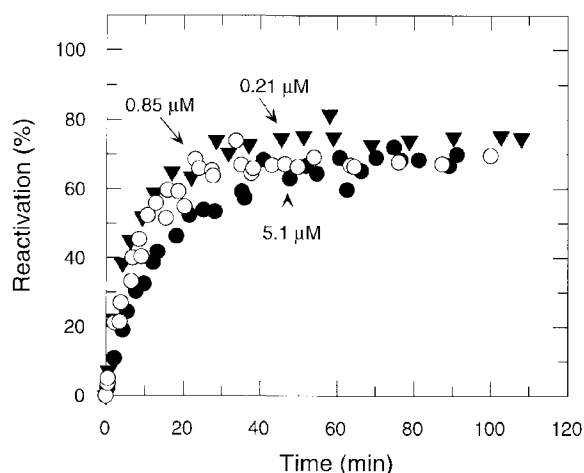


Fig. 5. Comparison between spontaneous refolding of HCA II_{pwt} at 25°C at 0.21 μM (▼), 0.85 μM (○) and 5.1 μM (●).

the presence of GroEL, a fraction of *I₁* binds to GroEL. This interaction with GroEL will prevent the misfolded state (*I_{mis}*) to form. At the same time, it will also lower the concentrations of intermediates, so that the rate of reactivation is lowered compared to the spontaneous refolding. Such a mechanism is consistent with the previously found heat inactivation of HCA II_{pwt} in the presence of GroEL; binding of *I₁* to GroEL at elevated temperatures will shift the equilibrium away from the native state. The suggested mechanism predicts a concentration-dependent rate of refolding. At higher concentrations of refolding HCA II_{pwt}, and GroEL, the equilibrium between *I₁* and GroEL-*I₁* is shifted towards the latter, and a larger fraction of *I₁* should be bound to GroEL. The faster rate of refolding in the presence of the complete chaperonin system (GroEL/ES+ATP), indicates a weaker interaction with the chaperonins, and thus leads to a higher refolding rate. There is ample evidence in the literature that the hydrophobic interaction between the refolding protein and the chaperonin is probably stronger when GroEL alone is present, compared to when the ATP hydrolysis-driven cycling between conformational states of the chaperonin is possible, which is the case in the presence of GroEL/ES and ATP [23,34,35]. Sparrer et al. have shown that a mutant of maltose-binding protein (MBP) is completely arrested by GroEL, whereas the wild-type reversibly can fold from GroEL without GroES and ATP [23]. ANS experiments showed that the amplitude of the decrease of ANS-fluorescence during refolding was three times higher for the mutant compared with wild-type MBP. The importance of hydrophobic interaction for the interaction with the chaperonin was further substantiated by the fact that the refolding of MBP could be arrested at high concentrations of salt.

Most interestingly, when the apparent activation energy for the refolding of HCA II_{pwt} is determined from an Arrhenius plot, it is evident that the apparent activation energy is substantially lower in the presence of GroEL than for the spontaneous reaction (Figs. 2 and 4). The apparent activation energy is, of course, the measure of the highest energy barrier of the complete reaction from the unfolded to the native state. Evidently, the chaperonin does interact with the refolding protein, and provides a route from the unfolded to the native state that has lower energy barriers than the spontaneous reaction. This can be envisioned as a less rough folding funnel, with fewer possibilities

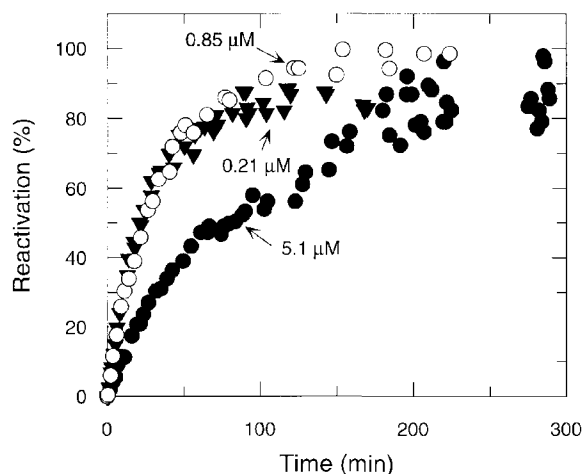


Fig. 6. Comparison between GroEL-assisted refolding of HCA II_{pwt} at 25°C at 0.21 μM (▼), 0.85 μM (○) and 5.1 μM (●). GroEL concentrations used were 0.43 μM, 1.7 μM and 5.1 μM, respectively.

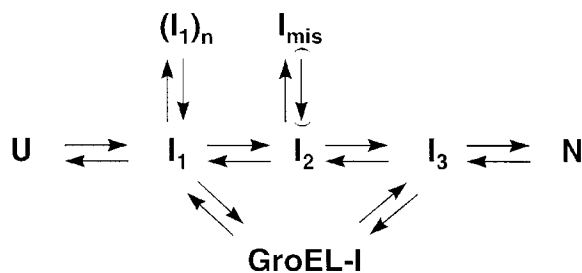


Fig. 7. Tentative mechanism for the refolding reaction of HCA II_{pwt} , with and without GroEL. I_{1-3} are different folding intermediates, I_{mis} is a misfolded state and $(I_1)_n$ is an aggregated state slowing down the refolding process.

for kinetic traps. Evidently, GroEL does not only bind an intermediate of the refolding HCA II_{pwt} , but the chaperonin influences the refolding reaction.

To address if the bimolecular binding step, when HCA II_{pwt} binds to GroEL, influences the apparent activation energy, the reactivations at different temperatures at two different concentrations of HCA II_{pwt} and chaperonin were measured. The apparent activation energy at a four-fold lower concentration of proteins is very similar (40 compared to 46 kJ/mol) (Figs. 2 and 4, ●). The bimolecular encounter is 16 times slower at this lower concentration, and one can conclude that the apparent activation energy is not influenced by the rate of association of HCA II_{pwt} to the chaperonin. Hence, the lowering in apparent activation energy is intrinsic to the interaction with the chaperonin, and is not changed by faster binding at higher concentrations of proteins.

When GroES and ATP is present, the apparent activation energy is only slightly lower than that of the spontaneous reaction (Fig. 2, ▲). Although the GroEL/ES complex does interact with HCA II_{pwt} to increase the yield, the rate of the reaction is very similar to that of the spontaneous process. This indicates rather a weak interaction between HCA II_{pwt} and GroEL/ES compared to the interaction with GroEL alone. A weak interaction will probably lead to a lesser influence on folding intermediates and therefore also on the complete folding pathway, i.e. the GroEL/ES complex does not appreciably change the apparent activation energy compared to that of the spontaneous reaction.

In spite of the astronomical number of potential pathways from the almost infinite number of denatured states to the single, or possible ensemble of native states, we have noted that the rate of reactivation exhibits a linear Arrhenius dependence. This indicates a substantial influence of GroEL on the folding pathway of HCA II_{pwt} . Since HCA II_{pwt} does not show a strong tendency to aggregate at varying protein concentrations, the chaperonin must provide assistance in a different manner than preventing aggregation in this case. Evidently, the chaperonin provides a pathway with a flatter energy landscape compared to the spontaneous reaction, thereby preventing off-pathway, dead-end reactions.

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