Trigger factor-assisted folding of bovine carbonic anhydrase II

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Abstract

Spontaneous refolding of GdnHCl denatured bovine carbonic anhydrase II (BCA II) shows at least three phases: a burst phase, a fast phase, and a slow phase. The fast and slow phases are both controlled by proline isomerization. However, we find that in trigger factor (TF)-assisted BCA II folding, only the fast phase is catalyzed by wild-type TF, suggesting that certain proline residues are accessible in folding intermediates. The refolding yields of BCA II assisted by wild-type TF and TF mutants which lack PPIase activity are about the same, which provides further experimental evidence that the PPIase and chaperone activities of TF are independent. The binding of TF to folding intermediates during BCA II refolding was characterized by chemical crosslinking and Western blotting. A scheme for TF-assisted BCA II folding is proposed and the possible role of the TF dimer as a “binding” chaperone in vivo is discussed.

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In the Escherichia coli cytosol, the ribosome-associated trigger factor (TF) binds at the exit site of the peptide tunnel via interaction with L23 protein of the ribosome [1], and acts as the first chaperone encountered by nascent chains [2,3]. After interaction with TF, most proteins fold rapidly upon synthesis, and only 20–35% of polypeptide chains require the further assistance of other chaperones [4,5]. Deletion of TF results in an increase in protein aggregation in the cytosol [5,6], while overproduction of TF depresses the formation of inclusion bodies and increases the productive yield of aggregation-prone proteins [7,8]. TF has an overlapping chaperone function with DnaK, and only if both are deleted is it lethal for bacteria [4,5]. TF is active in cooperating with GroEL and is involved in GroEL-dependent protein degradation [9,10]. When functioning as a molecular chaperone in vitro, TF has been shown to have a preference for random coil or loosely structured substrates [11–13], and the binding motif of TF has been identified as a short sequence enriched in hydrophobic and basic amino acids [14].

Here we investigate further the relationship of the PPIase activity of TF with its molecular chaperone function and the properties of target substrates that can be recognized by TF. A number of TF mutants lacking PPIase activity were prepared and bovine carbonic anhydrase II (BCA II) was chosen as a target protein in the present study. BCA II contains 16 trans- and 2 cis-prolines and is prone to aggregation during the refolding process [15,16]. Therefore BCA II, or its homologue human carbonic anhydrase II (HCA II), is widely used as a substrate for studying the function of PPIases and molecular chaperones [17–20]. Here, we explore the effect of the molecular chaperone and PPIase activities of TF on the refolding of BCA II in detail, in order to provide new clues towards identifying the role of TF in vivo.
Materials and methods

Materials. Bovine carbonic anhydrase II (BCA II), p-nitrophenyl acetate (p-NPA), chicken egg albumin, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight standards were purchased from Sigma. GdnHCl was from ICN. Tris was from Boehringer-Mannheim. Disuccinimidyl suberate (DSS) was from Pierce. Anti-TF polyclonal antibody was prepared by ShenZhen Bioinforbody (Shenzhen, China). All other chemicals were local products of analytical grade.

Plasmid pQE60 containing the wild-type tig gene that encodes E. coli TF was donated by Professor G. Fischer. TF site-specific mutants I195G, Y221G, F233G, and F233Y, in which the PPIase activity was effectively diminished, were generated using GeneEditor in vitro Site-Directed Mutagenesis System (Promega) and PCR. Wild-type TF and mutants were expressed in E. coli JM109 and purified as described [21]. The absorption coefficients of wild-type TF and mutants were calculated according to the method of Gill and von Hippel [22], and for bovine carbonic anhydrase II, $e_{280} = 53,070 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

Denaturation and renaturation. BCA II was denatured in 5 M GdnHCl for 12 h at 20°C. Reactivation was initiated by a rapid 100-fold dilution with 50 mM Tris sulfate buffer, pH 7.5, at 20°C to a final BCA II concentration of 4 µM. The buffer contained different concentrations of wild-type TF, mutant TF or chicken egg albumin. Reactivation kinetics and yields were determined by following the esterase activity of BCA II toward p-NPA by continuous or discontinuous assay in a Shimadzu UV-1601 spectrophotometer, as described previously [17]. Recovery of activity was complete 4 h after dilution and no further change was observed for at least 24 h. Aggregation of BCA II during refolding was monitored at 20°C by 90° light scattering at 488 nm in a Hitachi F4500 fluorescence spectrophotometer.

Crosslinking and electrophoresis. Reactivation of denatured BCA II was carried out in 15 mM sodium phosphate buffer, pH 7.5, at 4°C, and the final BCA II concentration was 4 µM. The buffer contained different concentrations of wild-type TF and 6 mM DSS. After 2 h incubation, the crosslinking reaction was quenched with 150 mM Tris–HCl, pH 7.0. Crosslinking products were analyzed by gradient SDS–PAGE and visualized by silver staining and Western blot using an anti-TF polyclonal antibody.

Results

Prevention of aggregation of BCA II by wild-type TF and mutants

As shown in Fig. 1A, aggregation occurred immediately after dilution of denatured BCA II into refolding buffer and aggregation was about 80% completed within the initial 20 s as monitored by light scattering. The extent and rate of aggregation declined markedly with increasing concentration of wild-type or mutant TF (Fig. 1B). It should be noted that wild-type TF, Y221G, and F233Y were more effective than I195G and F233G in prevention of aggregation of BCA II. In contrast, when hen egg white albumin is used in place of TF as a control, no effect on the BCA II aggregation is observed.

Effect of wild-type TF and mutants on the reactivation of denatured BCA II

Spontaneous reactivation of GdnHCl denatured BCA II does not precede completion, especially at high protein concentrations and in the presence of low concentrations of denaturant [16]. As shown in Fig. 2A, the spontaneous reactivation yield of BCA II is about 32% under the experimental conditions used here. With increasing concentrations of wild-type TF, the BCA II reactivation yield increased gradually, reaching a maximum of around 40% in the presence of 12 µM wild-type TF, after which no further increase was observed. In the case of the TF mutants lacking PPIase activity, Y221G showed the same effect on the reactivation yield of BCA II as wild-type TF, whereas I195G showed a lower
effect. Hen egg white albumin showed only a slight effect on the BCA II reactivation yield even at high concentration.

The reactivation kinetics of BCA II was also analyzed in the presence of various concentrations of wild-type and mutant TF (Fig. 2B). The reactivation kinetics of BCA II shows three phases: a burst phase \( t_{1/2} = 0.03 \) s, a fast phase \( t_{1/2} \approx 140 \) s, and a slow phase \( t_{1/2} \approx 600 \) s [23]; the two slower phases are controlled by isomerization of prolyl peptide bonds [15]. In this experiment, the time course of reactivation of BCA II was monitored by continuous assay over the time range 100–600 s and then by discontinuous assay from 10 to 120 min. Due to interference from aggregation when monitoring activity at 348 nm at early time points, time points less than 100 s were not monitored. The two phases are well resolved and the apparent rate constants could be obtained by fitting the data from each time range to a single-exponential function (Table 1). Fitting to a double exponential over the shorter time range by entering the slow phase rate constant as a fixed parameter improved the residual, but did not significantly alter the rate constant obtained for the fast phase. The rates of the slow phase are approximately the same within error for wild-type TF and the mutants I195G and Y221G. In contrast, the fast phase reactivation rate was clearly accelerated by wild-type TF. This suggests that isomerization of the proline residues that limit the rate of the fast phase of reactivation is catalyzed by wild-type TF. In support of this conclusion, the mutants I195G and Y221G, which lack PPIase activity, showed no effect on the rate constants for the fast phase of reactivation.

Identification of a complex between TF and BCA II intermediates

In the presence of wild-type TF or mutants, the increase of reactivation and the prevention of aggregation of BCA II suggest that interactions exist between TF and BCA II intermediates during refolding of BCA II. In order to identify the intermediate states that interact with TF, crosslinking was used. Since the reactivation and aggregation of BCA II are too fast to be followed at room temperature, we lowered the refolding temperature

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Table 1

<table>
<thead>
<tr>
<th>Assistant protein (µM)</th>
<th>Wild-type TF</th>
<th>I195G</th>
<th>Y221G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{fast} )</td>
<td>( k_{slow} )</td>
<td>( k_{fast} )</td>
</tr>
<tr>
<td>0 µM</td>
<td>1.84 ± 0.16</td>
<td>0.451 ± 0.072</td>
<td>1.84 ± 0.16</td>
</tr>
<tr>
<td>6 µM</td>
<td>3.32 ± 0.14</td>
<td>0.380 ± 0.065</td>
<td>2.21 ± 0.12</td>
</tr>
<tr>
<td>12 µM</td>
<td>4.77 ± 0.13</td>
<td>0.364 ± 0.053</td>
<td>2.37 ± 0.15</td>
</tr>
<tr>
<td>24 µM</td>
<td>6.47 ± 0.12</td>
<td>0.344 ± 0.047</td>
<td>1.99 ± 0.15</td>
</tr>
<tr>
<td>30 µM</td>
<td>6.51 ± 0.10</td>
<td>0.429 ± 0.068</td>
<td>2.43 ± 0.10</td>
</tr>
</tbody>
</table>
for crosslinking experiments to 4°C. At 4°C, the first intermediate of BCA II disappears within 2 h of dilution, at which time the relative population of the second intermediate is about 50% [24]. Thus, the possibility of interaction between TF and the BCA II intermediates at 4°C should be increased. By using the crosslinker DSS, the formation of dimers could be observed during refolding of denatured BCA II (Fig. 3A), while no other oligomers could be detected. In contrast, native BCA II itself could not be crosslinked by DSS. TF tends to show dimers when treated with DSS, especially at higher concentration, consistent with a monomer–dimer equilibrium [25]. When denatured BCA II was refolded in the presence of TF, a crosslinked complex with molecular weight above 230 kDa was observed, which most likely corresponds to two molecules of TF dimer and two molecules of BCA II. The quantity of crosslinked complex increased with increasing concentration of TF. The involvement of TF in the crosslinked complex was confirmed by Western blot using an anti-TF polyclonal antibody (Fig. 3B). That the 230 kDa complex contains two molecules of BCA II, which does not dimerize, is supported by the fact that native BCA II, which does not dimerize, is unable to form this complex. Other crosslinked complexes involving other ratios of TF and BCA II may also be present, but would co-migrate with the broad bands observed for TF or BCA II crosslinked dimmers [26].

Discussion

The fast phase folding of BCA II is catalyzed by wild-type TF

The refolding kinetics of BCA II contains at least three phases: a burst phase, a fast phase, and a slow phase. The first intermediate is a molten globule state and the second one has native-like structure [23]. Although isomerization of prolines is involved in both fast and slow phases of BCA II folding [15], we find that TF can only accelerate the fast phase of folding and has no effect on the slow phase. This is similar to cyclophilin-assisted folding of HCA II [18]. The catalysis of proline isomerization is only possible if the prolines are accessible to the prolyl isomerase during the rate limiting step of folding. In the molten globule intermediate of BCA II, the prolines located in the mobile side chains are likely to be accessible to the active site of TF and so their isomerization can be catalyzed. However, in the native like second intermediate of BCA II, access to the key prolines is presumably more restricted. TF mutants, Y221G and I195G, which lack PPIase activity, showed no effect on the refolding rates of BCA II even at high concentration (Table 1). This provides further evidence that the acceleration of the fast phase of BCA II folding is due to the PPIase activity of TF.

When comparing the refolding yields of BCA II assisted by wild-type TF and mutants, we found that the effect of mutant Y221G on the reactivation yield was identical to that of wild-type TF, although only the PPIase active wild-type could accelerate the fast phase of reactivation. Previous studies have shown that the mutants Y221G and F233Y have the same ability as wild-type TF in assisting folding of adenylate kinase in vivo [7] and in assisting refolding of glyceraldehyde-3-phosphate dehydrogenase in vitro (C.P.L and J.M.Z., unpublished data). These results suggest that the PPIase activity of TF alone can change the rate of protein folding, but does not increase the final yield of productive species, consistent with previous studies on other PPIases [17,18]. In fact, like TF, many PPIases also have chaperone activity, such as cyclophilin40 and FKBP52.
[27]. In terms of structure, there is a close relationship between the PPIase and chaperone activities of some PPIases. For example, earlier studies showed that as a molecular chaperone, the substrate-binding ability of TF is a key factor in its ability to catalyze isomerization of a protein substrate [11,28]. Another example is MtFKBP17, whose active site is necessary for chaperone function [29]. However, in terms of activity, our results suggest that the molecular chaperone and PPIase activities of TF are independent in assisting protein folding.

**TF binds intermediates with loosely defined structural properties**

In the presence of high concentrations of wild-type TF or the mutant Y221G, the reactivation yield of BCA II was 25% higher than spontaneous reactivation. However, this increase is principally limited to the initial 100 s of the folding reaction. There are many important events that occur within the initial 100 s after dilution of denatured BCA II. These include the formation of the molten globule intermediate, the transition from molten globule state to the native-like intermediate, and the association of molten globule intermediates, resulting in the formation of aggregation-prone dimers, trimers, and even insoluble aggregates [16,23]. Previous studies have shown that TF can interact with early-stage folding intermediates, which results in the prevention of aggregation and increases the yield of native protein [12,13]. Taken together, these results suggest that TF interacts with the molten globule state of BCA II to alter the proportions of the molten globule state that refold or aggregate, resulting in an increase in reactivation yield.

As a molecular chaperone, TF not only increases the reactivation yield, but also inhibits aggregation of folding intermediates. However, a simple correlation between the inhibition of aggregation and the increase of reactivation yield was not observed when the amount of TF was increased. Instead, the reactivation yield reached a plateau, while aggregation was further suppressed with increasing TF concentration. This suggests that in addition to the molten globule state, TF can interact with aggregation-prone species that are off the productive folding pathway. The presence of a stable complex consisting of BCA II dimer and TF dimer was identified by chemical crosslinking (Fig. 3). These results suggest that TF is a molecular chaperone that can recognize both on-pathway and off-pathway intermediates of BCA II.

The network of molecular chaperones in the cytosol includes Hsp90 (HtpG) and small Hsp’s (IbpA/B) (E. coli homologues shown in parentheses), which are referred to as “binding” chaperones [30]. These “binding” chaperones can bind aggregation-prone misfolded polypeptides, preventing their aggregation and serving as a reservoir of off-pathway species to be subsequently refolded by “folding” chaperones, such as DnaK and GroEL, or to be degraded by proteases [30–35]. Recent results show that TF has three states in vivo: the ribosome-associated state, the free monomer and the dimer [25]. The functional significance of the monomer–dimer equilibrium remains unclear. Here, we found that the TF dimer can associate with an aggregation-prone precursor of BCA II to form a stable soluble complex. This suggests that the TF dimer may serve as a “binding” chaperone like Hsp90 and small Hsp’s in the cytosol. It will be interesting to discover whether this complex can serve as a reservoir for further refolding or degradation of the off-pathway species.

Compared with the mutants I195G and F233G, wild-type TF and the mutants Y221G and F233Y possess stronger ability in increasing reactivation and suppressing aggregation (Figs. 1B and 2A). This suggests that mutation of residues F233 and I195 to glycine weakens the interaction of TF with folding intermediates compared to the other mutations. The same result was observed for the substrate glyceraldehyde-3-phosphate dehydrogenase (L.C.P and J.M.Z., unpublished data).

**Scheme for TF-assisted refolding of BCA II**

Taking into account the previous results for refolding of BCA II [16,23], a scheme for TF-assisted refolding of BCA II is proposed (Fig. 4). In the spontaneous refolding pathway of BCA II, TF can reversibly, transiently associate with the early-formed intermediate (molten globule state) and prevent self-association of this species. At the same time, binding of TF to the
intermediates enables the accessible prolines of BCA II to be isomerized by TF. The chaperone activity of TF favors the on-pathway folding of denatured BCA II. However, the intermediates that escape from the protection of TF will associate to form aggregation-prone oligomers. As a molecular chaperone that binds substrates with loosely defined structural properties, TF can also interact with those oligomers to form a stable complex and prevent further aggregation.

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References


