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 ($K_{\rm d} \approx 41\ {\rm nM}$).

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^{1,2}. 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^{3,4}. 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 rRNA^{5,6}. 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 ribosomeassisted 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^{8,9}. HRP contains a single tryptophan (Trp)

residue and the heme moiety^{10,11}, an intrinsic fluorescence energy transfer acceptor¹², 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 ribosome, 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 13,14. 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^{4,15}. HRP concentration was determined spectrophotometrically⁹. 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 holo-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 earlier9. The loss of secondary stucture 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 spectrofluorometer using 10 mm pathlength quartz cuvettes. Trp fluorescence was measured upon excitation at a

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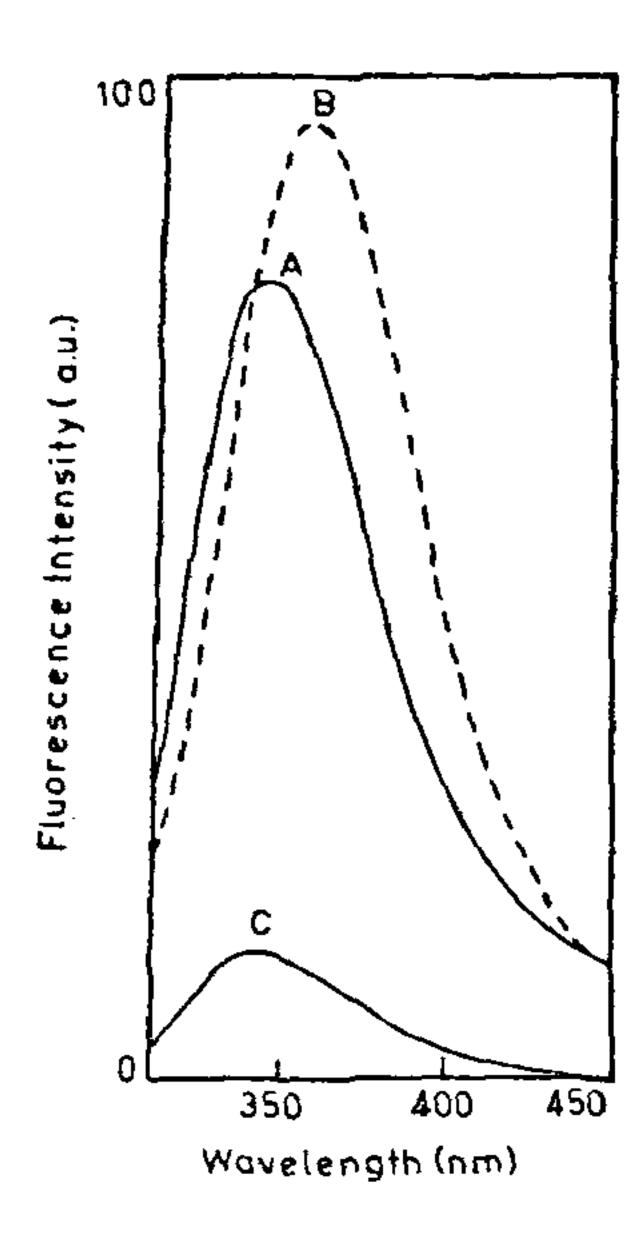


Figure 1. Fluorescence emission spectra of native HRP, 1.3 μM (A); denatured HRP in presence of 6M GdmCl (B); and 70S ribosome from E. coli, 0.02 μM (C).

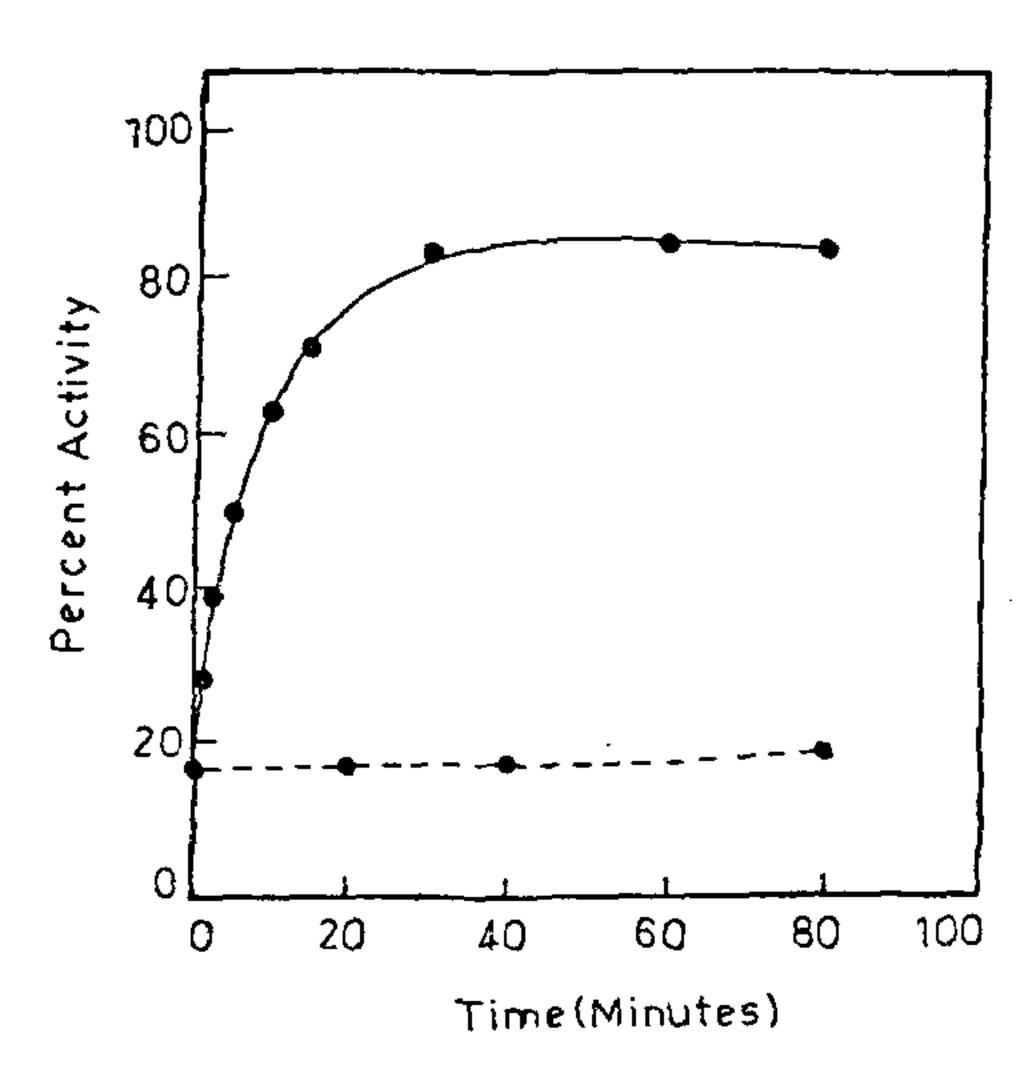


Figure 2. Time-dependent reactivation of enzymic activity during ribosome-assisted folding (solid line) and folding of denatured HRP in the absence of ribosomes (dotted line). Denatured HRP (0.8 μ M) was reactivated with or without ribosomes (0.2 μ M) for different times as indicated in the abscissa prior to assay.

wavelength of 295 nm. A nominal bandwidth of 5 nm and 10 nm for the excitation and emission slits, respectively, were used for measurements. Only ~ 10% self-quenching of ribosomal fluorescence was observed over a time-period of one hour with no change in the 90° light scattering. Sample temperature was maintained at 22°C.

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^{16,17} 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,

$$E = 1 - F_{\rm DA}/F_{\rm D},\tag{1}$$

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,

$$E = R_0^6 / (r^6 + R_0^6), (2)$$

where R_0 is the distance at which E = 0.5. R_0 for Trp and heme in HRP has been previously determined¹² 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 $F_{\rm DA}$. 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

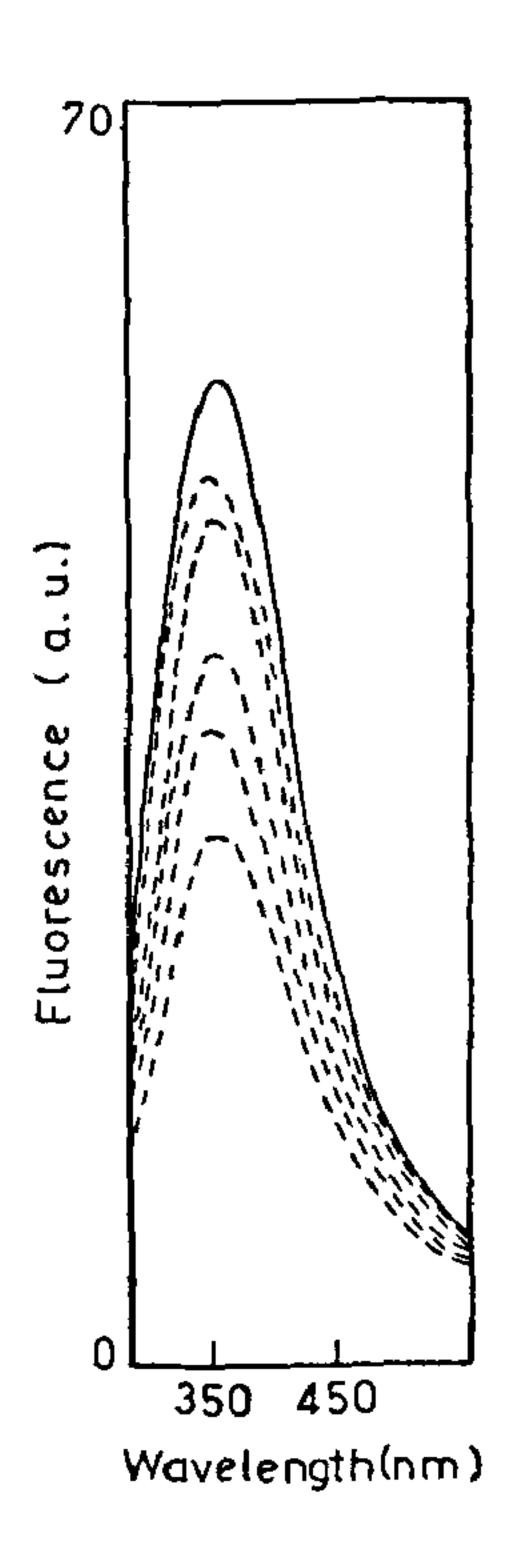


Figure 3. Tryptophan fluorescence spectra of denatured HRP $(1.1 \,\mu\text{M})$ at different time-points in the presence of 70S ribosome $(0.015 \,\mu\text{M})$. The top spectra in solid line is the control in the absence of ribosome. Corrected fluorescence emission spectra in dashed lines correspond 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.

Table 1. Energy transfer efficiencies and estimated distances between Trp and heme at different time-points

Trp/Heme in HRP	E _{2 min}	E _{10 min}	E _{20 min}	E _{30 min}
Unassisted folding of denatured HRP in absence of ribosome	0.029 (43.1 Å)	0.196 (30.4 Å)	0,270 (28.3 Å)	0.272 (28.3 Å)
Ribosome-assisted folding of denatured HRP	0.024 (44.5 Å)	0.143 (32.3 Å)	0.357 (26.5 Å)	0.470 (24.4 Å)

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 18. 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

Arg, His and the Trp. More recently, the structural model has been refined by energy minimization and molecular dynamics calculations¹⁹. 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_d) of binding of denatured fluorescein-HRP with ribosomes, obtained from concentrathe 70S

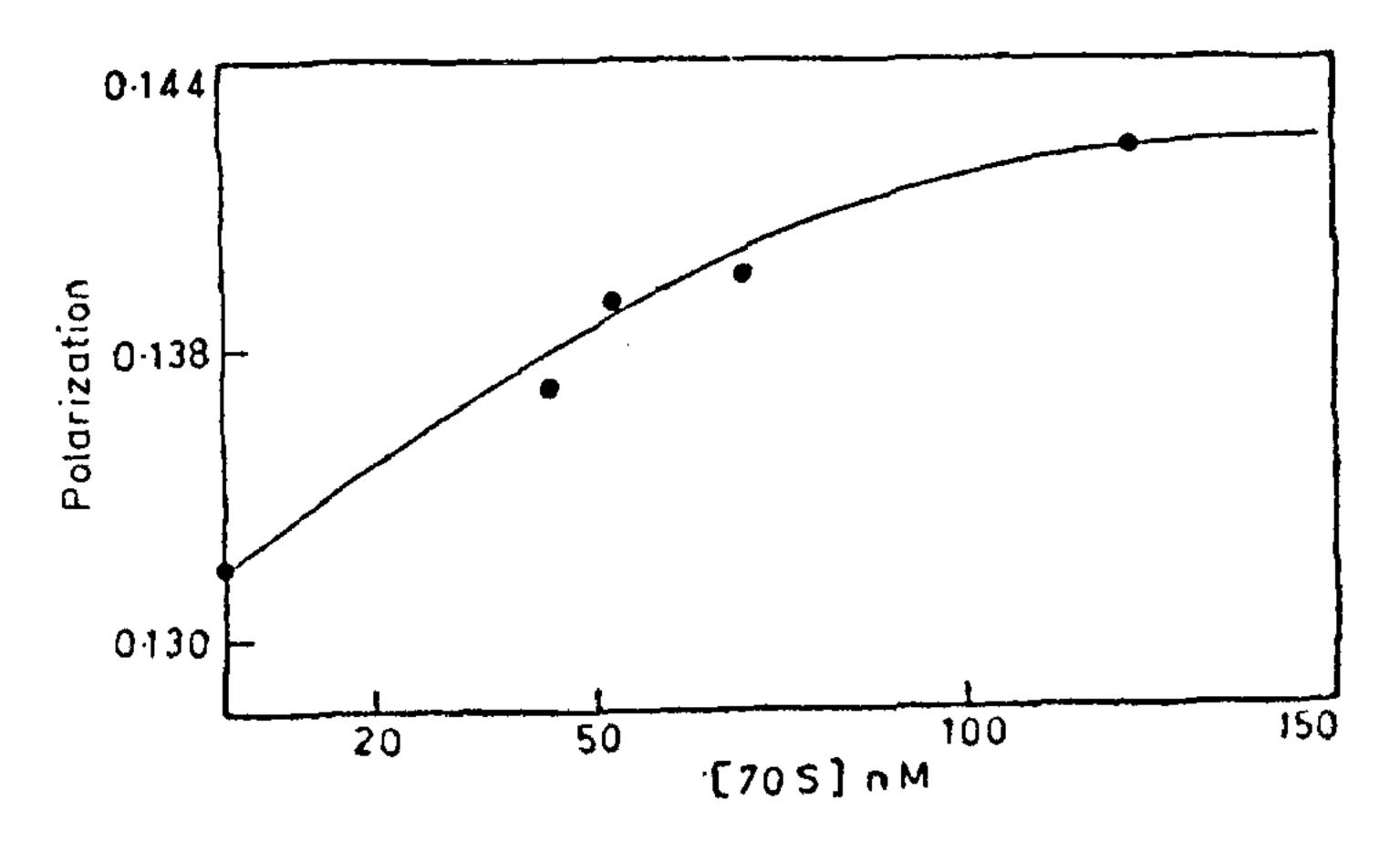


Figure 4. Change in fluorescein polarization in denatured fluorescein-conjugated HRP (1.0 μ M) upon interaction with E. coli ribosomes. The concentration of 70S ribosomes corresponding to the 50% change in the total amplitude determines the K_0 being 41 nM.

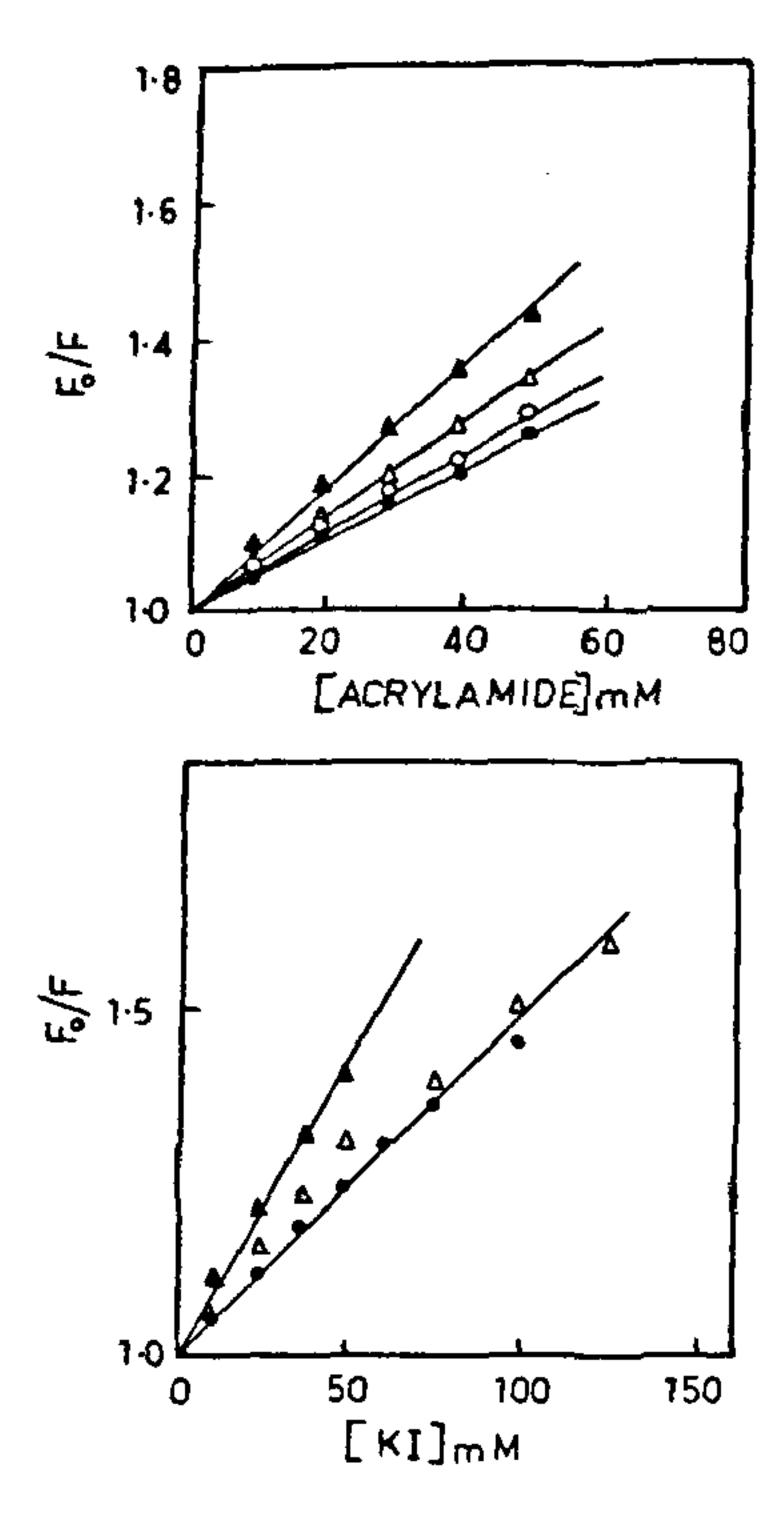


Figure 5. Stern-Volmer plots of acrylamide and iodide quenching of Trp fluorescence of native and denatured HRP (1.1 μ M). The figure shows plots of F_0/F against concentration of acrylamide and iodide in the native HRP (\bullet); native HRP after 30 min incubation with ribosomes (O); and denatured HRP after 30 min refolding in absence (Δ); and presence (Δ) of ribosomes.

tion corresponding to the 50% change in the total amplitude was 41 nM.

The emission intensities of HRP both in the presence and absence of ribosome in 20 mM Tris-HCl, 10 mM MgCl₂, 50 mM KCl, pH 7.5 were measured as a function of increased concentration of acrylamide and iodide which are widely used as quenchers of Trp fluorescence 17 . For iodide quenching experiments the same buffer contained 2 mM sodium thiosulphate in order to prevent the formation of I_3 . The fluorescence intensity in the absence of acrylamide and iodide (F_0) was quenched by increasing concentration of both the quenchers (F), as shown in Figure 5. The linear range of the plot of F_0/F versus the final quencher concentration (Stern-Volmer plot) shows that the extent of quenching was larger in case of denatured HRP after refolding in the absence of ribosomes. The quenching pattern of the

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 nonnative 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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