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# Chaperone Activity of Cyp18 through Hydrophobic Condensation That Enables Rescue of Transient Misfolded Molten Globule Intermediates†

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ABSTRACT: The single-domain cyclophilin 18 (Cyp18) has long been known to function as a peptidyl-prolyl *cis*/*trans* isomerase (PPI) and was proposed by us to also function as a chaperone [Freskgård, P.-O., Bergenhem, N., Jonsson, B.-H., Svensson, M., and Carlsson, U. (1992) *Science 258*, 466−468]. Later several multidomain PPIs were demonstrated to work as both a peptidyl-prolyl *cis*/*trans* isomerase and a chaperone. However, the chaperone ability of Cyp18 has been debated. In this work, we add additional results that show that Cyp18 can both accelerate the rate of refolding and increase the yield of native protein during the folding reaction, i.e., function as both a folding catalyst and a chaperone. Refolding experiments were performed using severely destabilized mutants of human carbonic anhydrase II under conditions where the unfolding reaction is significant and a larger fraction of a more destabilized variant populates molten globule-like intermediates during refolding. A correlation of native state protein stability of the substrate protein versus Cyp18 chaperone activity was demonstrated. The induced correction of misfolded conformations by Cyp18 likely functions through rescue from misfolding of transient molten globule intermediates. ANS binding data suggest that the interaction by Cyp18 leads to an early stage condensation of accessible hydrophobic portions of the misfolding-prone protein substrate during folding. The opposite effect was observed for GroEL known as an unfoldase at early stages of refolding. The chaperone effect of Cyp18 was also demonstrated for citrate synthase, suggesting a general chaperone effect of this PPI.

Although protein folding is a spontaneous process, many proteins in vivo appear to require assistance by two kinds of protein factors: foldases such as peptidyl-prolyl *cis*/*trans* isomerase (PPI)[1] and protein disulfide isomerase (PDI) as well as molecular chaperones. PPIs act by accelerating the *cis*−*trans* isomerization of X−Pro peptide bonds (*1*, *2*), and PDI catalyzes the formation and exchange of disulfide bonds (*3*, *4*). A chaperone, on the other hand, binds to unfolded states and prevents the polypeptide chain from making illicit associations with other unfolded proteins or being trapped in misfolded conformations.

In 1989, the original 18 kDa PPI was found to be identical to cyclophilin (Cyp), a high-affinity receptor for the immunosuppressive drug cyclosporin A (CsA) (*5*−*7*). Subsequently, several forms of Cyps were discovered. Moreover, two additional families of PPIs have been found: the FK506-binding proteins (FKBPs) and the parvulins. Now a large number of PPIs have been found to be present in all organisms and in all subcellular compartments and participate not only in de novo protein folding but also in a variety of cellular processes (*1*).

In 1992, we presented data indicating for the first time that a PPI (the 18 kDa PPI, Cyp18) could, in addition to its peptidyl-prolyl *cis*/*trans* isomerase activity, act as a chaperone (*8*). However, there has been a controversial discussion about whether this PPI can also exhibit chaperone activity (*9*, *10*), and it has long been considered that the function of small single-domain PPIs is restricted to peptidyl-prolyl *cis*/*trans* isomerase activity.

However, it was later well established that large multidomain PPIs can also exert chaperone function. This has been shown for FKBP51, FKBP52, and Cyp40, which all by themselves exhibit chaperone activity in vitro, and in addition, they are important components of the Hsp90 chaperone complex (*11*−*13*).

Furthermore, the periplasmic *Escherichia coli* PPIs FkpA (FKBP-family) and SurA have been shown to possess chaperone activity independent of their isomerase activity (*14*, *15*). The *E. coli* trigger factor, a PPI of the FKBP family, can also act as a chaperone (*16*). In plants, the dual PPI and chaperone functions have been found for wFKBP73 (wheat) (*17*) and in *Archea* for the two-domain PhFKBP29 (*18*).

In addition, the archeal single-domain PPIs TcFKBP18 (*19*) and MTFK (homologous to FKBP16) (*20*) have been shown to exhibit chaperone activity. Another PPI with chaperone and isomerase activity in the same domain is LdCyP (from *Leishmania donovani*) (*21*). Recently, this was also demonstrated for the FKBP-like N-terminal domain of SlyD from different bacterial

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[1]Abbreviations: CsA, cyclosporin A; Cyp18, 18 kDa cyclophilin; HCA $II_{pwt}$, pseudo-wild-type human carbonic anhydrase II with a Cys206 → Ser mutation; H107F, HCA $II_{pwt}$ with a His107 → Phe mutation; H107N, HCA $II_{pwt}$ with a His107 → Asn mutation; PPI, peptidyl-prolyl *cis*/*trans* isomerase.


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species (*22*). Thus, single-domain PPIs are also capable of acting as chaperones for their protein substrates.

Previously, we have presented data showing that Cyp18 can act as a chaperone by using human carbonic anhydrase II (HCA II) as a protein substrate (*8*). The noted increase in refolding yield of denatured HCA II has, however, been questioned (*9*, *10*), despite several examples of PPIs (for the most part multidomain PPIs) that later were shown to possess this activity as presented above. To determine whether the single-domain PPI Cyp18 also can act as a chaperone, we have repeated our experiments on Cyp18-assisted refolding of HCA II, this time using human recombinant Cyp18 instead of porcine kidney PPI. We also used HCA II mutants in position 107 linked to disease as substrates that are more prone to misfold than the wild type in order to be able to register larger chaperone effects.

Moreover, the fluorescent hydrophobic probe ANS was used to monitor the modulation of molten globule intermediates formed in the presence of Cyp18 during early stages of folding. Another protein substrate was also included in this study, namely citrate synthase that has been shown to need chaperone assistance for correct folding (*23*, *24*).

Altogether, Cyp18 was demonstrated to possess both peptidyl-prolyl *cis*/*trans* isomerase and chaperone activity.

## MATERIALS AND METHODS

*Chemicals*. Citrate synthase (porcine heart), acetyl coenzyme A, dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid), oxaloacetate, ANS, and cyclosporin A were purchased from Sigma. Ultra pure guanidine hydrochloride was obtained from ICN Biomedicals.

*Cloning, Production, and Purification of Cyp18*. The human cyclophilin A (Cyp18) gene was isolated from a human lymphocyte cDNA library (CD4 positive Jurkat T) in cloning vector lambda gt11 from Clontech and cloned into expression vector pACA (*25*). The expression of Cyp18 in *E. coli* and purification followed the procedure described by Zydowsky et al. (*26*) with some modifications. Thus, Dnase I (a spatula tip) treatment of the cell solution was performed prior to the protamine sulfate precipitation of nucleic acids. The buffers in the chromatographic steps were complemented with 0.5 mM DTT. In the final gel filtration step, Sephadex G-50 Fine was used instead of Sephadex G-75.

The purity of Cyp18 was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Twenty-one micrograms of Cyp18 was applied and stained with Coomassie Brilliant Blue R-250. No significant impurity bands could thereby be detected.

One known prevalent PPI of *E. coli*, Sly D, could directly be ruled out as a contaminant, because the PPI activity of Sly D cannot be inhibited by CsA (*22*), and the PPI activity was completely abolished by CsA in our preparations.

Since *E. coli* also has a carbonic anhydrase enzyme (*27*), any contaminating carbonic anhydrase activity in the Cyp18 preparation was measured in the $CO_2$ hydration activity assay for a Cyp18 concentration used in the reactivation experiments (8.3 $\mu$M). This control did not exhibit any $CO_2$ hydration activity.

*Site-Directed Mutagenesis, Expression, and Purification of HCA II Variants*. To prevent formation of unwanted intermolecular disulfide bridges, a pseudo-wild-type variant of HCA II (HCA $II_{pwt}$) containing the C206S mutation was used in all measurements and as a template for engineering of the HCA II mutants. This pseudo-wild-type form of the enzyme has previously been shown to have properties that are indistinguishable from those of wild-type HCA II (*28*, *29*). Mutagenesis, expression, and purification of the HCA II variants were performed as described previously (*30*).

Although HCA II was purified by a selective affinity chromatography method (with the specific carbonic anhydrase inhibitor *p*-aminomethylbenzene sulfonamide coupled to CM-agarose), the presence of proline isomerase activity in the prepared HCA $II_{pwt}$ was controlled. A concentration of HCA II equivalent to that used in the reactivation experiments (0.83 $\mu$M) was used in the proline isomerase activity assay with the chromogenic peptide substrate succinyl-Ala-Leu-Pro-Phe-4-nitroanilide as described by Scholtz et al. (*31*). No proline isomerase activity could be detected here.

*Reactivation of Denatured HCA II Variants with and without Cyp18*. The HCA II variants were denatured in 4 M GuHCl for 1 h. Reactivation was achieved by dilution of the denaturant to different concentrations depending on the stability of the HCA II variant, whereas the enzyme concentration during refolding was the same in all cases (0.83 $\mu$M). Reactivation of HCA $II_{pwt}$ was performed by dilution to 0.3 M GuHCl at 21 °C, a GuHCl concentration that has previously been found to be optimal for reactivation of denatured HCA II (*32*). The reactivations of the destabilized H107N and H107F variants were achieved by dilution to 0.1 M GuHCl. For H107N, this denaturant concentration is below that causing partial denaturation according to the stability curve, midpoint concentration of GuHCl denaturation [$C_m$ = 0.41 M (*33*)]. Since H107F is less stable ($C_m$ = 0.08 M), 80% of the native state at equilibrium becomes denatured at 0.1 M GuHCl. Nevertheless, the sample was diluted to 0.1 M GuHCl to yield a reasonable dilution of the denatured H107F enzyme.

As a comparison, the H107N variant was also reactivated during a denaturing condition that like H107F gave rise to 80% inactivation. To determine the GuHCl concentration that leads to this degree of inactivation, H107N was incubated at different GuHCl concentrations [buffered with 0.1 M Tris-$H_2SO_4$ (pH 7.5)] for 24 h. From these stability measurements, we observed that 0.28 M GuHCl led to an 80% loss of enzyme activity. Therefore, renaturation of H107N was also conducted at 0.28 M GuHCl.

The measurements of the regain in $CO_2$ hydration activity during refolding were performed as described previously (*34*). The concentration of the HCA II variant during refolding was 0.83 $\mu$M, and the Cyp18 concentration was 8.3 $\mu$M. Cyclosporin A (CsA) was added to a concentration of 25 $\mu$M. This concentration of CsA did not have any effect on the $CO_2$ hydration activity of HCA II. All solutions were buffered with 0.1 M Tris-$H_2SO_4$ buffer (pH 7.5). The double-exponential zero-intercept equation $y = A_1[1 - \exp(-k_1 t)] + A_2[1 - \exp(-k_2 t)]$ was fitted to the data (TableCurve, Jandel Scientific), where $y$ is the refolding yield in percent, $t$ denotes the time in minutes, $A_1$ and $A_2$ denote the amplitudes of the first and second phases, respectively, and $k_1$ and $k_2$ are the rate constants for the first and second phases, respectively.

*Refolding of Citrate Synthase*. Refolding was conducted essentially as described previously (*35*). Briefly, citrate synthase (CS, 10 $\mu$M) was denatured in 6.0 M GuHCl in 0.1 M Tris-HCl buffer (pH 8.1) and 20 mM DTT for at least 1 h at room temperature. Refolding was conducted in a glass test tube, where the denatured CS was diluted 1:100 to 0.1 $\mu$M in the refolding buffer [0.1 M Tris-HCl (pH 8.1)] by being vigorously mixed for

30−60 s. Refolded samples were incubated at room temperature until the completion of the reaction. The Cyp18 concentration was 1.0 $\mu$M when present. The CS activity was measured to follow the formation of native CS molecules at room temperature. The reaction mixture contained 0.1 M Tris-HCl, 0.1 mM DTNB, 0.047 mM acetyl coenzyme A, and 0.23 mM oxaloacetate (*36*). The activity was registered as the difference in absorption at 412 nm ($\Delta A_{412}$ per second). The activity of the native CS, treated in the same way as the refolded sample but without denaturation, was taken to be 100%, for calculation of the relative refolding yields.

*ANS Binding Assessed by Stopped-Flow Measurements.* The time course of binding of ANS to the protein upon binding was measured with an SX.18MV-R stopped-flow reaction analyzer (Applied Photophysics) at 21 °C. Excitation was performed at 360 nm, with a bandwidth of 5 nm in a 20 $\mu$L cell. The emission was collected through the cell fluorometric port, via an optical filter with a cutoff at wavelengths of $<$455 nm. Prior to the measurements, the photomultiplier tube voltage and offset were adjusted using folded HCA II (0.85 $\mu$M) in 0.3 M GuHCl to set the signal to zero mixed with (8.5 $\mu$M) ANS in the cell. The HCA II variants (9.35 $\mu$M) were denatured for 2 h at 21 °C with 3.3 M GuHCl. To study the molten globule formation in the early events of refolding, we performed a 1:10 dilution of the denatured protein to a final HCA II concentration of 0.83 $\mu$M and 0.3 M GuHCl with a 0.1 M Tris-$H_2SO_4$ buffer (pH 7.5) containing ANS and Cyp18 (final concentrations of both were 8.3 $\mu$M). The binding of ANS to the folding protein was monitored for 100 ms, and the data reported for the kinetics of the interaction between the folding protein and ANS originated from the first data point. The dead time between the mixing of protein and ANS and the collection of the first data point with this setup was 2 ms. For each protein sample, seven consecutive runs were made, of which the first two were discarded because they often showed signs of tube diffusion; the remaining five runs were averaged. We recorded data by monitoring the fluorescence signal in real time from the end of the stopped-flow push. All data were collected with the drive pressure held constant through 2 s measurements.

## RESULTS

In this study, we aimed to investigate the debated chaperone activity of Cyp18. Herein, we chose to use the recombinant human Cyp18 to obtain a homogeneous preparation and misfolding-prone HCA II mutants as substrate proteins to maximize the chaperone effects (see below). In Figure 1, the time courses of the regain in the $CO_2$ hydration activity of denatured HCA $II_{pwt}$, H107N, and H107F are shown. HCA $II_{pwt}$ was as previously refolded at 0.3 M GuHCl, which has been demonstrated to lead to optimal reactivation of HCA II (*32*). H107N and H107F were, however, refolded at lower GuHCl concentrations (0.1 M) because of the lower stability of these variants (*33*). At this denaturant concentration, the native state of H107N is stable, whereas we found that 80% of H107F becomes inactivated in 0.1 M GuHCl. The GuHCl concentration was kept at 0.1 M also for H107F to prevent too much dilution of the enzyme in the refolding step (for a more detailed background, see Materials and Methods).

As demonstrated earlier, the presence of Cyp18 accelerated the reactivation process for HCA $II_{pwt}$, as well as for the two HCA II variants (Figure 1 and Table 1). Hence, our system appears to be suitable for reassessment of the debated chaperone activity by

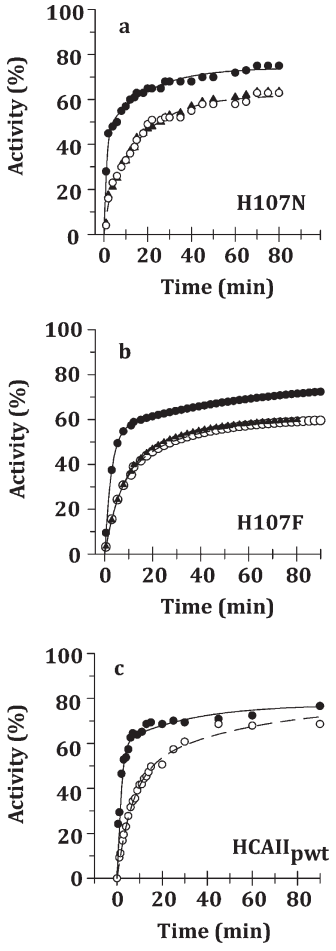


FIGURE 1: Time courses for reactivation of unfolded HCA II variants in the presence and absence of Cyp18: (a) H107N, (b) H107F, and (c) HCA $II_{pwt}$. The assisted and unassisted reactivation curves were recorded in parallel to eliminate effects caused by the varying conditions of the protein samples. The kinetic data were best fitted to two exponential terms: (○) without Cyp18, (●) with Cyp18, and (▲) with CsA. Activity for each enzyme variant was compared to the reference enzyme incubated at the same final concentration of GuHCl.

Table 1: Kinetic Data for the Reactivation of HCA II Variants with and without the Assistance of Cyp18[a]

| protein | $k_1$[b] (min$^{-1}$) | $A_1$[b] | $k_2$[b] (min$^{-1}$) | $A_2$[b] | $t_{1/2,total}$[c] (min) |
|---|---|---|---|---|---|
| HCA $II_{pwt}$ | 0.15 | 0.45 | 0.02 | 0.32 | 8.8 |
| HCAII$_{pwt}$ and Cyp18 | 0.71 | 0.60 | 0.03 | 0.18 | 1.5 |
| HCAII$_{pwt}$, Cyp18, and CsA | 0.14 | 0.34 | 0.03 | 0.33 | 9.9 |
| H107N | 0.44 | 0.15 | 0.05 | 0.47 | 8.3 |
| H107N and Cyp18 | 0.98 | 0.45 | 0.05 | 0.29 | 1.4 |
| H107N, Cyp18, and CsA | 0.51 | 0.14 | 0.05 | 0.51 | 9.3 |
| H107F | 0.17 | 0.32 | 0.04 | 0.29 | 7.8 |
| H107F and Cyp18 | 0.37 | 0.55 | 0.02 | 0.22 | 2.6 |
| H107F, Cyp18, and CsA | 0.15 | 0.35 | 0.04 | 0.26 | 7.8 |

[a]The kinetic parameters were obtained from one series of experiments run at one occasion for each HCA II variant to eliminate the effects caused by the varying conditions of the protein samples. [b]The rate constants and amplitudes were calculated using a nonlinear fit program (see Materials and Methods). [c]The half-time of the total reactivation process.

Cyp18, i.e., the effect of Cyp18 on the reactivation yield of the denatured HCA II variants.

In our original study of the PPI-mediated recovery of the $CO_2$ hydration activity of unfolded HCA $II_{wt}$, we obtained an increase (30%) in the yield of the active enzyme after it had refolded for

Table 2: Reactivation Yields after 24 h with and without the Assistance of Cyp18[a]

| protein | unassisted (%)[b] | with Cyp18 and CsA (%)[b] | with Cyp18 (%)[b] | chaperone effect (%)[c] |
|---|---|---|---|---|
| H107N | 63.0 ± 0.9[d] | 64 ± 3[d] | 74 ± 3[d] | 11 ± 2 |
| H107N | 52 ± 2[e] | not determined | 69 ± 3[e] | 18 ± 3 |
| H107F | 61 ± 3[f] | 63 ± 1[g] | 79 ± 2[f] | 18 ± 3 |
| HCA $II_{pwt}$ | 69 ± 1[h] | 69 ± 1[i] | 76 ± 1[h] | 7 ± 2 |
| citrate synthase | 36 ± 3[j] | 32[k] | 46 ± 3[j] | 10 ± 5 |

[a]These values were obtained from one series of experiments run at one occasion when the assisted and unassisted reactivation yields were determined in parallel to eliminate the effects caused by the varying conditions of the protein samples. The reactivation yield for each enzyme variant was compared to the reference enzyme incubated at the same final concentration of GuHCl. [b]The standard errors of the mean are also given. [c]The calculated pooled standard deviations are also given. [d]Reactivation in 0.1 M GuHCl ($n$ = 6). [e]Reactivation in 0.28 M GuHCl ($n$ = 5). [f]Reactivation in 0.1 M GuHCl ($n$ = 6). [g]Reactivation in 0.1 M GuHCl ($n$ = 3). [h]Reactivation in 0.3 M GuHCl ($n$ = 5). [i]Reactivation in 0.3 M GuHCl ($n$ = 3). [j]Reactivation in 0.06 M GuHCl ($n$ = 3). [k]From one experiment. In parallel, the unassisted reactivation gave a yield of 33%.

1 h compared to that obtained in the uncatalyzed reaction (*8*). At that time, a complete recovery of the PPI-assisted reactivation was observed, whereas now 76% was recovered (Table 2); however, it is important to note that in the previous study we used PPI that was prepared from porcine kidney whereas human recombinant Cyp18 (PPI) was used in this study to obtain a more homogeneous protein and in addition allowed us to engineer an active site mutant, $Cyp18_{R55A}$, to determine the effects on chaperone function.

The different forms, as well as the different sources from which PPI is isolated, might explain the different absolute yields that were obtained. During the chromatographic purification of porcine PPI, we observed several fractions with PPI activity, and all fractions containing PPI activity also exhibited chaperone activity. Additionally, PPIs tested with molecular masses of 17 and 22 kDa exhibited the same chaperone activities (*8*). Despite extensive chromatographic separation steps, the possibility that PPIs other than Cyp18 contributed to the chaperone activity noted in the previous work cannot be excluded.

Furthermore, the condition of the protein substrate is also of importance, since the denaturation−renaturation process for an initially partially denatured protein can give rise to apparently higher yields. Therefore, it is always important not to compare the refolding yield with an absolute final value; instead, comparisons of yields should be done from refolding reactions run in parallel in the presence and absence of Cyp18 on the same protein substrate preparation, to be able to eliminate such side effects. Therefore, the refolding experiments in this work were performed at the same time on freshly prepared HCA II variants or citrate synthase with and without human recombinant Cyp18 to accurately obtain the extent of the increase in the yield of the active enzyme.

When Kern et al. (*9*) used a spectrophotometric assay of the esterase activity to monitor the reactivation process, they found a slow kinetic phase in the spontaneous reactivation of the enzyme, showing that the uncatalyzed reaction is not complete after 1 h. According to their measurements, the same final yields were obtained after reactivation for 4 h in the presence and absence of Cyp18. Here we reexamined the reactivation of unfolded HCA II with and without Cyp18 and compared the yields after prolonged (24 h) reaction times to ensure completion of the reaction.

The same analysis was also performed on two destabilized HCA II variants, H107N and H107F, that are more prone to misfolding than the wild-type protein (*33*). After reactivation of HCA $II_{pwt}$ for 24 h, we still observed a small increase in $CO_2$ hydration activity (≈7%) when the reaction was assisted by human Cyp18 (Table 2). Interestingly, for the two other destabilized variants, there was a larger increase in the yield of the reactivated enzyme in the Cyp18-mediated refolding process. The recovery of the $CO_2$ activity was on average 11% higher for H107N and 18% higher for H107F than that without Cyp18 (Table 2). It can also be pointed out that the $CO_2$ hydration activity for H107N and H107F during uncatalyzed reactivation remains constant from 4 to 24 h.

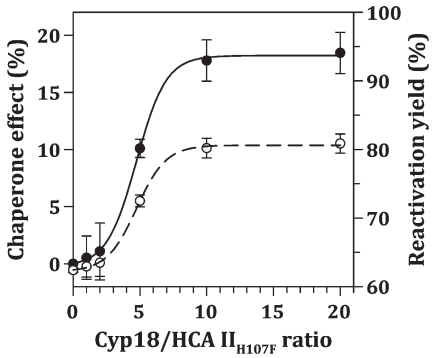


FIGURE 2: Reactivation yield of H107F as a function of Cyp18 concentration. The final yield of refolding of H107F (0.83 $\mu$M) was recorded after 24 h in the presence of Cyp18 in 0−20-fold molar excesses over H107C. The data are presented as the chaperone effect (●), i.e., the increase in yield in the presence of Cyp18, and as the total yield of the refolded enzyme (○). The data fit well to a sigmoidal function ($r$ = 0.999). The standard deviations were calculated as a function of the mean of triplicate samples for each condition.

As in the previously published work, a 10-fold molar excess of Cyp18 over HCA II was used in these reactivation experiments (*8*). For the HCA II variant (H107F) for which the reactivation yield by Cyp18 was most efficiently increased, we also showed the dependence of the concentration of Cyp18 on the obtained reactivation yield (Figure 2).

From this titration curve, one can see that a 10-fold molar excess of Cyp18 (8.3 $\mu$M) appears to give rise to a maximal increase in the reactivation yield. Interestingly, the reactivation data were very well fitted to a sigmoidal function, indicating a cooperative process of reactivation as a function of Cyp18 concentration.

Previously, various effects by the Cyp18 inhibitor CsA on the Cyp18-mediated reactivation of HCA II were investigated. The presence of CsA in the refolding buffer abolished the Cyp18-mediated accelerated reactivation of HCA II. In this study, the same observation was made for H107N and H107F (Figure 1a,b and Table 1). When the Cyp inhibitor CsA was included, not only the acceleration of the Cyp18-mediated reactivation process but also the increase in yield of the reactivated enzyme was offset (Table 2).

The H107F mutant is considerably destabilized, and from stability measurements performed in GuHCl at 21 °C, we

observed that at 0.1 M GuHCl, the concentration to which the denaturant was diluted when refolding was initiated, already 80% of the native state had become inactivated at equilibrium (after incubation for 24 h); i.e., a significant population of the mutant was unfolded to the molten globule light state, which easily becomes trapped in a misfolded state (*37*). Therefore, it was interesting to see how the H107N variant behaves during refolding under a similar denaturing pressure, i.e., at a GuHCl concentration that at equilibrium inactivates the enzyme to 80%. Incubation at various concentrations of GuHCl [in 0.1 M Tris-$H_2SO_4$ buffer (pH 7.5)] showed that 0.28 M GuHCl was required to reach this degree of inactivation. By performing the reactivation of H107N at 0.28 M GuHCl, we observed that the Cyp18 increased the yield of the active enzyme to a significantly greater degree than at 0.1 M GuHCl (18% compared to 11%), and thus, Cyp18 under these conditions could invoke the same refolding efficiency as for H107F at 0.1 M GuHCl (Table 2 and Figure 1).

In addition, we studied the reactivation of unfolded citrate synthase to investigate whether Cyp18 can also assist in the refolding of another well-documented misfolding-prone protein. This enzyme has been shown to form aggregates to a large extent during spontaneous refolding which could be prevented by the action of the chaperonin GroEL/ES (*23*, *24*) and osmolytes (*35*). As one can see in Table 2, Cyp18 was also able to increase the yield of reactivated citrate synthase by 10%, an effect that was completely counteracted by the presence of CsA in refolding buffer (Table 2).

It is obvious from the results given above that Cyp18 is able to increase the yield of the native protein during the refolding process of both HCA II variants and citrate synthase. In an attempt to gain some insight into the mechanism behind this effect, we performed a stopped-flow ANS fluorescence study. The hydrophobic ANS dye is known to bind to hydrophobic patches that are exposed in a protein structure (*38*). As a reference, we also performed an ANS binding study during GroEL chaperone-mediated refolding of HCA II. From our previous studies, it is known that GroEL functions as an unfoldase, i.e., is capable of stretching the protein apart during the folding process and thereby potentially facilitating rearrangements of misfolded structure. This action has also been demonstrated for HCA II as a substrate by various parameters (*39*, *40*). The interaction by the chaperonin GroEL initially led to a lower degree of ANS binding compared to that observed for the unassisted refolding of HCA II (Figure 3C). Thus, from the ANS data, GroEL also initially appears to extend the protein substrate, leading to fewer ANS-binding hydrophobic patches and a less compact structure, an observation that is in agreement with the unfoldase mechanism.

The interaction with Cyp18 on the HCA II substrates (HCA $II_{pwt}$ and H107N), on the contrary, initially led to an increase in ANS fluorescence compared to that in the spontaneous reaction (Figure 3A,B). Since there was no binding of ANS to Cyp18 alone (Figure 3A−C), this fluorescence increase is due to ANS binding to both substrates and was most prominent for H107N. This effect must originate from remodeling of HCA II by Cyp18. Since GroEL-mediated refolding decreased ANS binding and GroEL has unfoldase activity (*39*, *40*), the increased ANS binding in the Cyp18-mediated refolding most likely shows that Cyp18 condenses hydrophobic regions in the HCA II molecule during the initial phases of folding. Interestingly, the small-amplitude increase mediated by Cyp18 for ANS binding for HCA $II_{pwt}$ is in agreement with its modest chaperone effect,

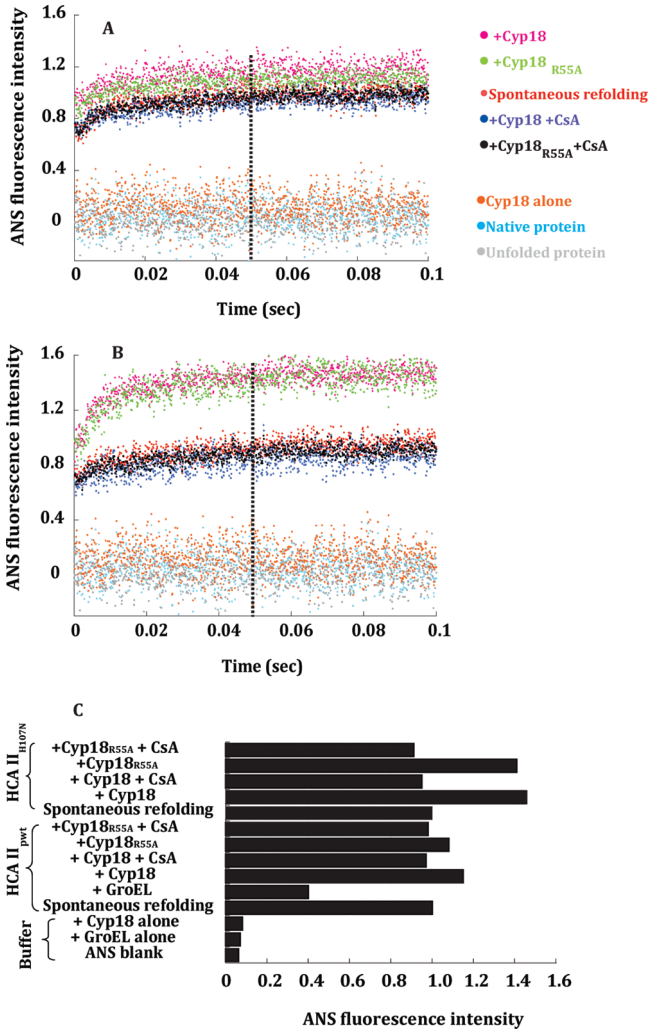


FIGURE 3: Initial formation of hydrophobic patches in the presence or absence of Cyp18 and GroEL. ANS binding to HCA II variants was assessed by stopped-flow fluorescence intensity enhancement: (A) HCA $II_{pwt}$ and (B) H107N. Spontaneous refolding (red), with Cyp18 (magenta), with $Cyp18_{R55A}$ (green), with Cyp18 and CsA (blue), with $Cyp18_{R55A}$ and CsA (black), with the native protein substrate (cyan), with the unfolded protein substrate (gray), and with Cyp18 alone (orange). (C) Comparisons of ANS fluorescence reached after 50 ms (indicated in the time courses in panels A and B) in the presence or absence of GroEL and Cyp18 with and without CsA.

whereas the substantial increase in ANS binding for H107N appeared to be in accordance with the stronger effect of the chaperone on the mutant.

In a recent report, we showed that the $Cyp18_{R55A}$ mutant was fully active in catalyzing the rate-limiting peptidyl-prolyl *cis*/*trans* isomerase steps in the refolding reaction of HCA II (*41*), although this mutant is almost completely inactive (0.1% remaining activity) toward model peptide substrates (*26*). The kinetics of reactivation of HCA $II_{pwt}$ catalyzed by the $Cyp18_{R55A}$ mutant was identical to that of Cyp18, and this is also the case for the H107N mutant (data not shown). Moreover, the reactivation yield was increased to the same degree by $Cyp18_{R55A}$ as by Cyp18 [increases of 7 and 13% after reactivation for 24 h for HCA $II_{pwt}$ and H107N, respectively (cf. Table 2)]. The presence of CsA also inhibited the ability of $Cyp18_{R55A}$ to catalyze and increase the yield of reactivation of the HCA II variants. For comparative purposes, we also studied how the $Cyp18_{R55A}$ mutant affected the binding of ANS to the refolding HCA II variants (HCA $II_{pwt}$ and

H107N). Clearly, the replacement of the Arg55 residue located in the catalytic site did not change the Cyp18-mediated modulation of the binding of ANS to the refolding HCA II molecule (Figure 3A,B). Therefore, in conclusion, both the wild type and this active site mutated variant of Cyp18 have equal ability (i) to catalyze the rate-limiting peptidyl-prolyl *cis*/*trans* isomerase, (ii) to increase the final yield of misfolding-prone mutants during refolding, and (iii) to condense hydrophobic substructures in the very early stages of refolding.

## DISCUSSION

As surveyed in the introductory section, there has been a debate about whether PPIs can have a dual folding role, i.e., act as both a foldase and a chaperone. In particular, this concerns the single-domain PPI, Cyp18, which was the first PPI that was suggested to possess these two characteristics (*8*). For the time being, there is an agreement that larger multidomain PPIs in addition to their peptidyl-prolyl *cis*/*trans* isomerase activity also can act as chaperones and thereby prevent aggregation and increase the yield of the native protein during the folding process (*1*). When we originally showed that a PPI could also act as a chaperone, it was based on the observation that the yield of active HCA II was increased by the action of Cyp18 (*8*). However, Kern et al. (*9*) later showed the presence of a slow kinetic phase in the spontaneous reactivation of HCA II. By taking this slow phase into account, they claimed that no significant increase in the level of Cyp18-mediated reactivation of HCA II was noticed. When a two-phase kinetic analysis was performed on our recent data, kinetic parameters very similar to those reported in the cited work above were obtained, and it can be added that in both kinetic studies, recombinant human Cyp18 has been used. However, after reactivation for 24 h, a 7% increased reactivation yield in the presence of Cyp18 was observed. Since the original work on the effect of Cyp18 on HCA II, we have employed engineered mutants of the enzyme during our folding studies on HCA II that are considerably less stable than the wild-type enzyme and that consequently promote populations of molten globule intermediates that are prone to misfolding. To determine if the chaperone effect of Cyp18 could be detected more clearly, we chose two such destabilized HCA II mutants. From the reactivation experiments performed on these protein substrates, H107N and H107F, we can undoubtedly conclude that Cyp18 is capable not only of accelerating the reactivation process but also of increasing the yield of the active enzyme and thus also of acting as a chaperone. Interestingly, an inverse correlation between the stability of the native state of the tested HCA II variants and the chaperone effect was observed (Figure 4). For the destabilized H107N and H107F variants, partial inactivation also occurred at the GuHCl concentration (0.1 M) at which they were reactivated. By 24 h, an equilibrium value appeared to have been reached for this inactivation, which was also the case for the reactivation process at this GuHCl concentration. This degree of inactivation as a function of the stability of the native state is congruent with the chaperone effect (Figure 4). In addition, the graph indicates a limiting chaperone effect at approximately 1 kcal/mol of native state stability of HCA II.

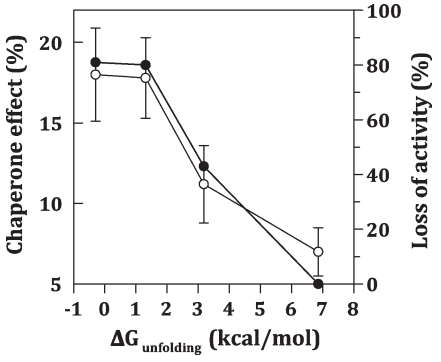


FIGURE 4: Chaperone activity of Cyp18 and loss of activity of the HCA II variants as a function of stability of the HCA II variants. The thermodynamic stability of the HCA II variants has been calculated for the concentration of GuHCl at which the reactivation was performed by using the equation $\Delta G^{GuHCl} = \Delta G^{H_2O} - m[GuHCl]$. The stability of the native state was measured at 4 °C so that we could accurately determine the stability of the destabilized variants, H107N and H107F. The $\Delta G^{H_2O}$ and $m$ values for the calculations were taken from Almstedt et al. (*33*): (○) chaperone effect of Cyp18 and (●) loss of activity of HCA II variants. The standard deviations of the chaperone effect were calculated as a function of the mean of five or six samples (cf. Table 2).

Previously, we have shown that the molten globule intermediate formed upon equilibrium unfolding of the native state of HCA II is very prone to aggregation. The same holds true for the kinetic molten globule (designated $MG_k$ in Figure 5). However, the molten globule light intermediate (designated $MGL_e$ in Figure 5) that appears during the first equilibrium unfolding transition of the H107N and H107F variants is not prone to

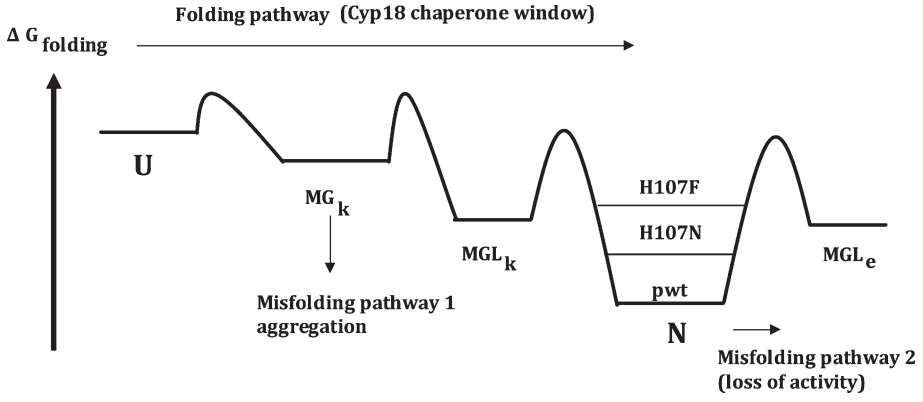


FIGURE 5: Schematic energy landscape for HCA II under "folding permissive conditions" (0.1 M GuHCl and 21 °C). U represents the unfolded state, $MG_k$ the kinetic molten globule intermediate, $MGL_k$ the kinetic molten globule light intermediate, N the native state, and $MGL_e$ the equilibrium molten globule light intermediate.

aggregation but becomes trapped in a misfolded state, thus also leading to decreased yields of the active enzyme upon storage (*37*). Cyp18 apparently has the ability to partly circumvent kinetic misfolding traps and is consequently acting as a chaperone by raising the refolding yield. How is Cyp18 performing this chaperone action? A schematic energy diagram (Figure 5) illustrates how Cyp18 might function in this process. We have observed that the more destabilized the variants are, the larger is the fraction of the protein molecules to be rescued. The loss of activity and the chaperone effect correlate very well and are dependent on protein stability (Figure 4), indicating that the stability of the native state influences both processes. The total rate of reactivation does not, however, differ significantly for these variants [$t_{1/2,total}$ = 7.8−8.8 min (Table 1)]. Thus, the rate-limiting activation barrier for folding appears to be of the same height for the variants. This indicates that there is a common folding intermediate that filters the expected kinetic refolding effect from stability. This insensitivity to mutations (H107N and H107F) on the refolding kinetics indicates that this intermediate appears to lack major tertiary interactions. In retrospect, the same kinetic stability indifference on the global refolding was exhibited by the W16F, W97C, and W245C mutants (*42*). These properties resemble those of the recently characterized molten globule light state under equilibrium conditions, the $MGL_e$ state (*33*, *37*). Hence, this kinetic counterpart is here designated $MGL_k$.

Then, the energy level of the native states should be responsible for the increased yields induced by Cyp18, and if the unfolding step is considered, this should be slowest for the most stable variant and fastest for the most destabilized variant. Since the reactivation reaction is studied under conditions that bring H107N and H107F into the unfolding−refolding transition zone, the unfolding reaction also comes into play. This means that during reactivation, a larger fraction of a more destabilized variant will be partially unfolded. These partly folded states (of the molten globule light type) could then be approached by Cyp18, leading to correction of misfolded conformations. Thus, by this energetic partitioning, the refolding protein substrate is given a new chance to fold correctly. Cyp18 is probably capable of acting on only folding intermediates that have not become too compact; it has previously been shown that a fraction of prolines become inaccessible to Cyp18 interaction during the later stages of folding (*8*, *10*). Thus, Cyp18 appears to be able to act only transiently as a chaperone which is in line with the observed varying chaperone effects. When the native state of H107N is lifted toward the unfolding barrier by the presence of 0.28 M GuHCl, a larger fraction can unfold back to the state that is accessible to the Cyp18 chaperone action and the yield of the correctly folded protein is accordingly increased. Probably, the maximum increase in the yield of the natively refolded protein by Cyp18 is ~20% for HCA II, since H107F (in 0.1 M GuHCl) and H107N (in 0.28 M GuHCl) during refolding seem to be rather close to the indicated limiting chaperone effect (Figure 4). This was further corroborated in the titration experiment with H107F showing that a 10-fold molar excess of Cyp18 was the limiting concentration and a 20-fold molar excess of Cyp18 did not further elevate the chaperone effect (Figure 2). HCA $II_{pwt}$ is located at the other end (stable) of the native state stability transition curve which appears to explain the weak chaperone effect for this variant.

It should also be noted that kinetic models have been published that show that an increased rate of enzyme-catalyzed refolding reactions can explain an increase in the refolding yield (*43*). Since the Cyp18-catalyzed rate of reactivation does not differ significantly between the HCA II variants, and the reactivation yield nevertheless increases when the stability of the variant decreases, the increased yield cannot be explained as a kinetic effect. Notably, the rate increase by Cyp18 on the H107F mutant, showing the most significant chaperone effect, is actually the least efficiently accelerated variant in this study.

Like the mitigation of misfolding of the molten globule by Cyp18, we have demonstrated that the GroEL chaperonin can facilitate reactivation of $HCAII_{pwt}$ and severely destabilized mutants by capturing the molten globule intermediate (*44*). The GroEL effect was demonstrated to work through unfolding of the molten globule, giving the protein a new chance to fold after release from the chaperonin (*39*, *40*). We have also shown that suppression of the molten globule intermediate of HCA II during refolding leads to increased yields of the reactivated enzyme (*45*).

That the presence of CsA inhibits both the isomerase and chaperone activities of Cyp18 shows that both these activities are localized in the same region of the molecule, i.e., in the active site. The multidomain PPIs on the other hand have been shown to conduct the chaperone activity in domains different from the catalytic one.

The presence of Arg55 in Cyp18, which is critical for catalytic activity on smaller peptide substrates, does not have any impact on either catalysis of rate-limiting Pro isomerization during refolding or the final yield of native HCA II. The only thing that is important is access of the protein substrate to the hydrophobic active site cavity of Cyp18 (*41*). Interestingly, the wild type as well as the R55A mutant of Cyp18 has recently been shown to considerably prolong the lag phase of amyloid fibril formation for stefin B, also strongly indicating binding to the hydrophobic active site in Cyp18 and supporting chaperone activity of Cyp18 also on this protein (*46*). Structural data for Cyp18 in a complex with the barely water soluble inhibitor CsA clearly reveal the hydrophobic character of the active site cavity (*47*). There are at least 11 hydrophobic residues both buried in the cavity and flanking the entrance to Cyp18. We hypothesize that during interactions with a partially folded protein substrate Cyp18 can function as a hydrophobic basket that condenses accessible hydrophobic portions of the substrate during folding. This is supported by our stopped-flow ANS binding data on HCA II showing correlating binding magnitudes with observed chaperone effects even on distinctly different kinetic intervals of the folding reaction coordinate. The conclusion from the effects shown by Cyp18 on creatin kinase (*48*), citrate synthase, and HCA II, in particular on the destabilized variants, is that Cyp18 indeed hereby can act as a chaperone. It is moreover interesting to note that the chaperone activity was dependent on native state stability even though the molten globule is insensitive to tertiary structural interactions (*33*). Taken together, our data lead us to propose that (i) interactions of Cyp18 with the protein substrate even at very early stages of folding ($MG_k$) favorably partition the protein on the productive folding route likely functioning as a hydrophobic collector basket for misfolding-prone regions and (ii) Cyp18 can at later stages of folding ($MGL_k$) interact with HCA II and protect it from misfolding. These findings might be important for understanding the in vivo quality control function of foldases and chaperones.

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