THE EFFECT OF HSP70 BINDING PEPTIDES ON PROTEIN SYNTHESIS

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NOMENCLATURE

BCIP	5-bromo-4-chloro-3-indolyl phosphate
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetra acetic acid
eIF-2	eukaryotic initiation factor-2
eIF-2B	eukaryotic initiation factor-2B
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HDJ2	DnaJ homolog in human (~48 KD)
HEPES	(N-[2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic]) acid
HRI	heme-regulated eIF-2α kinase or heme-regulated inhibitor
hsc	heat shock cognate protein
hsp	heat shock protein
kD	kilodaltons
Leu	Leucine
Met	Methionine
NBT	Nitro blue tetrazolium
PF	FYQLALT
PN	NIVRKKK
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER I

INTRODUCTION

The response of cells or organisms to environmental stress such as heat shock or chemicals is connected to the induction or enhancement of the synthesis of a member of proteins, called heat shock proteins (hsps). Based on the molecular weight and amino acid sequence homologies, hsps are grouped into families; the most abundant hsps are: hsp90, hsp70 and hsp60. Hsps are not only induced during stresses, some family members are constitutively expressed. Up to 5% of the cellular protein might be hsps and they are highly conserved during evolution (1). The above facts indicate that hsps fulfill important tasks in cells under normal conditions. One of the functions of hsps is chaperoning proteins during synthesis, folding, assembly and degradation (1).

Chaperones are broadly defined as proteins that bind the otherwise unstable non-native conformations of proteins, mainly by shielding hydrophobic surfaces exposed to solvent, and facilitate correct folding by releasing the bound polypeptide in a controlled way (2). It is generally believed

that chaperones do not recognize a condensed sequence motif, therefore they have the ability to prevent the incorrect intra- and intermolecular folding and association of many different proteins.

Hsp70 and hsp60 are involved in the early stages of protein biogenesis, while Hsp90 may be involved in the later stages of protein biogenesis when extensive secondary structure has already formed (3,4,5). Chaperones function as heteromeric chaperone machines. The affinity of chaperones for their substrates is modulated by their associated proteins (cohorts) and the binding and hydrolysis of ATP (6,7). This thesis is mainly focused on the role of hsp70 and its cohorts in regulation of protein synthesis. Therefore, the structure and function of hsp70, and the regulation of protein synthesis will be discussed in the following sections.

Heat shock protein 70 (hsp70)

The Hsp70 family of heat shock proteins is a multi-gene family, coding for a number of proteins with related structures and functions. Genes for hsp70 are widely distributed through the genome, on the chromosomes 1,5,6,14 and 21 (8). Hsp70 are found in almost all cellular compartments of eucaryotes including nuclei, mitochondria, chloroplasts, the endoplasmic reticulum and the cytosol. In

addition, they are found in all bacteria examined so far. Hsp70 family members share high amino acid sequence homology, having at least 50% identity (9). The structure of hsp70 consists of two domains: ~44KD N-terminal ATPase domain and ~25KD C-terminal peptide-binding domain (10,11).

Hsp70s function widely in different aspects in eukaryotic cells under normal conditions as well as during stress (12,13). They maintain the translocation-competent state of proteins destined for endoplasmic reticulum and mitochondria (14) and transiently interact with nascent polypeptides during translation (15). Hsp70 is a crucial component in targeting proteins to lysosomes for degradation (16) and importing cytoplasmic proteins into the nucleus (17). Hsp70 cooperates in defined folding(18) and may be involved in targeting proteins to the ubiquitin/proteasome machinery for degradation (19). In cooperation with hsp90, hsp70 is involved in steroid receptor signaling (20). Hsp70 also binds to retinoblastoma protein (21) and to a mutant form of tumor suppresser protein p53(22). In addition, hsp70 suppresses protein aggregation (23) and facilitates reactivation of heat-denatured proteins (24).

Hsp70 participates in these diverse cellular processes through the binding and release of protein substrates at the expense of ATP hydrolysis. Hsp70 recognizes short extended peptide sequence motifs enriched in either

aromatic/hydrophobic or hydrophobic/basic residues, which are exposed during translation and membrane translocation, or following stress-induced damage (25,26). Peptide binding is regulated by the binding of ATP. Nucleotide binding induces conformational changes in the conserved ATPase domain which are transmitted to the C-terminal peptide-binding domain, and modulate the intrinsic peptide affinity. The ATP-bound form of Hsp70 binds and releases peptide rapidly, whereas the ADP-bound form binds and releases peptides slowly (27,28,29). Flexibility in the hsp70 peptide binding domains can only explain part of the diverse functions of hsp70. Recent research has suggested that specific accessory proteins, such as DnaJ and GrpE in E.coli, may help to determine the diverse chaperoning activities of hsp70 (30). These two accessory proteins have different actions on bacterial hsp70 homolog DnaK. DnaJ can present certain substrates to DnaK and stimulate the ATPase activity of DnaK, while GrpE seems to act as a nucleotide exchange factor, allowing rebinding of ATP that in turn promotes substrate dissociation (29,31,32).

Specific regulators of hsp70--DnaJ

Although a eukaryotic GrpE homolog has only been found in mitochondria (33), DnaJ homologs have been identified in all compartments of eukaryotic cells that contain hsp70 (34). All DnaJ-homologs contain a J-domain, which is proposed to mediate interactions with hsp70 that regulate ATPase

activity. Interactions with polypeptides are proposed to be mediated through regions in the C-terminus of the protein, which is much less conserved.

DnaJ-homologs and hsp70 have different substrate specificities. Hsp70 proteins bind unfolded proteins, recognizing short stretches of amino acids with extended conformation; while DnaJ-homologs bind protein substrates exhibiting secondary and tertiary structure (35). These differences in substrate specificity allow DnaJ-homologs to facilitate the interaction of nascent proteins with hsp70, which would not normally be bound. DnaJ-homologs are able to facilitate both the dissociation and the stabilization of complexes between hsp70 and polypeptides substrates. If DnaJ-homologs can form a complex with a substrate protein, they will probably stabilize complex formation between hsp70 and that substrate protein (36). However, if a DnaJ-homolog has a low affinity for polypeptide substrate of hsp70, or lacks the conserved regions in the C-terminus of E.coli DnaJ, it is likely to stimulate the dissociation of complexes between hsp70 and polypeptides by stimulating hsp70 ATPase activity (37).

Regulation of protein synthesis by HRI

When reticulocyte lysates are deprived of hemin, inhibition of protein synthesis occurs after about a 5-min

lag (38). This inhibition is caused by the heme-regulated inhibitor (HRI), a protein kinase specific for the α-subunit of eIF-2. When eIF-2 completes a round of initiation, it is ejected from the ribosomes as an eIF-2•GDP complex. For the factor to recycle and promote another round of initiation, GDP must be replaced by GTP in a reaction catalyzed by eIF-2B. Phosphorylated eIF-2 does not undergo the eIF-2B-catalyzed guanine nucleotide exchange reaction but rather inhibits eIF-2B by sequestering it (39). Since eIF-2B is present in cells at relatively low level, about 30% phosphorylation of eIF-2α is sufficient to inhibit all of the eIF-2B and thus to severely inhibit protein synthesis.

HRI is present in heme-supplemented reticulocyte lysates in an inactive form. Activation of HRI is accompanied by its autophosphorylation (40). Hemin binds to HRI and prevents its autophosphorylation and eIF-2α kinase activity (41). The inhibition by hemin may be due to the formation of disulfide cross-links between the subunits of the native dimer (42).

Hsps and regulation of protein synthesis

Activation of HRI also occurs in hemin-supplemented lysates in response to a variety of conditions that include oxidative stress, heat stress and heavy metal ion treatment (43). Three heat shock proteins, hsp90 and hsp70 and a 56KD

protein, have been shown to interact with HRI in reticulocyte lysates (43).

The amount of hsp70 in any given lysate is variable and higher concentrations of hsp70 correlate closely with more active protein synthesis by lysates. Poorer protein synthesis in lysates with low hsp70 appears to result from an elevated level of eIF-2α phosphorylation. The presence of hsp70 seems to correlate with low eIF-2α phosphorylation. It has been demonstrated that protein synthesis in reticulocyte lysate is inhibited by denatured protein but not by much higher amounts of native proteins (44). Addition of denatured protein to lysates leads to dissociation of hsp70 from HRI, elevation of HRI activity, phosphorylation of eIF-2α and inhibition of protein synthesis. Based on above observations, we propose that the stress-induced translational repression and recovery may be regulated through the ability of hsp70 to suppress the activation of HRI; the accumulation of denatured or unfolded nascent polypeptides during stress conditions would compete with HRI for hsp70, and lead to the activation of HRI and the cessation of protein synthesis.

This thesis is to investigate further the relation of hsp70 and the regulation of protein synthesis. Rabbit reticulocyte lysate (RRL) was used as the model system not only because of its unique capabilities for in vitro protein synthesis, but also because it contains high amounts of

molecular chaperones which are involved in protein folding. Two peptides which have high affinity for hsp70 were used, one is a basic peptide NIVRKKK (PN) , the other is a hydrophobic peptide FYQLALT (PF). The effects of these two peptide on protein synthesis in RRL, as well as their effects on eIF-2α phosphorylation and eIF-2B activity were examined. The results are quite surprising, that PN inhibits protein synthesis in RRL, induces eIF-2α phosphorylation and inhibits eIF-2B activity; while PF have no effect on protein synthesis or eIF-2α phosphorylation or eIF-2B activity. Therefore, we further compared the chaperone binding abilities of these two peptides and their effects on interaction of HRI with different chaperones by immunonprecipetation. Our results indicate that the hsp70 cohort DnaJ-homolog is also required to suppress HRI activation and the function of DnaJ-homolog is to recruit hsp70 binding to HRI. During the investigation, we also found that although PF did not inhibit protein synthesis, it inhibited [^{14}C]leu incorporation into acid-perceptible protein and the inhibition was due to the reduced [^{14}C]leu-tRNA charging.

CHAPTER II

Materials and Methods

Materials

Reticulocyte lysate was prepared from anemic rabbits, using buffered saline containing 5 mM glucose to wash the reticulocytes before their lysis (45). TnT coupled reticulocyte lysate was obtained from Promega. Nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), bovine tRNA, p-nitrophenyl chloroformate-activated Agarose, bromoacetic acid N-hydroxysuccinimide ester, and aminoethyl-Sepharose were obtained from Sigma. Alkaline phosphate conjugated to goat anti-rabbit IgG and rabbit anti-mouse IgG was obtained from ICN. Chicken IGY was obtained from Jackson ImmunoResearch. L-[^{14}C]Leucine and L-[35S]Methionine were obtained from Du Pont-New England Nuclear. Autofluor was obtained from National Diagnostics. eIF-2 was isolated from reticulocyte lysate obtained from Green Hectares as described previously (46). Rabbit 4322 anti-hsp90/hsp70 polyclonal antiserum was provided by Dr. Stephen J. Ullrich in National Cancer Institute (47). Anti-DnaJ was provided by Dr. William Welch at University of

California. YDJ-1 was provided by Dr. David Toft at Mayo Medical School. PN (NIVRKKK) and PF (FYQLALT) were obtained from Sarkeys Biotechnology Research Laboratory at OSU.

Buffers

TB, 10 mM Tris-HCl (pH 7.5); TB/50, 10 mM Tris-HCl (pH7.5) containing 50 mM NaCl; TBS, 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl; TB/500, 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl; TB/50/Tween, TB/50 containing 1% Tween-20; gradient buffer, 25 mM Tris-HCl (pH 7.4) containing 100 mM KCl, 1.5 mM $Mg(OAc)_2$ and 2 mM EDTA; dilution buffer, 40 mM Tris-HCl, pH 7.4, 100 mM KCl, 50 mM KF, 2 mM $Mg(OAc)_2$, 10% glycerol, 40 µM GDP; wash Buffer, 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM Mg(OAc)2; 1xSDS sample buffer, 62.5 mM Tris-HCl (pH 6.8), 5% SDS, 90 mM dithithreitol, 5% glycerol and 0.005% Bromophenol Blue; alkaline phosphatase reaction buffer, 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl, 100 mM Mg(OAc)2, 300 µg/ml NBT and 150µg/ml BCIP.

Methods

Protein Synthesis Assay- Protein synthesis assay was carried out at 30°C in standard reticulocyte lysate reaction mixture with or without the addition of [^{14}C]Leucine or [^{35}S]Methionine as described by Ernst et al (48). Hemin-supplemented lysates contained 10 µM hemin-HCl. Protein

synthesis was determined by liquid scintillation, measuring the incorporation of [^{14}C]Leucine or [^{35}S]Methionine into the acid precipitable protein at 30°C in standard reticulocyte reaction mixture (48).

Polyribosomal profiles- Protein synthesis mixes (100 µl) were pretreated with or without PN or PF at 30°C for 15 min. These reaction mixes were diluted with 150 µl of ice cold gradient buffer and laid over a 15%-40%(w/v) sucrose gradient and centrifuged at 45,000 rpm in a Sorvall AH-650 rotor for 1 hour. Polyribosomal profile in the gradient was analyzed as decried previously (45).

Polyribosomal run off assays- Protein synthesis mixes were incubated at 30°C for 5 min with or without PN or PF, a protein synthesis initiation inhibitor ATA (120 µM) was added to the protein synthesis mixes, and mixes were incubated for an addition 1.5 min at 30°C. 100 µl of these protein synthesis mixes were diluted and applied to polyribosomal gradient, and polyribosomal profile were analyzed. Since initiation of protein synthesis was completely stopped by ATA, failure to run off polyribosome indicates an inhibition of elongation phase of translation.

Polyacrylamide gel for Separating Low Molecular Weight polypeptides- Separation gels for resolving low molecular weight proteins contained 20% polyacrylamide with 0.5%

crosslinking (200:1 acrylamide:bis), 10% glycerol, 0.1% SDS, and 0.75 M Tris-HCl (pH 9.3). Stacking gel contained 10% polyacrylamide (20:1 acrylamide:bis), 10% glycerol, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8). Gels were run at constant current over-night. After electrophoresis, the gels were fixed for 1 hour in 50% ethanol and 10% acetic acid, and then fixed in 10% glutaraldehyde for 12 hours. Gels were then washed for 3 hours with water, incubated in Autofluor for 2 hours, vacuum dried and exposed at -70°C.

eIF-2α Phosphorylation assays- Protein synthesis mixes were incubated at 30°C for 15 min with or without PN or PF. Two µl of these mixes were separated on an isoelectric focusing gel, western blotted and analyzed for phosphorylation of eIF-2α, as described previously by Maurides (49).

Guanine nucleotide exchange assay for measuring eIF-2B activity - RRL were incubated under protein synthesizing conditions at 30°C for 20 min with or without PN or PF. The eIF-2B activity was measured as described previously (50). Briefly, at the end of 20 min, 50 µl of protein synthesis mix was mixed with 130 µl dilution buffer, and 20 µl of performed eIF-2 • [^{3}H]GDP complex. Reaction mixes were incubated at 30°C for additional 2 min. Exchange assays were stopped by the addition of 1 ml ice cold wash buffer, followed by filtration of reaction mixture through nitrocellulose filters (HAWP

02500, millipore) which rapidly binds the remaining eIF-2•[^{3}H]GDP complex. Filters were washed with 15 ml of ice cold wash buffer to remove the unbound [^{3}H]GDP. The amount of remained eIF-2•[^{3}H]GDP bound on the filters was determined by liquid scintillation counting.

Cross-linking PN to agarose- Because PN (NIVRKKK) has 3 basic residues in the sequence, it can not be cross-linked through its N-terminus to agarose. Therefore a Cys and three Gly were added to the C-terminal of PN (PNC), so that it can by cross-linked through SH- group of Cys. First aminoethyl-Sepharose was activated with bromoacetic acid N-hydroxysuccinimide ester (BAHE) by incubating 2 ml of packed resin together with 34 mg BAHE (dissolved in 250 µl of DMF) in 0.1 M $NaPO_4$ buffer (pH 7.5) containing 1 mM EDTA at 4°C for 2 hours. Then the resin was washed with ice cold 0.1 N NaCl, followed by washing with 0.1 M $NaPO_4$ buffer (pH 7.0) containing 1 mM EDTA (PBSE 7.0). PNC (10 µmol) was added into the resin with PBSE 7.0 buffer and incubated at room temperature with continuous shaking. The uncoupled resin was blocked by incubation with 0.2 M ß-Mercaptoethanol at room temperature for 24 hours with continuous shaking. Finally, the resin was washed with PBSE 7.0 buffer and stored in TBS. A negative control resin (NCR) for PN-Sepharose was prepared by activating the aminoethyl-Sepharose with BAHE, followed by blocking with 0.2 M ß-Mercaptoethanol.

Affinity adsorption with PN-SepharosE- RRL was incubated under protein synthesis conditions at 30°C for 20 min with or without addition of PN or PF. PN-sepharose (8 µl) or NCR was then added to 16 µl protein synthesis mixes, and incubation in 30°C was continued for an additional 10 min. The resin was then washed 5 times with TB/50, and boiled in 1xSDS sample buffer for 5 min, and analyzed by 10% SDS-PAGE and western-blotting.

Immunoadsorptions with anti-HRI antibody- Chicken anti-HRI IgY and non-immune chicken IgY were cross-linked to agarose (CaH-agarose and NIC-agarose) as previously described (43). The resin was washed with TB/50/Tween, TBS, TB/50/Tween, TBSx2 prior to use. RRL was incubated under protein synthesis conditions at 30°C for 20 min with or without PN or PF, washed resin (20 µl) was then added to 50 µl of protein synthesis mix and incubated on ice for 90 min. The resin was then washed 3 times with TB/50/Tween, boiled in 1xSDS sample buffer for 5 min and applied to 10% SDS-PAGE and western-blotted.

Western-blot analysis and autoradiography- After SDS-PAGE, proteins were transferred to PVDF membrane as previously described by Matts et al (43). Hsp70 and DnaJ were detected with 4322 anti hsp70/90 and anti-DnaJ antiserum, respectively. The radioisotope labeled protein was detected by exposure to X-ray film at -70°C.

Assay for tRNA charging- RRL was incubated under protein synthesis conditions at 30°C for 5 min with or without PN or PF in the presence of ATA. Bovine tRNA and [^{14}C]Leu or [^{35}S]Met were then added to the protein synthesis mixes. The mixes were incubated at 30°C for an additional 20 min. tRNA charging was determined by measuring the incorporation of [^{14}C]Leu or [^{35}S]Met or [^{3}H]lys into acid-precipitable tRNA by liquid scintilation counting.

Pulse-chase incorporation of[^{35}S]Met - Protein synthesis mixes were incubated at 30°C in the presence of [^{35}S]Met for the time indicated. Unlabeled Met was then added to the protein synthesis mixes, and incubation was continued at 30°C. Protein synthesis mix samples were withdrawn at times indicated. Samples were analyzed by 10% SDS-PAGE or 20% SDS-PAGE as described for analysis of low molecular weight proteins, followed by radioautography.

Peptide mapping of synthesized firefly-luciferase-- Firefly-luciferase was synthesized in the presence of [^{35}S]Met in TnT lysate with or without PF for 40 min at 30°C. ATA was added to stop initiation of translation, and lysate was incubated at 30°C for an addition 30 min, to ensure the synthesis of full length firefly-luciferase. Various amounts of trypsin were added to the protein synthesis mixes, which were then incubated on ice for 6 min. The reactions were

stopped by addition of an equal amount of 2x sample buffer and immediately boiled for 5 min. Samples were applied to 12% SDS-PAGE and analyzed by radioautography.

CHAPTER III

RESULTS

PART I

Effects of PN and PF on protein synthesis

It has been proposed that hsp70 is necessary to suppress the activation of HRI. Therefore, it would be predicted that peptides PN and PF would compete for the binding of hsp70 with HRI, and inhibit protein synthesis. Addition of PN inhibited protein synthesis in hemin-supplemented lysate. With the addition of increasing amounts of PN to protein synthesis mixes, the inhibition of incorporation of [^{14}C]Leu (fig.1) or [^{35}S]Met into hemoglobin was enhanced (fig.2). However, PF had no inhibitory effect on protein synthesis, measured as incorporation of [^{35}S]Met into acid-precipitable protein (fig.2). Curiously PF appeared to arrest translation completely when protein synthesis was measured by incorporation of [^{14}C]Leu (fig.3) (fig.4). The mechanism of this apparent inhibition of translation will be addressed in the second part of this Chapter.

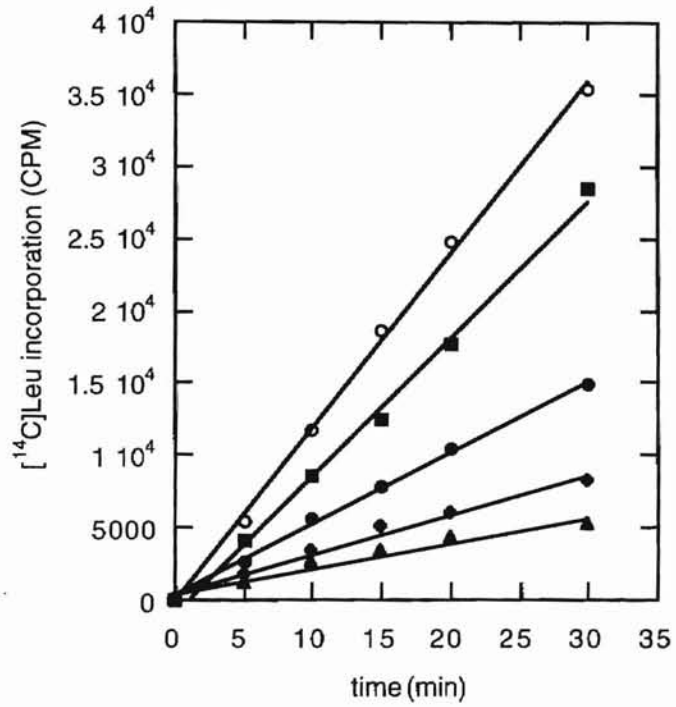


Figure 1. Effect of PN on protein synthesis measured by [^{14}C]leu incorporation

Protein synthesis mixes supplemented with 0 mM (o), 1.25 mM (■), 2.5 mM (●), 3.75 mM (◆), or 5 mM (▲) PN, were incubated at 30°C. protein synthesis was measured by following incorporation of [^{14}C] leucine into acid-precipitable protein by liquid scintilation at the times indicated.

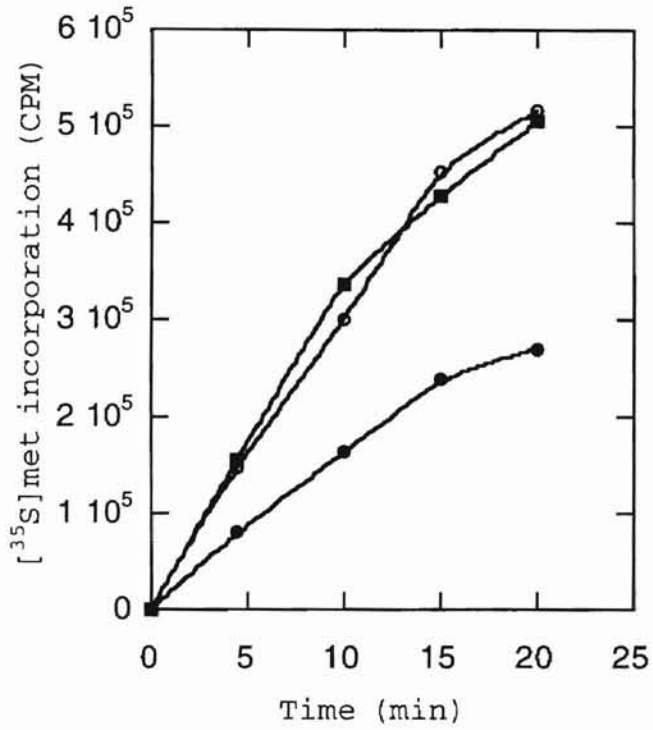


Figure 2. Effect of PN and PF on protein synthesis measured by [^{35}S]met incorporation

Protein synthesis mixes supplemented with DMSO (o), 5mM PN (●), or 0.25 mM PF (■), were incubated at 30°C. Protein synthesis was measured by following incorporation of [^{35}S]met into acid-precipitable protein by liquid scintillation at times indicated.

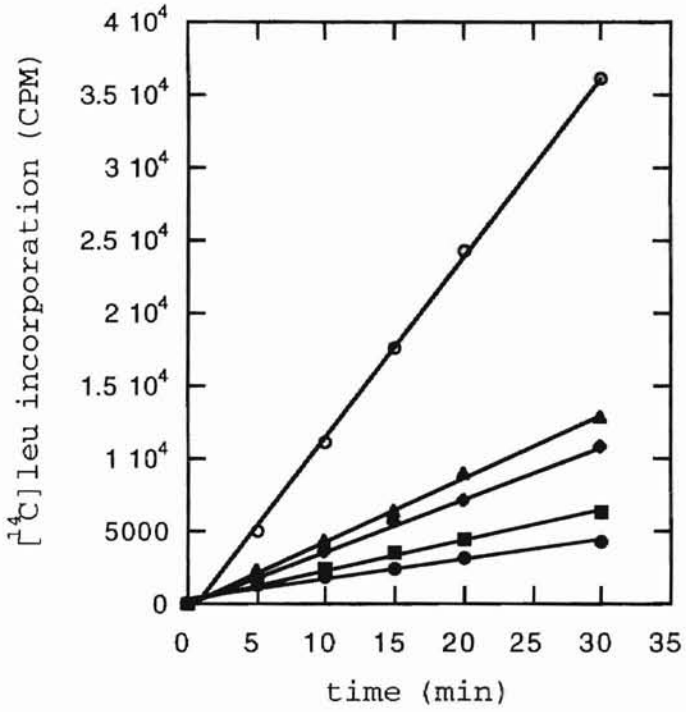


Figure 3. Effect of PF on protein synthesis measured by [^{14}C]leu incorporation

Protein synthesis mixes supplemented with 0 mM (o), 0.02 mM (▲), 0.025 mM (◆), 0.05 mM (■), or 0.1 mM (●) PF, were incubated at 30°C. Protein synthesis was measured by following incorporation of [^{35}S]met into acid-precipitable protein at times indicated.

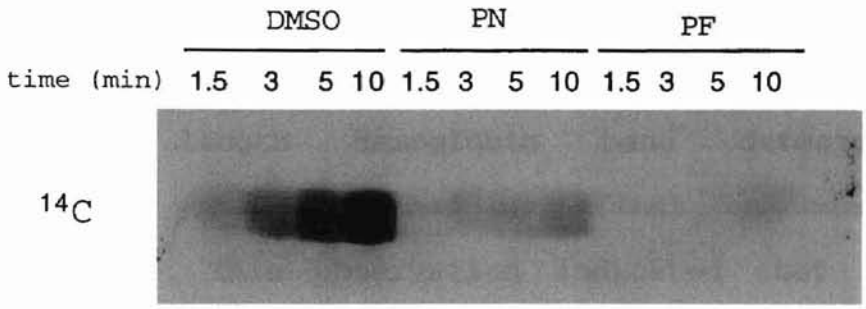


Figure 4. The effect of PN and PF on protein synthesis measured by [^{14}C]leu incorporation followed by SDS-PAGE.

Protein synthesis mixes were supplemented with DMSO (DMSO), 5mM PN (PN), or 0.25mM (PF), and incubated at 30°C.At the times indicated, aliquots (3µl) of protein synthesis mixes were denatured in SDS-sample buffer. Samples were analyzed by SDS-PAGE and autoradiography

The effect of the addition of PN on the rate of protein synthesis was immediate suggesting PN was inhibiting elongation. To verify that PN was inhibiting the elongation rate, the effect of PN on the rate at which pulse-labeled nascent chains of hemoglobin were chased into full length protein was examined. PN dramatically lowered the intensity of the full length hemoglobin band detected by autoradiography, while PF had no effect on hemoglobin synthesis (fig.5). This observation indicated that in the presence of PN, less [^{35}S]Met was incorporated during the pulse time, and/or the rate at which full length hemoglobin was synthesized was remarkably inhibited.

Since hemoglobin is a small protein with molecular weight of approximately 16kDa, differences in length of the polypeptides during protein synthesis can not be observed in 10% SDS-PAGE. Therefore, the pulse-chase samples were applied to a 20% acrylamide gel for separation of short polypeptides. Full length hemoglobin was synthesized within 1.5 min in control protein synthesis mix and in the protein synthesis mix containing PF (fig.6), while no full length hemoglobin was synthesized within 1.5 min in the protein synthesis mix containing PN. (fig.6). This suggests that PN inhibited the rate of elongation, while PF has no effect on protein synthesis.

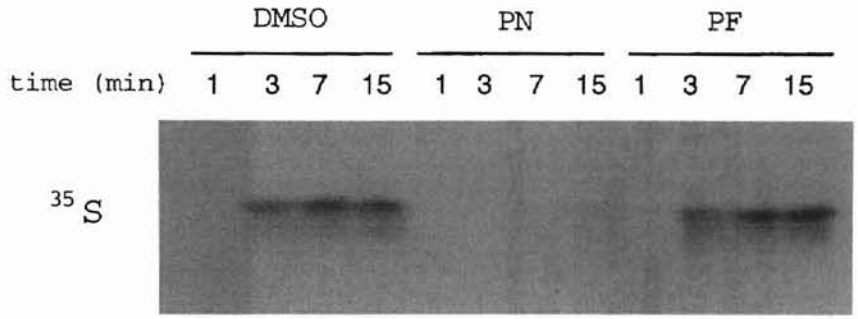


Figure 5. The effect of PN and PF on protein synthesis determined by pulse-chase with [^{35}S]met and SDS-PAGE

Protein synthesis mixes, supplemented with DMSO (DMSO), 5mM PN (PN), or 0.25mM PF (PF), were incubated in the presence of [^{35}S]met, at 30°C for 1 min. Cold met was then added, and aliquots(0.3µl) of protein synthesis mixes were denatured in SDS-sample buffer at times indicated and analysed by SDS-PAGE and autoradiography.

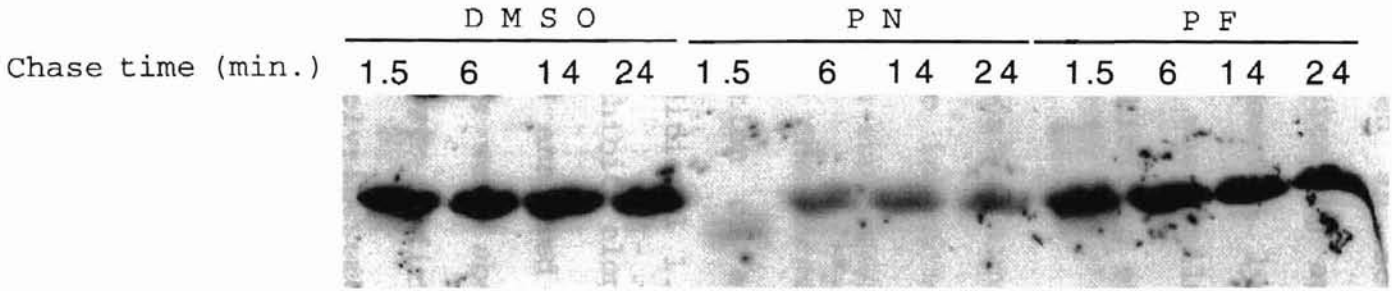


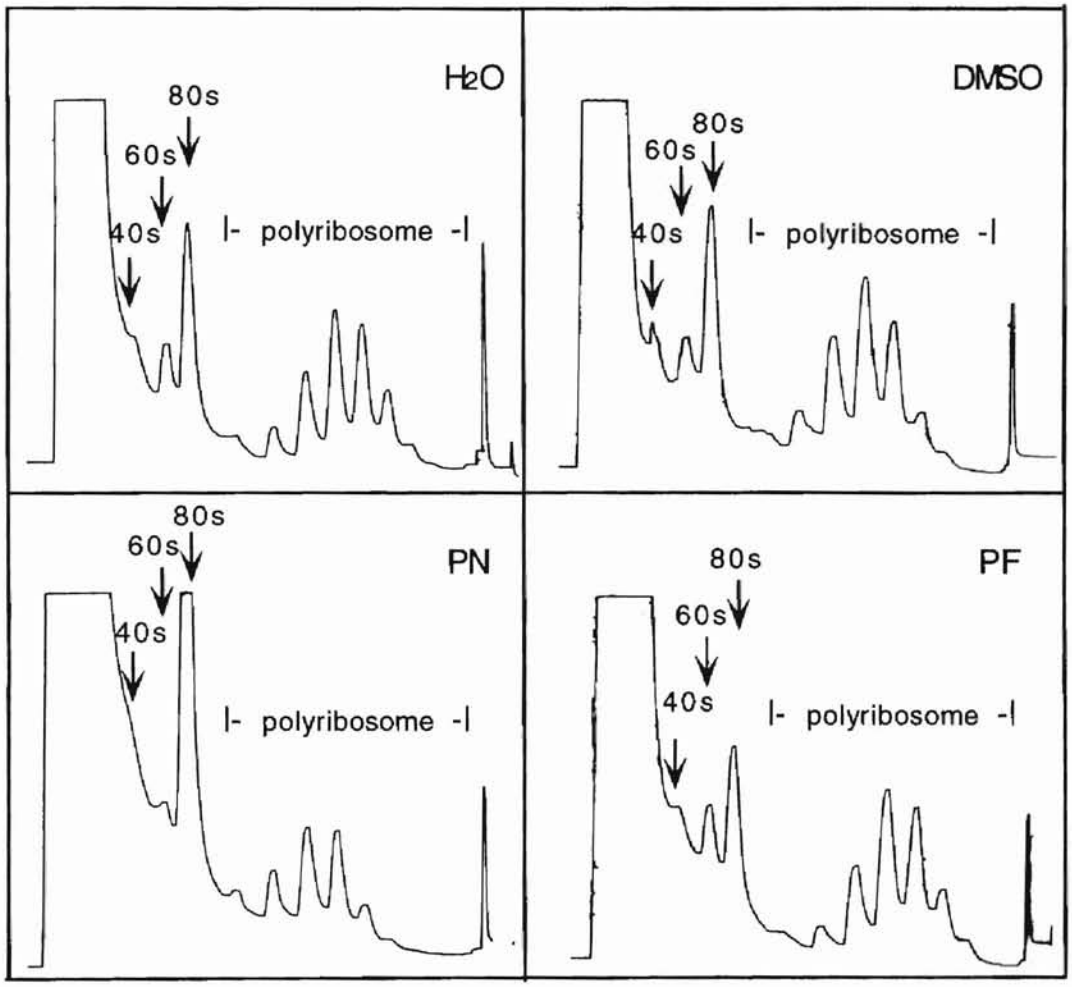
Figure 6. The effect of PN and PF on protein synthesis determined by pulse-chase with [^{35}S]met followed by analysis on a 20% SDS-gel.

Protein synthesis mixes, supplemented with DMSO (DMSO), 5mM PN (PN), or 0.25mM PF (PF), were incubated in the presence of [^{35}S]met at 30°C for 1 min. Cold met was then added, and aliquots (10µl) of protein synthesis mixes were denatured in SDS sample buffer at times indicated and analysed by SDS-PAGE and autoradiography

To more fully characterize the effects of PN and PF on protein synthesis in hemin-supplemented lysate, polyribosomal profiles were analyzed. Addition of PN to protein synthesis mixes, caused an accumulation of inactive 80S ribosome (fig.7), indicating an inhibition of initiation of protein synthesis. However, lysate containing PF had a polyribosomal profile that was similar to the DMSO control, confirming that PF has no inhibiting effect on protein synthesis (fig.7).

Polyribosomal run-off assays were carried out to confirm that PN also inhibited protein synthesis at the elongation stage. Hemin-supplemented protein synthesis mixes were pre-incubated in the presence of DMSO (controls), PN or PF for 5 min. Initiation of translation then was arrested by ATA. After an additional 1.5 min of incubation, cycloheximide was added to inhibit elongation and the extent of polyribsome runoff was analyzed by sucrose density centrifugation. Polyribosomes were absent from control lysates and lysates containing PF, confirming PF had no effect on elongation. However, polyribosomes were still present in lysate containing PN, indicating that polyribosome run off was delayed (fig. 8).

These results indicate that PN inhibited protein synthesis at both the initiation and elongation stages, while PF had no effect on protein synthesis.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60s
40s
I- polyribosome -I
DMSO
80s
60s
40s
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PN
80s
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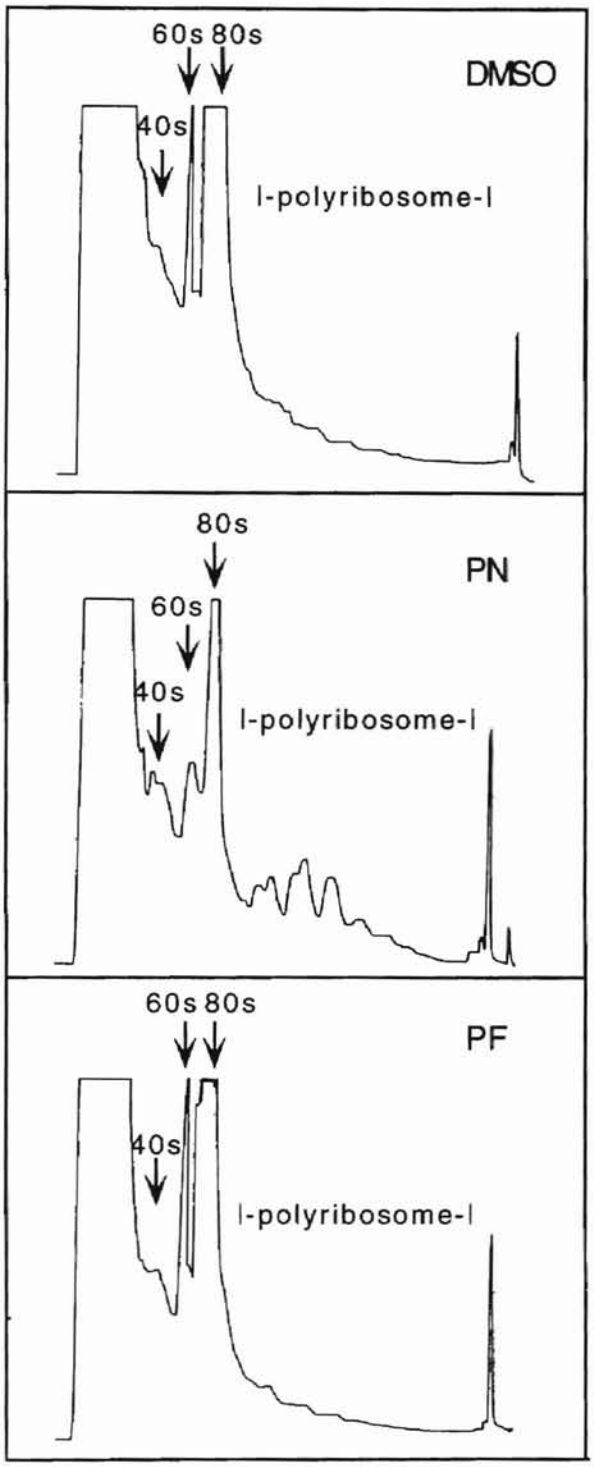
I- polyribosome -I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40s
I-polyribosome-I

The effects of PN and PF on eIF-2α phosphorylation and eIF-2B activity

According to our hypothesis, agents that compete for the binding of hsp70, will block the binding of hsp70 to HRI, leading to the activation of HRI, the phosphorylation of eIF-2α and inhibition of eIF-2B activity. Hence, the effects of PN and PF on eIF-2α phosphorylation and eIF-2B activity were examined.

Protein synthesis mixes were incubated in the presence of PN and PF. Samples were then analyzed by isoelectric focusing, followed by western blotting to determine the phosphorylation state of eIF-2α. Phosphorylated eIF-2α focuses at a more acidic pH than non-phosphorylated eIF-2α. Incubation of hemin-supplemented lysate in the presence of PN induced the phosphorylation of eIF-2α (fig.9), while no eIF-2α phosphorylation appeared in control lysates or lysates containing PF (fig.9). This indicated that PN induces the activation of HRI and the phosphorylation of eIF-2α, while PF has no effect on activation of HRI.

Phosphorylation of eIF-2α impairs the ability of eIF-2B to catalyze guanine nucleotide exchange. The activity of eIF-2B to exchange GDP for GTP from the eIF-2•GDP complex was examined to determine whether the level of eIF-2α phosphorylation induced by PN was sufficient to impair the

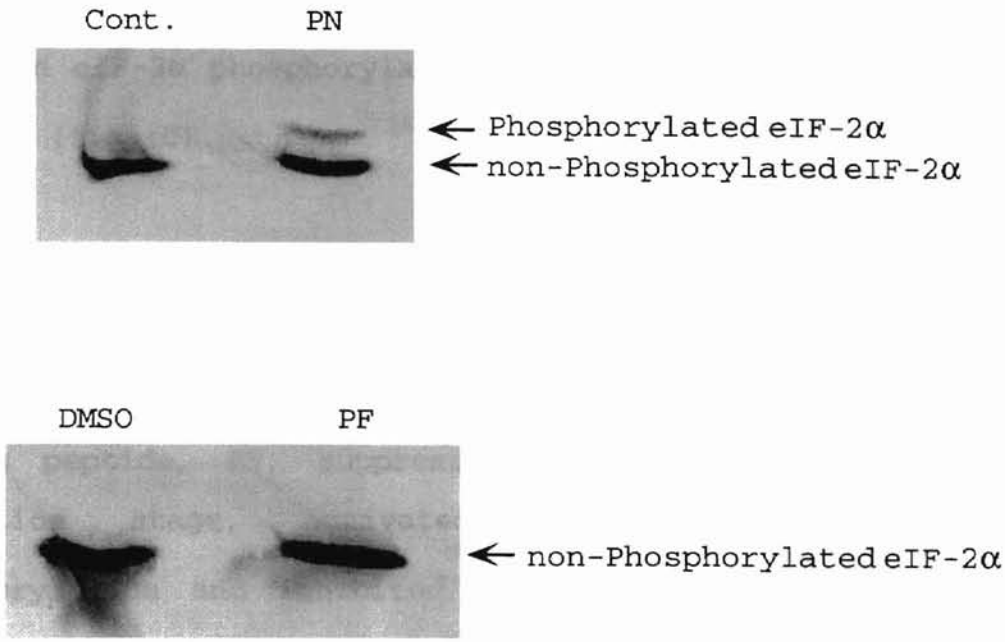

Figure 9. The effects of PN and PF on eIF2-α phosphorylation.

Protein synthesis mixes were supplemented with no addition (cont.), 5 mM PN (PN), DMSO (DMSO), or 0.25 mM PF (PF), and incubated at 30°C for 15 min. Aliquots (2 μl) of protein synthesis mixes were applied to IEF gels and analyzed as described in Materials and Methods.

activity of eIF-2B. In the presence of PN, eIF-2B was decreased to approximately 30% as active as the control (fig.10). Consistent with the observation that PF had no effect on eIF-2α phosphorylation, PF had no effect on eIF-2B activity (fig.10).

Identification of the PN and PF associated hsps

Consistent with our hypothesis, the putative hsp70 binding peptide, PN, suppressed protein synthesis at the initiation stage, activated HRI, stimulated eIF-2α phosphorylation and inhibited eIF-2B activity. However, it was surpassing that the hsp70 binding peptide, PF, had no effect on protein synthesis and HRI activity. To elucidate the difference between the properties of these two peptides on protein synthesis, we investigated what chaperones were actually bound by PN and PF. PN was modified at the C-terminus by adding 3 Gly and 1 Cys, so that this modified PN could be cross-linked to Sepharose. Proteins were then adsorbed to the PN-Sepharose. Bound proteins were eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting to identify bound proteins. The results showed that in addition to hsp70, which has been previously reported to bind PN, PN also bound to two DnaJ-homologs (fig.11). The binding was specific, since when soluble PN was added to the protein synthesis mix, both hsp70 and DnaJ-like protein were competed off the PN-Sepharose (fig.11). When soluble PF was

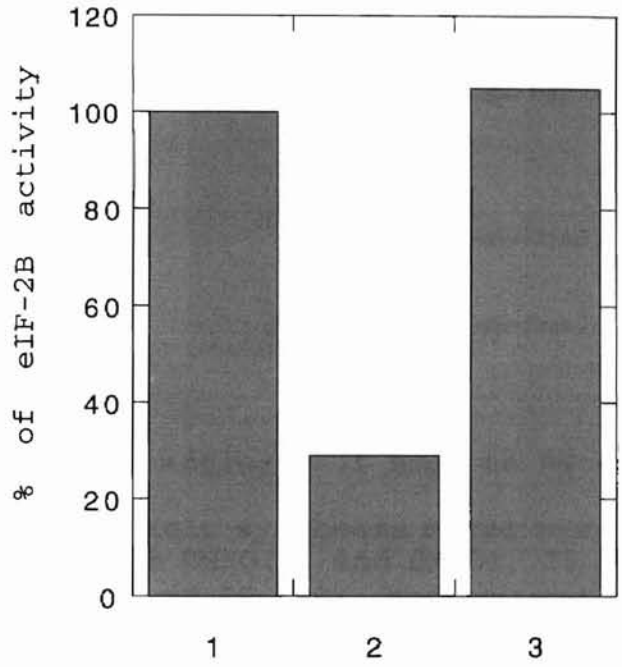

Figure 10. The effects of PN and PF on eIF-2B activity

Lysates were preincubated with no additions (1), 5 mM PN (2), or 0.25 mM PF (3) for 20 min at 30°C. The activity of eIF-2B was determined as described in Materials and Methods.

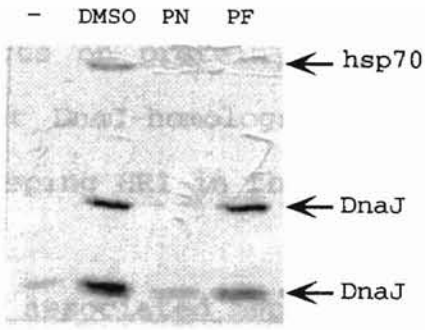

Figure 11. The affinity of hsps to PN and PF

Protein synthesis mixes were supplemented with DMSO(-, and DMSO), 25 mM PN (PN), or 2.5 mM PF (PF), and incubated at 30°C for 20 min.Proteins from the incubations were then adsorbed to negtive control resin (-), PN-Sepharose [(DMSO), (PN), or (PF)] as described in Materials and Methods.

added to protein synthesis mix, only the binding of hsp70 to PN-Sepharose was blocked (fig.11). This observation suggests that PF had affinity specifically to hsp70 and lacked affinity to the DnaJ-homologs. The differences in the interaction of PN and PF with DnaJ-homologs may contribute to their different effects on protein synthesis and HRI. These results suggested that DnaJ-homologs might interact with HRI and play a role in keeping HRI in the inactive form.

Identification of HRI associated hsps

In order to confirm whether a DnaJ-homolog was associated with HRI, anti-HRI chicken IgY was cross-linked to agarose and used to immunoadsorb HRI. Samples were analyzed by SDS-PAGE and western blotted with anti-hsp70 and anti-DnaJ antiserum. The results showed that in addition to hsp70, a 40kDa DnaJ-homolog was associated with HRI (fig.12). When PN was added to the protein synthesis mix, both hsp70 and DnaJ-like protein were competed off from HRI (fig.12). However, PF did not compete off hsp70 or the DnaJ-homolog from association with HRI (fig 12). This result suggests that both hsp70 and the DnaJ-homolog may be required to suppress HRI from activation.

The effects of hsp70 and DnaJ on protein synthesis in heme-deficient lysate

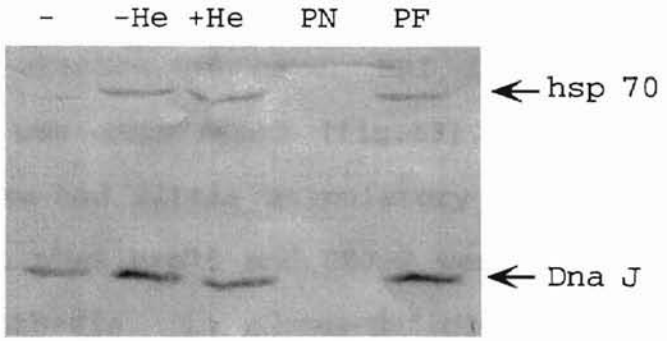

Figure 12. Detection of HRI associated hsps

Protein synthesis mixes were incubated in the absence of heme (-He), or in the presence of heme with no additions (+He), with added PN (PN), or with added PF (PF). HRI from each incubation was immunnoadsorbed with control NIC-agarose(-), or CaH-agarose [(-He), (+He),(PN),or (PF)]as described in Materials and Methods. Proteins co-adsorbing with HRI were detected by western blotting.

Hsp70 has been demonstrated to suppress the activation of HRI (44). DnaJ-homologs have been reported to stimulate hsp70 function. Therefore, we examined the effects of hsp70 and HDJ-2, a human DnaJ homolog, on protein synthesis in hemin-deficient lysate, when added alone or when added together. In the absence of heme, HRI is activated, and protein synthesis was suppressed (fig.13). The addition of hsp70 or HDJ-2 alone had little stimulatory effect on protein synthesis, however, when hsp70 and HDJ-2 were added together, the protein synthesis in heme-deficient lysate was dramatically enhanced (fig.13). These results suggested that hsp70 and DnaJ homologs may act in concert to keep HRI inactive.

PART II

Effect of PF on [^{14}C]Leu incorporation

The results in the first part of this chapter showed that PF had no effect on hemin-supplemented protein synthesis at initiation stage (fig.7) or elongation stage (fig.8), and had no effect on [^{35}S]Met incorporation (fig.2), while PF dramatically inhibited [^{14}C]Leu incorporation into hemoglobin (fig.3). In order to address the mechanism of this inhibition, the amino acid sequence of hemoglobin was carefully studied. Within the 142 amino acids, there were

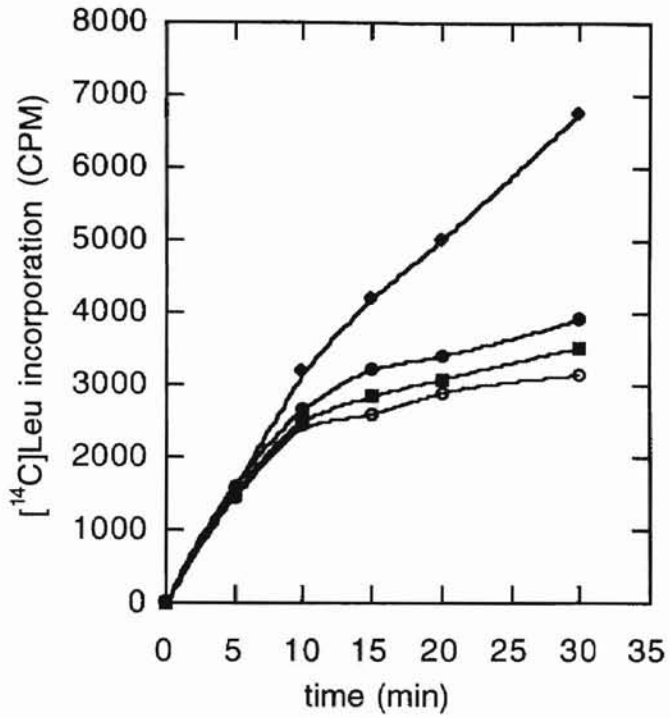

Figure 13. The effect of hsp70 and HDJ-2 on protein synthesis in heme-deficient lysate

Protein synthesis mixes were incubated at 30°C, in the absence of heme with no additions (o), or with added hsp70 (●), HDJ-2 (■), or Hap70/HDJ-2 (◆), [^{14}C]leu incorporation into acid-precipitable protein at times indicated as described in the Materials and Methods.

only 2 Methionines, one coded by the start codon and one at position 32. However, there were 17 Leu, and 14 of these 17 Leu were located in the C-terminal half of the hemoglobin from position 70 to 141. Therefore, it was possible that PF might cause premature termination of protein synthesis. PF might not affect the rate of [^{35}S]Met incorporation, since Met is located near the N-terminal of hemoglobin, but might inhibit [^{14}C]Leu incorporation, because most of the Leu are located in the C-terminal half of hemoglobin. To test this hypothesis a shorter time course pulse-chase experiment was carried out. Samples were prepared every 15 seconds in the first 1 min of incubation, and analyzed on 20% acrylamide gel. Protein synthesis in the presence of PF showed similar polypeptide bands distribution and intensity compared to the control at the corresponding time (fig.14). This result suggested full length hemoglobin was synthesized in the present of PF. Therefore, PF did not cause premature termination of protein synthesis.

These observations confirm that PF had no effect on translation. Thus, the inhibition of [^{14}C]Leu incorporation into hemoglobin might be due to the ability of PF to inhibit [^{14}C]Leucyl-tRNA charging, a step prior to incorporation. To test this hypothesis the effect of PF on [^{14}C]Leu-tRNA and [^{35}S]Met-tRNA charging was examined and compared with that of PN. PF dramatically inhibited the [^{14}C]Leu-tRNA charging (fig.15), while PN which also inhibited [^{14}C]Leu incorporation

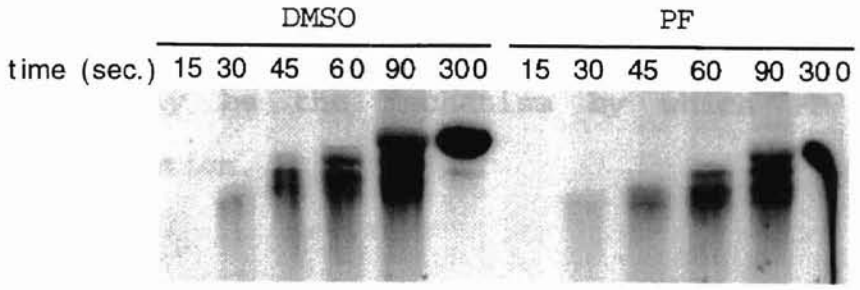

Figure 14. The effect of PF on protein synthesis determined by short time course pulse-chase of [^{35}S]met, followed by 20% SDS-Gel.

Protein synthesis mixes, supplemented with DMSO (DMSO), or 0.25mM PF(PF) were incubated at 30°C for 75 sec, in the presence of [^{35}S]met, Cold met was then added, and aliquots (12µl) of protein synthesis were denatured in SDS sample buffer at times indicated and analysed by 20% SDS-Gel followed by autoradiography.

(fig.1), did not significantly affect [^{14}C]Leu-tRNA charging (fig.15). Neither PN nor PF had an effect on [^{35}S]Met-tRNA charging (fig.16). These results confirm that the inhibition on [^{14}C]Leu or [^{35}S]Met incorporation into hemoglobin that was caused by PN occurred at the translation stage of protein synthesis, and it was not due to inhibition of tRNA charging. However, PF specifically inhibited [^{14}C]Leu-tRNA charging, and this might likely be the mechanism by which PF reduced [^{14}C]Leu incorporation.

While [^{14}C]Leu-tRNA charging was inhibited by PF and the incorporation of [^{14}C]Leu into hemoglobin was reduced, full length hemoglobin was synthesized at a normal rate. This observation suggested that PF might stimulate the mis-incorporation of some amino acids in place of Leu into hemoglobin. Specifically, the CUG codone coding for Leu in some cases can be utilized by the Met-tRNA. In order to test this hypotheses, peptide mapping of firefly luciferase synthesized in the presence of PF was analyzed. Full length firefly luciferase was synthesized in the presence of [^{35}S]Met with or without PF. Various amounts of trypsin were added to the protein synthesis mixes to cut the firefly luciferase into small segments. Samples were analyzed by 12% SDS-PAGE and autoradiography. The distribution and intensity of peptide bands of firefly luciferase in the presence of PF were identical with that of the control (fig.17). This observation indicates that PF does not cause mis-

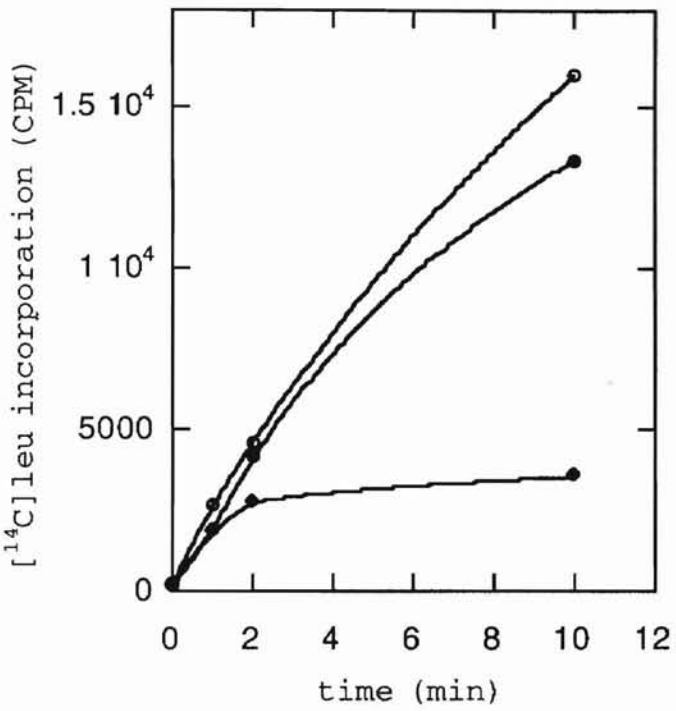

Figure 15. The effect of PN and PF on leu-tRNA charging

Lysates were pre-incubated at 30°C for 5 min, in the presence of ATA. Lysates were supplemented with no additions (o), or 5mM PN (●), or 0.25 mM PF (◆). and tRNA. Leu-tRNA charging was determined at the times indicated, as described in Materials and Methods.

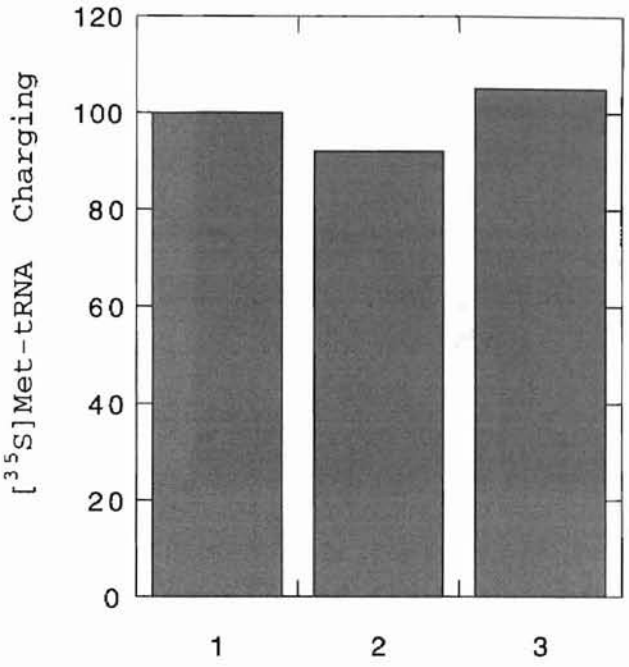

Figure 16. The effect of PN and PF on Met-tRNA Charging

Lysates were incubated at 30°C for 5 min in the precence of ATA. Lysates were then supplemented with tRNA and [^{35}S]met with no additions (1), 5mM PN (2), or 0.25 mM PF(3). and incubated at 30°C for 20 min. The Met-tRNA charging was determined by [^{35}S]met incorporated into acid-precipitable tRNA as described in Materials and Methods.

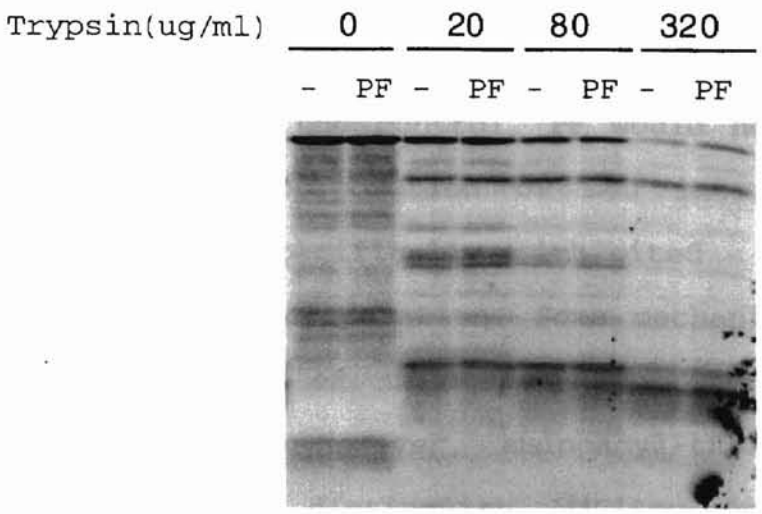

Figure 17. Peptide mapping of firefly-luciferase synthesized in TnT-lysate containing PF

Protein synthesis mixes were incubated at 30 °C for 40 min in the presence of [^{35}S]met, and DMSO (-), or 0.25 mM PF (PF). ATA was then added to each lysates and incubated at 30°C for an additional 30 min. Peptide-mapping was performed as described under Materials and Methods.

incorporation of Met for Leu, as the protein synthesized in the presence of PF had a peptide map identical to the control.

Since the protein synthesized in the presence of PF was apparently identical to the control, PF would not appear to inhibit the overall incorporation of Leu into hemoglobin. However, since PN specifically inhibited [^{14}C]Leu-tRNA charging and [^{14}C]Leu incorporation. Some mechanism by which selective incorporation of unlabeled Leu into hemoglobin would appear to exist. However, aminoacyl-tRNA synthetase should not be able to distinguish [^{14}C]Leu from [^{12}C]Leu. Therefore, the concentration of [^{14}C]Leu might be significantly diluted with [^{12}C]Leu upon hydrolysis of the added PF. Since PF contains two Leu. Partial degradation of PF might then provide additional [^{12}C]Leu, so that [^{14}C]Leu charging and incorporation are reduced upon addition of PF. In order to determine whether this was a feasible hypothesis, the degree to which quantitative hydrolysis of PF would dilute [^{14}C]Leu and inhibit the subsequent incorporation of [^{14}C]Leu into acid-precipitable protein was calculated (fig.3). Addition of 0.02 mM PF inhibited [^{14}C]Leu incorporation more than 60% (fig.3). Standard protein synthesis mixes contain 0.1 mM each of exogenously added amino acids, to supplement for any shortage of endogenous amino acids for protein synthesis. If we assume there was no endogenous Leu in RRL, then there will be 0.1 mM [^{12}C]Leu in

the protein synthesis mixes. If all the 0.02 mM PF was degraded into amino acids, there would be an additional 0.04 mM [^{12}C]Leu in the protein synthesis mix. Thus, the maximum % by which PF could inhibit [^{14}C]Leu incorporation due to PF degradation and dilution of the specific activity of [^{14}C]Leu can be calculated; set:

* the amount of [^{14}C]Leu incorporated in control as X.
* the amount of [^{14}C]Leu incorporated in the presence of PF as Y.
* the efficiency of Leu incorporation as a constant C.
* the concentration of [^{14}C]Leu added as 0.08 mM;
* the concentration of [^{12}C]Leu in control is 0.1 mM.
* the concentration of [^{12}C]Leu in the protein synthesis mixes in the presence of PF is (0.1 + 0.04) = 0.14 mM.

Then:

$$X = C * (0.08/(0.1 + 0.08)) = 0.444C$$

$$Y = C* (0.08/(0.14 + 0.08)) = 0.364C$$

$$\text{maximum \% of inhibition} = 1 - (Y/X)$$

$$= 1 - (0.364C/0.444C)$$

$$= 18.0\ \%$$

Therefore, if all PF added in RRL were immediately degraded, and the inhibition of PF on [^{14}C]Leu incorporation was simply due to the dilution of the [^{14}C]Leu pool, the maximum inhibition of [^{14}C]Leu incorporation by 0.02 mM PF would be 18.0 %. Since the actual inhibition of [^{14}C]Leu incorporation by 0.02 mM PF was more than 60 %, the

inhibition of [^{14}C]Leu-tRNA charging and incorporation into protein caused by PF was not merely due to its degradation.

With the above hypotheses excluded, we proposed that there is either more than one Leucine pool in the lysate for the charging of $tRNA^{Leu}$ or more than one pool of charged Leu-tRNA. Addition of PF may cause the lysate to switch to a pool of Leucine or charged Leu-tRNA, which is not used under normal conditions. To partially test this hypothesis, the effect of PF on protein synthesis in the absence of exogenous Leu was examined. In the absence of exogenous Leu, protein synthesis was markedly reduced when measured by [^{35}S]Met incorporation into acid precipitable protein (fig.18). This observation suggested that exogenously added Leu is the primary Leu pool used under normal conditions. According to our hypothesis, addition of PF to the lysate would switch the translation machine to use another pool of Leu or charged Leu-tRNA, so that absence of exogenous Leu would have less of an effect on the protein synthesis rate in the presence of PF. Addition of PF to protein synthesis mixes in the absence of exogenous Leu, stimulated protein synthesis in the absence of exogenously added Leu to the control level (fig.18). This observation supports the hypothesis that PF induces reticulocyte lysate to switch to the utilization of a pool of Leu or Leu-tRNA, which is different from the pool of Leu or Leu-tRNA that is used to synthesize proteins under the normal conditions.

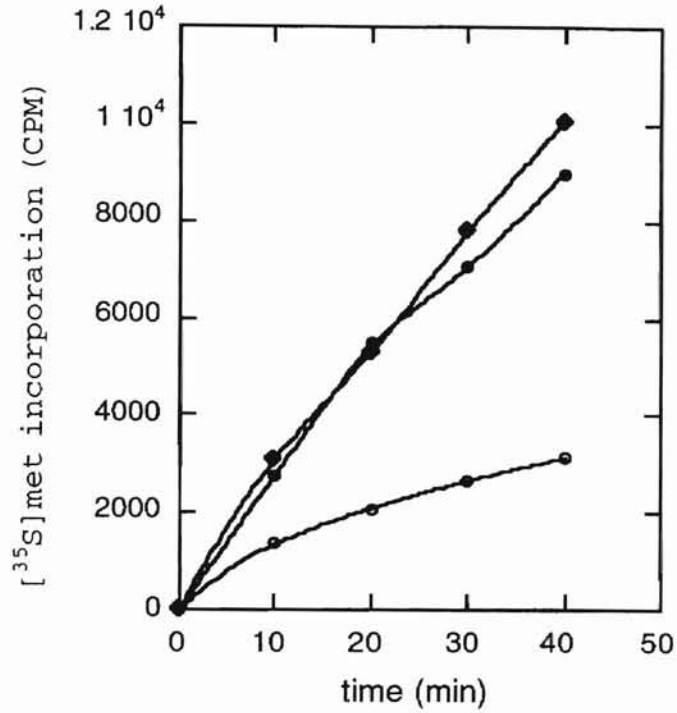

Figure 18. The effect of PF on protein synthesis in leu-deficient hemin-supplemented lysate.

Protein synthesis mixes were incubated at 30°C, in the presence of DMSO with (◆) or without (o) the addition of exogenous leu or in the presence of 0.025mM PF without exogenous added leu(●).Protein synthesis was determined at the times indcated by following [^{35}S]met incorporation into acid-precipitable protein as described in Materials and Methods.

CHAPTER IV

DISCUSSION

The Role of DnaJ-homolog and hsp70 in regulation of protein synthesis at initiation stage through HRI

During the examination of the effects of hsp70 binding peptides on regulation of protein synthesis, we found that DnaJ-homolog was associated with HRI, in addition to previously reported hsp70, hsp90 and p56 (43). Hsp70 and DnaJ-homolog from RRL bind specifically to PN (NIVRKKK) (fig.11), and block the association of hsp70 and the DnaJ-homolog with HRI (fig.12). This results in the activation of HRI as evidenced by elevated eIF-2α phosphorylation (fig.9), inhibited eIF-2B activity (fig.10) and suppressed protein synthesis (fig.1,2,3,4,6,7). In contrast, PF (FYQLALT) only specifically blocked the binding of hsp70 to PN-agarose (fig.11), and had no effect on the interaction of hsp70 or the DnaJ-homolog with HRI (fig.12), and had no effect on protein synthesis. The differences in the binding affinity of the DnaJ-homolog for PN versus PF may contribute to the differences observed between these two peptides on blocking the association of hsp70 and DnaJ-homolog with HRI, and on

the regulation of protein synthesis. These observations suggested that DnaJ-homolog together with hsp70 may be required to suppress HRI activity and to mediate stress induced inhibition of protein synthesis.

PN and PF have been reported to bind different conformations of hsp70. PN contains hydrophobic residues followed by basic residues, while PF contains only hydrophobic residues. Moreover, in a non-denaturing polyacrylamide gel, the complex of hsp70 and PF moves faster than uncomplexed hsp70, and uncomplexed hsp70 moves faster than the complex of hsp70 and PN (26). Furthermore, PF stimulates the ATPase activity of hsp70 while PN does not (26). These observations indicated that a comformational change of hsp70 occurred upon binding to these peptides and the interactions of these two peptides with hsp70 may be fundamentally different, and therefore may affect the hsp70 function differently.

There are two possible explanations for the relationship between hsp70 and the DnaJ-homolog, and their interactions with peptide substrates and their effect on the functions of hsp70. (i) Hsp70 may recognize two different amino acid sequence motifs by having two different substrate binding sites, in order to serve its different role in chaperoning proteins. PF-like binding site may be used in binding hydrophobic segments of proteins where relatively rapid

binding and release of the substrate must occur in conjunction with cycles of nucleotide exchange and hydrolysis, such as with the refolding of denatured proteins. While, the PN-like binding site may be used for transportation or chaperoning purpose, such as association with HRI, where longer-lived interactions are required and the interaction is mediated by its cohorts, such as DnaJ-homolog. This hypothesis is supported by the observation that PF had a greater ability to inhibit firefly-luciferase renaturation (IC50 0.3 mM) than PN (IC50 3 mM) (V. Thulasiraman, personal conmunication), where direct interaction between hsp70 and hydrophobic segments as well as rapid binding and release cycle may be required. However, PF had no inhibitory effect on the folding of nascent firefly-luciferase, where DnaJ-homologs interact with the luciferase peptides emerging from ribosomes first, followed by the binding of hsp70 which is directed to the nascent peptides through DnaJ-homolog (51). On the contrary, PN inhibited the folding of nascent firefly luciferase. Thus PN may bind to hsp70, which may require DnaJ-homolog to mediate its interaction and stabilize its binding. Therefore, PN may block the interaction of both hsp70 and DnaJ-homolog with substrate. HRI may bind to hsp70 at the same site as PN and may also require a DnaJ-homolog to mediate the formation and stabilization of the complex.

Alternately, PN may directly block the binding of the DnaJ-homolog to HRI. Since Coomassie blue staining of SDS-PAGE analysis of proteins bound to PN-agarose indicated that PN binds more DnaJ-homolog than hsp70. Hence, PN is a better competitor for the binding of DnaJ-homolog with HRI than PF. Inhibition of the interaction of DnaJ-homolog with HRI may block the subsequent binding of hsp70.

There are two kinds of mechanisms by which hsp70 binds to its substrate: first hsp70 may directly recognize and bind the peptide substrate (e.g. PF-like), and may or may not be required to associate with DnaJ-homolog to regulate the binding affinity of hsp70 for these substrates. Secondly, DnaJ-homolog may bind to the peptide substrate first, and subsequently direct the binding of hsp70 to bind the peptide (e.g. PN-like). Hsp70 may use these two different mechanisms to bind different substrate for different functions.

Another possible explanation for the differential effect of PN and PF in HRI activation is that the population of hsp70 that PN and PF both bind do not associate with HRI. Thus, the effect of PN on HRI would be solely due to its ability to bind DnaJ-homolog. Although hsp70 proteins have been shown to be highly conserved during evolution, they are not functionally interchangeable. For example, neither the endoplasmic reticulum hsp70 protein, Bip, nor the bacterial hsp70 protein, DnaK, can substitute for hsc70 in binding to

peptide sequences that target proteins for lysosomal degradation (52), or in facilitating in vitro translocation of proteins into mammalian microsomes (53). Peptide binding specificities of three different hsp70 family members, E.coli DnaK, bovine liver Bip, and bovine brain hsc70 have been compared by screening more than 50 synthetic peptides by Fourie et al(54). They concluded that hsp70 proteins have common, but also exclusive, specificities that are highly sensitive to peptide sequence; the functional diversity of hsp70 molecular chaperones may result from variations in their ability to bind to different target proteins. A number of cytoplasmic hsp70s have been identified. Since PF does not block the association of the hsp70 with HRI (fig.12), the hsp70 bound to HRI may not be the same hsp70 whose binding to PN-agarose is blocked by PF. However, since both PN and PF block renaturation of luciferase, the hsp70 family member to which PF and PN both bind probably participate in protein refolding.

Based on previous observations, we propose that both DnaJ-homolog and hsp70 are required to associate with HRI, in order to keep it inactive. The ability of PN to block the association of hsp70 and DnaJ-homolog with HRI may be mainly due to the ability of PN to sequester the DnaJ-homolog. Recognition and binding of the DnaJ-homolog to HRI may occur first, and then direct the association of hsp70 with HRI. Therefore, inhibition of DnaJ-homolog to HRI would result in

the inability of hsp70 to associate with HRI, and the activation of HRI.

The effect of PF on [^{14}C]Leu-tRNA charging and incorporation

PF inhibits the incorporation of [^{14}C]Leu into protein, but not [^{35}S]Met. Several mechanisms by which this inhibition might occure were tested. The observations suggest that the inhibition of PF on [^{14}C]Leu incorporation is not due to asymmetric incorporation, premature termination (fig.14), or mis-incorporation. Inhibition of PF on [^{14}C]Leu-tRNA charging appears to be the mechasm by which PF inhibits the incorporation of [^{14}C]leu into protein.

The inhibition of PF on [^{14}C]Leu-tRNA charging and incorporation into hemoglobin is not simply due to the rapid degradation of PF upon addition to RRL. If all 0.02 mM PF added in RRL were rapidly degraded, and the inhibition of [^{14}C]Leu incorporation was simply due to dilution of the [^{14}C]Leu pool, the maximum inhibition of [^{14}C]Leu incorporation by 0.02 mM PF would be 18.0%. The percent inhibition of [^{14}C]Leu incorporation by 0.02 mM PF actually observed was more than 60%. Thus, inhibition of PF on [^{14}C]Leu-tRNA charging and incorporation is not mearly due to PF degradation.

Although the inhibition of PF on [^{14}C]Leu incorporation is not solely because of its degradation, we can not rule out the possibility that part of PF is degradated. While part of PF may be degraded, it is not degradated rapidly, since PF inhibits luciferase refolding for up to 20 min of incubation. However, it is possible that the amino acids from protein degradation may be the primary source for charging tRNA. Thus, PF may be a more potent inhibitor of [^{14}C]Leu-tRNA charging than calculated, if $tRNA^{Leu}$ is preferently charged with amino acids derived from proteolysis.

Another hypothesis is that there is more than one pool of Leu-tRNA in the RRL: one that is used under the normal conditions, and one which is used under stress conditions. It has been documented that at least 9 mammalian aminoacyl-tRNA synthetases, namely those for Arg, Asp, Gln, Glu, Ile, Leu, Lys, Met and Pro exist in high-Mr complexes (55). It has been found that increased protein synthesis correlated with high stability of high-Mr aminoacyl-tRNA synthetase complexes. However, reduced protein synthesis correlated with partial or full dissociation of aminoacyl-tRNA synthetases from the high-Mr complex (56). Furthermore, it has been reported that the profile of Leucyl-tRNA isoacceptors obtained with the full complex was different from the one obtained with partially distrupted complex. One of the $tRNA^{Leu}$ species was especially poorly aminoacylated at low Leucine and enzyme concentration. Therefore, a mechanism may exist to coordinate

protein synthesis, with aminoacyl-tRNA synthetase complex stability and Leucyl-tRNA charging. The protein that may mediate this regulation might be hsp70. Hsp70 may be required to assemble or stabilize the aminoacyl-tRNA synthetase complex. The addition of PF to protein synthesis mixes may block the interaction of hsp70 with the aminoacyl-tRNA synthetase complex, which may lead to the disassociation of Leucyl-tRNA synthetase from the complex. This may alter the charging specificity of the Leucyl-tRNA synthetase from the prefered $tRNA^{Leu}$ species or in the prefered pool of Leucine, and may result in the preferential use of a Leucyl-tRNA pool that is fully charged but is not usually utilized.

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