4.0 SUMMARY

PHOSPHORYLATION OF WHEAT GERM eIF-2 BY PURIFIED KINASES *in vitro*

Purified wheat germ eIF-2 was resolved into three subunits on 10% SDS-PAGE. Molecular weights of these three polypeptides are 36 kDa, 41-42 kDa and 52 kDa. Purified reticulocyte kinases, HRI or PKR which can phosphorylate the small subunit (38 kDa) of reticulocyte eIF-2 (eIF-2a), phosphorylated the p41-42 doublet subunit of wheat germ eIF-2 *in vitro*. The small subunit, p36 subunit, of wheat germ eIF-2 however was not phosphorylated under those conditions. Impure HRI preparations or highly purified multipotential kinase like CK II were found to phosphorylate both the p36 and p41-42 doublet subunits of wheat germ eIF-2. These findings suggest that the phosphorylation of small subunit of wheat germ eIF-2 can be carried out by CK II or by a contaminant CK II like activity in impure HRI.

We have also analyzed the ability of native reticulocyte eIF-2α kinases (which get activated during heme-deficiency or poly IC or dsRNA treatment in reticulocyte lysates) to phosphorylate wheat germ eIF-2. The results are consistent with the ones obtained by purified kinases *in vitro*.

GUANINE NUCLEOTIDE EXCHANGE ACTIVITY OF WHEAT GERM eIF-2 *in vitro* AND THE EFFECT OF PHOSPHORYLATION

Purified wheat germ eIF-2 was able to bind added labeled GDP (2 μM) in presence of 2.5 mM magnesium. In the absence of magnesium, eIF-2 could not bind to GDP efficiently. However, the binary complex was found to exchange its labeled GDP for unlabeled GDP in the reaction mixture very efficiently in the absence of any eIF-2B-like factor being added. The labeled complex was fairly stable in the absence of unlabeled GDP. It is not known however, if the guanine nucleotide exchange activity associated with wheat germ eIF-2 is due to a contaminant eIF-2B-like activity in eIF-2 preparation. Alternatively, it is possible that the ability of wheat germ eIF-2 to bind GDP or GTP is not markedly different and the guanine nucleotide exchange may be occurring independent of an eIF-2B-

like protein. A recent publication that appeared after we have taken up these studies suggests the latter possibility based on the estimation of dissociation constants of eIF-2.GDP and eIF-2.GTP.

Purified eIF-2 from mammalian sources co-migrates with eIF-2 during several steps of purification. Hence the possibility of such an eIF-2B-like contamination with wheat germ eIF-2 preparation has been tested here. In mammalian systems such a contaminant eIF-2B activity is known to be inhibited upon phosphorylation of serine 51 residue in the wild type eIF-2a. The p41-42 doublet subunit of wheat germ eIF-2 can also be considered to be equivalent of mammalian eIF-2α since it is phosphorylated by reticulocyte eIF-2α kinases. So, the guanine nucleotide exchange activity of wheat germ eIF-2 was tested when the latter is phosphorylated by purified or native reticulocyte eIF-2a kinases. As it is not known if the heterologous eIF-2α kinase phosphorylates the wheat germ eIF-2 at the right site we have also determined the guanine nucleotide exchange activity of wheat germ eIF 2 in presence of phosphorylated reticulocyte eIF-2α in which the serine 51 is known to be phosphorylated. The findings indicate that

- phosphorylation of wheat germ eIF-2 by HRI could not inhibit the guanine nucleotide exchange activity associated with wheat germ eIF-2,
- reticulocyte lysates in which HRI or PKR was activated and could inhibit the exchange of guanine nucleotides on reticulocyte eIF-2 however were unable to inhibit the exchange of GDP on wheat germ eIF-2,
- addition of phosphorylated reticulocyte eIF-2a *in vitro* could not inhibit the guanine nucleotide exchange activity associated with wheat germ eIF-2.

These findings suggest that the guanine nucleotide exchange activity of wheat germ eIF-2 is not because of a contaminant eIF-2B-like activity and support the idea that guanine nucleotide exchange activity proceeds under sufficiently high GTP/GDP ratio.

WHEAT GERM eIF-2 MITIGATES PROTEIN SYNTHESIS INHIBITION IN RETICULOCYTE LYSATES MEDIATED BY RETICULOCYTE eIF-2a PHOSPHORYLATION

Addition of wheat germ eIF-2 to inhibited reticulocyte lysates was found to mitigate the protein synthesis inhibition mediated by eIF-2a phosphorylation in the present studies. Hence we have studied further the mechanism by which wheat germ eIF-2 is able to overcome the protein synthesis inhibition in reticulocyte lysates under those conditions. Our findings indicate that wheat germ eIF-2 did not decrease the level of reticulocyte eIF-2a phosphorylation that was required for the protein synthesis inhibition to occur and the conditions in inhibited reticulocyte lysates also facilitated the phosphorylation of p41-42 doublet subunit of wheat germ eIF-2. Consistent with the decrease in the inhibition of protein synthesis, addition of wheat germ eIF-2 to inhibited reticulocyte lysates also protected partially the lysate eIF-2B activity. This is possible if wheat germ eIF-2 is exchanging the phosphorylated reticulocyte eIF-2a from the 15S complex [eIF-2(aP).eIF-2B] and the phosphorylated wheat germ eIF-2 is unable to interact with reticulocyte eIF-2B as efficiently as reticulocyte eIF-2(aP). This possibility is also tested here by monitoring the 15S complex's in inhibited reticulocytes treated with or without wheat germ eIF-2. These results indicate that 15S complex formation is reduced in inhibited lysates treated with wheat germ eIF-2 compared to lysates carrying protein synthesis without the addition of wheat germ eIF-2.

Since the eIF-2 in the 15S complexes is probed by reticulocyte eIF-2a monoclonal antibodies, the results do not reveal if phosphorylated wheat germ eIF-2 is able to interact with reticulocyte eIF-2B. Hence we have measured the eIF-2B activity of reticulocyte lysates under those conditions and also probed the 15S complex with polyclonal anti-wheat germ eIF-2 antibodies.

Reticulocyte eIF-2B activity was partially protected and the 15S complexes did not show any wheat germ eIF-2 signal when probed with a wheat germ eIF-2 polyclonal antibody, suggesting that phosphorylated wheat germ eIF-2 can

exchange reticulocyte eIF-2(aP) from the 15S complexes but however it cannot interact as efficiently as reticulocyte eIF-2(aP) with eIF-2B. Hence this should make briefly the eIF-2B available before it is complexed again with reticulocyte eIF-2(aP). The free eIF-2B can immediately catalyze the exchange of GDP on reticulocyte eIF-2 so as to enable the latter to enter into the initiation cycle. The ability of wheat germ eIF-2 to escape the effect of phosphorylation of reticulocyte eIF-2a draws similarities with mutant mammalian eIFB 2a in which serine 48 residue, adjacent to phosphorylated serine 51 residue, is replaced by alanine. This mutant eIF-2 can still be phosphorylated in its serine 51 residue. The expression of such a mutant eIF-2 is shown to overcome the inhibition in protein synthesis and eIF-2B activity in Chinese hamster ovary (CHO) cells that is mediated by wild type eIF-2a phosphorylation. Based on this information presented, it is likely that wheat germ eIF-2 is different from mammalian eIF-2 and the differences may have occurred in evolution due to a modification of some amino acid(s) in and around the phosphorylation site of eIF-2.

A polyclonal anti-wheat germ eIF-2 antibody raised in these studies was found to recognize the p36 and p41-42 subunit of wheat germ eIF-2. The polyclonal antibody was also found to cross react with the reticulocyte eIF-2 (3-, *y*- subunits. This suggests that although these systems are phylogenitically placed distinctly wheat germ and rabbit reticulocyte eIF-2s still carry conserved sequences among them. This antibody is also found useful to determine the phosphorylated wheat germ eIF-2 interaction with reticulocyte eIF-2B as has been described earlier.

Further the antibody has been used here to determine if wheat germ eIF-2 can enter into the protein synthesis cycle of inhibited reticulocyte lysates. Our findings indicated that polysomes were completely disiaggregated in inhibited reticulocyte lysates and were partially disaggregated in inhibited lysates treated with wheat germ eIF-2. These findings correlate with the reduced inhibition of protein synthesis under those conditions. When these polysomes were analyzed for

the presence of wheat germ eIF-2, it was observed that the 80S subunits and to some extent the disomes and trisomes were found to carry wheat **germ ell-Z'** These observations suggested that wheat germ eIF-2 can probably enter into the reticulocyte initiation cycle.

ANALYSIS OF PHOSPHOPEPTIDES OF RETICULOCYTE eIF-2α (p38) AND p36 AND p41-42 SUBUNITS OF WHEAT GERM eIF-2:

Since the p41-42 doublet subunit of wheat germ eIF-2 can be phosphorylated by reticulocyte eIF-2a kinases, we have tested here the phosphopeptides that are generated by limited proteolytic digestion from phosphorylated wheat germ eIF-2 and reticulocyte eIF-2. The findings suggest that there are more than one phosphorylation site in the p41B42 doublet subunit of wheat germ eIF-2. Also the phosphorylation of small subunit of wheat germ eIF-2, that is p36 by CK II is compared to the phosphorylation of small or a subunit of reticulocyte eIF-2 by HRI (please note that CK II cannot phosphorylate the small subunit of reticulocyte eIF-2 and hence we have used HRI to compare the phosphopeptides of the small subunits in both the preparations). All these studies reveal that wheat germ eIF-2 subunits have multiple phosphorylation sites whether that be in p36 or p41-42 subunits, whereas reticulocyte eIF-2a appears to have a single site of phosphorylation. The latter is consistent with the earlier reports