Binding and conformation of denatured horseradish peroxidase during E. coli ribosome mediated folding

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Denatured horseradish peroxidase (HRP) refolded in the presence of intact 70S E. coli ribosome. Fluorescence spectroscopic evidence of direct physical association between the ribosome particles and the denatured HRP during refolding has been detected. The efficiency of energy transfer from the single tryptophan (Trp) to the heme moiety and the quenching patterns of the Trp fluorescence by iodide and acrylamide differed with time while folding in the presence and absence of ribosome. An estimate of the binding of denatured fluorescein-conjugated HRP with ribosome was obtained from polarization measurements ($K_a = 41 \text{nM}$).

FOLDING of denatured proteins to their biologically active forms has been shown to be assisted by a class of proteins collectively known as the molecular chaperones. We have previously shown that ribosomes from E. coli could fold a large number of proteins to their active forms when denatured with 6 M guanidinium chloride (GdmCl) prior to incubation with ribosomes. Subsequently it was found that the protein folding activity remained in the 50S subunit and the 23S rRNA of the E. coli ribosome. The 30S subunit and the 16S rRNA were devoid of such activity and the protein folding activity in the 23S rRNA could be inhibited by the antibiotics, chloramphenicol and erythromycin which interact with the large loop of domain V of the 23S RNA. These observations were later confirmed by Hardesty and coworkers. In the present study we have detected the physical association between denatured horseradish peroxidase (HRP) and the intact 70S ribosomes from E. coli in the course of ribosome-assisted folding. We have also determined the affinity constant of the association of the 70S ribosome with the denatured fluorescein-conjugated HRP.

The folding pathways in the monomeric protein, HRP and its interaction with the denaturant, GdmCl, are well studied. HRP contains a single tryptophan (Trp) residue and the heme moiety, an intrinsic fluorescence energy transfer acceptor, making HRP suitable for fluorescence spectroscopic studies. The time-dependent changes in proximity between the Trp residue and the heme moiety were different when denatured HRP was allowed to refold in the absence and presence of ribosomes. The acrylamide and iodide quenching behaviour, used as an index of overall conformation of different states of the protein after refolding in the presence and absence of ribosomes, also indicated that the denatured HRP after ribosome-assisted folding assumed conformation similar to that of the native HRP.

HRP (Type VI, Sigma) at a concentration of 44 μM was incubated with 6 M GdmCl at 22°C for 2.5 h for denaturation. Refolding was initiated by 40-fold dilution of the denatured HRP in the buffer containing 20 mM Tris·HCl, 50 mM KCl, 10 mM MgCl₂, pH 7.5, with or without ribosome. After 30 min of refolding at 22°C the enzyme was assayed in 0.1 M phosphate buffer, pH 6.0 with 0.005% freshly prepared solution of o-dianisidine and 2.2 mM hydrogen peroxide. The mixture was incubated with the enzyme for 3 min at 20°C and the absorbance of the coloured solution was measured at 460 nm. 70S ribosomes were purified from E. coli MRE 600 cells, which has been described in our earlier publications. HRP concentration was determined spectrophotometrically. An HRP concentration of 1.1 μM and 0.015 μM of ribosome was used throughout the course of study in a final volume of 0.8 ml, unless otherwise mentioned.

Denaturation of HRP by 6 M GdmCl was associated with ~30% enhancement in fluorescence intensity (Figure 1). The emission maximum was red-shifted from 342 nm to 356 nm upon denaturation in 6M GdmCl. Figure 1 also shows the fluorescence spectrum of 70S ribosome preparation from E. coli. Both enhancement in fluorescence intensity and red shift occurred immediately after mixing the denaturant with HRP. Removal of GdmCl by 40-fold dilution restored the emission maximum immediately back to 345 nm and the fluorescence slowly quenched up to ~25% in 15-20 min before reaching a plateau. It has been reported that heme dissociates from the halo-enzyme when HRP was denatured by overnight incubation in 6 M GdmCl (ref. 8). However, in our denaturing condition there was no dissociation of heme as indicated from absorbance measurements of both native and denatured HRP in the Soret region with a maximum absorption at 402 nm. CD spectra of native and denatured HRP have been reported earlier. The loss of secondary structure was complete in less than 5 min as indicated by the decrease in the negative peak at 222 nm (not shown). No further change was observed after incubation for 3 h. The steady-state fluorescence was measured in a Hitachi F3010 spectro-photometer using 10 mm pathlength quartz cuvettes. Trp fluorescence was measured upon excitation at a...
Denatured HRP was diluted 40-fold in the buffer containing 70S ribosome, incubated for different times and assayed at 20°C, the optimum temperature for its activity. Figure 2 shows time-dependent reactivation of denatured HRP in the presence and absence of E. coli ribosome.

The Forster type of fluorescence resonance energy transfer has been shown to be useful in measuring distances in the range of 10 to 60 Å. The efficiency of transfer, \( E \), from the donor Trp to the acceptor heme is defined by,

\[
E = 1 - \frac{F_{DA}}{F_D},
\]

(1)

where \( F_{DA} \) and \( F_D \) are fluorescence intensities of the donor at 345 nm in the presence and absence of the acceptor respectively. From \( E \), the distance between the donor and the acceptor, \( r \), can be calculated using the following expression,

\[
E = \frac{R_0^6}{r^6 + R_0^6},
\]

(2)

where \( R_0 \) is the distance at which \( E = 0.5 \). \( R_0 \) for Trp and heme in HRP has been previously determined to be 24 Å.

Time-dependent changes in HRP fluorescence in the presence of ribosome particles were monitored at different time-points by spectral subtraction of ribosome from the mixture of HRP and ribosome. The ribosome concentration chosen was found to impart no inner filter effect. The control spectrum of denatured HRP, for the measurement of \( F_D \), was obtained after immediate dilution of 6M GdmCl-treated HRP in buffer without ribosome. During the process of refolding, Trp fluorescence of denatured HRP quenched with time both in the presence and absence of ribosome. Quenching of fluorescence from the single Trp residue of the denatured HRP was attributed to energy transfer from Trp to the heme. Such time-dependent quenching of fluorescence during ribosome mediated folding of denatured HRP is shown in Figure 3. Fluorescence emission spectra, upon subtracting the contribution of ribosome, are shown at each of the time-points and used for the measurement of \( F_{DA} \). Contribution from the change in ribosomal fluorescence, if any, was neglected because of the weak fluorescence signal from 70S ribosome (Figure 1) compared to HRP.

The blue-shift in the emission maximum from 356 nm to 345 nm occurred immediately when the denatured enzyme was diluted 40-fold to initiate refolding both in the presence and absence of ribosomes. No further change in the position of the emission maxima was observed during 30 min of refolding. Fluorescence intensity at 345 nm was used for the evaluation of \( E \). The efficiencies of transfer (\( E \)) and the distances (\( r \)) between the Trp and heme were evaluated from eqs (1) and (2) respectively at different time-points during the folding of
Figure 3. Tryptophan fluorescence spectra of denatured HRP (1.1 μM) at different time-points in the presence of 70S ribosome (0.01S μM). The top spectra in solid line is the control in the absence of ribosome. Corrected fluorescence emission spectra in dashed lines correspond to the same at different time-points (from top to bottom) of 5, 10, 15, 20 and 30 min respectively, in the presence of ribosomes.

Arg, His and the Trp. More recently, the structural model has been refined by energy minimization and molecular dynamics calculations. From energy transfer measurements we have determined a distance of 24.4 Å between Trp and heme when the denatured HRP was refolded for 30 min in the presence of ribosome restoring biochemical activity in the enzyme. The distance between the Trp and heme was ~ 44 Å in the denatured enzyme, measured at the earliest time-point after the initiation of folding by the 40-fold dilution of 6M GdmCl. On the other hand, Trp and heme were 28.3 Å apart when denatured HRP was refolded in the absence of ribosome showing poor biochemical activity in the enzyme (Figure 2). The data on energy transfer efficiency and distances between Trp and heme are summarized in Table 1.

Binding of denatured fluorescein-conjugated HRP (Sigma) with intact 70S ribosome particles was monitored by measuring the increase in fluorescein polarization with increasing concentrations of the ribosomes. A fixed concentration of the denatured fluorescein-HRP was added to different increasing concentrations of ribosomes and the polarization parameters were quickly measured. The ribosomes showed no detectable absorbance at 495 nm. The scatter contributions were subtracted from each data-point. For polarization measurements with fluorescein-conjugated HRP (EX: 495 nm; EM: 515 nm), excitation and emission slits with a nominal bandpass of 3 nm and 5 nm respectively were used. Polarization measurements were performed using Hitachi polarization accessory. An estimate of binding between the 70S particle and denatured HRP was obtained by measuring the increase in fluorescein polarization of fluorescein-conjugated HRP with increasing concentration of 70S ribosomes (Figure 4). The apparent (K_{app}) of binding of denatured fluorescein-HRP with 70S ribosomes, obtained from the concentra-

<table>
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<tr>
<th>Trp/Heme in HRP</th>
<th>E_{2 min}</th>
<th>E_{10 min}</th>
<th>E_{20 min}</th>
<th>E_{30 min}</th>
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</thead>
<tbody>
<tr>
<td>Unassisted folding of denatured HRP in absence of ribosome</td>
<td>0.029</td>
<td>0.196</td>
<td>0.270</td>
<td>0.272</td>
</tr>
<tr>
<td>(43.1 Å)</td>
<td>(30.4 Å)</td>
<td>(28.3 Å)</td>
<td>(28.3 Å)</td>
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<td>Ribosome-assisted folding of denatured HRP</td>
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<td>0.143</td>
<td>0.357</td>
<td>0.470</td>
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<tr>
<td>(44.5 Å)</td>
<td>(32.3 Å)</td>
<td>(26.5 Å)</td>
<td>(24.4 Å)</td>
<td></td>
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</tbody>
</table>

Values in parenthesis are distances between Trp and heme in HRP during the process of refolding; E is the efficiency of energy transfer.

denatured HRP in the presence and absence of 70S ribosomes. E and r values are summarized in Table 1.

Amino acid sequence of horseradish peroxidase revealed the single Trp positioned at 117th residue from the N-terminus. As the X-ray structure of HRP was not available, the structural model of the protein was built by aligning the amino acid sequence of HRP on that of yeast cytochrome c peroxidase. The model consists of two domains of the peroxidase enclosing the central heme prosthetic group. Of the 10 helical regions (35% overall helicity), two heme-attached helices include the proximal His and three proposed distal catalytic residues
Figure 5. Stern-Volmer plots of acrylamide and iodide quenching of Trp fluorescence of native and denatured HRP (1.1 μM). The figure shows plots of $F_0/F$ against concentration of acrylamide and iodide in the native HRP (●); native HRP after 30 min incubation with ribosomes (○); and denatured HRP after 30 min refolding in absence (▲); and presence (△) of ribosomes.

denatured protein after ribosome-assisted folding and a mixture of the native HRP and ribosomes was similar to that of the native HRP. No quenching in ribosomal fluorescence was observed using either acrylamide and iodide. The quenching experiments indicated that the denatured HRP assumes native conformation after ribosome-assisted folding. Refolding in the absence of ribosomes led denatured HRP to assume a misfolded non-native conformation as indicated by the larger extent of quenching by both acrylamide and iodide along with the marked decrease in the enzymic activity.


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