CHAPTER 4

STUDIES ON GUANINE NUCLEOTIDE EXCHANGE ON WHEAT GERM eIF-2
At the end of protein chain initiation, eIF-2 is released as eIF-2 GDP in mammalian systems and this complex cannot join the initiator tRNA (Met-tRNA<sub>i</sub>) and enter into the initiation pathway unless the GDP is exchanged for GTP. This is because eIF-2 has greater affinity for binding to GDP than GTP (Merrick, 1992). Further, phosphorylation of eIF-2α impairs the eIF-2B activity in vitro and in translating lysates (Clemens et al., 1982, Matts and London, 1984). The affinity of eIF-2B for eIF-2(aP).GDP is higher than that for eIF-2.GDP. And also, eIF-2B constitutes a small portion of eIF-2 (Rowlands et al., 1988). Consequently, phosphorylation of a limited portion of eIF-2a (10-20%) sequesters all the available eIF-2B into a 15S complex [eIF-2(aP).eIF-2B], in which eIF-2B becomes non-functional. This can lead to the inhibition in protein synthesis (Thomas et al., 1984, 85).

The eIF-2B like activity is apparently conserved in other systems like yeast (Dever et al., 1992) and insects (Chefalo et al., 1994). However it is not known whether a similar protein is required for the exchange of guanine nucleotides from wheat germ eIF-2. Hence labeled binary complex, eIF-2.[3H]GDP, was prepared here with wheat germ eIF-2 in vitro and studied the exchange of the labeled GDP complexes under a variety of conditions that have been observed to affect wheat germ eIF-2 phosphorylation and lysate protein synthesis.

4.1 Binary complex, eIF-2.GDP, formation requires Mg<sup>2+</sup>: Wheat germ eIF-2 can bind to added labeled GDP to form a binary complex, eIF-2.[3H]GDP. The binary complex was formed as described (Ramaiah et al., 1994). The formation of the binary complex is highly dependent on Mg<sup>2+</sup> (Fig. 27).

The efficiency of binary complex formation was found better with carboxy-methyl-Sephadex (CMS, highly purified) purified eIF-2 than with phosphocellulose (P11, one step less purified than CMS-eIF-2) eIF-2 (Fig. 28).
Fig. 27 Mg$^{2+}$ is required for efficient binary complex formation.

Wheat germ binary complex was formed using purified wheat germ eIF-2 (2 µg) as described in 'Materials and Methods'. The requirement of Mg$^{2+}$ for stabilization of the wheat germ binary complex was studied using different concentrations of Mg$^{2+}$ (as indicated in the figure) during the formation of binary complex. The figure indicates the pmols of labeled wheat germ binary complex formed.
4.2 Dissociation of the preformed binary complex:

In order to assess whether the dissociation of preformed binary complex or exchange of unlabeled GDP for labeled GDP present in the reaction mixture, requires any additional protein factors, the guanine nucleotide exchange in the presence of excess unlabeled GDP (40 μM) and physiological concentration of Mg²⁺ (2.5 mM) was measured as described (Ramaiah et al., 1994). Interestingly, the exchange reaction occurred without the addition of any protein factor (Fig. 29).

This finding suggests that

- the wheat germ eIF-2 preparation may be contaminated with small amounts of eIF-2B like activity,
- one of the eIF-2 subunits may be serving the function of eIF-2B or, the exchange reaction may not require an eIF-2B like protein and occurs probably due to mass action.

4.3 Guanine nucleotide exchange on wheat germ eIF-2:

The above observation that the exchange of unlabeled GDP for labeled GDP in the preformed wheat germ binary complex, eIF-2.[³H]GDP, can proceed without the addition of any protein factor is consistent with the previous report on the guanine nucleotide exchange on wheat germ eIF-2 (Osterhout et al., 1983; Shaikin et al., 1992). Despite rigorous purification, earlier workers however could not purify eIF-2B like activity from wheat germ (Osterhout et al., 1983; Lax et al., 1982). In many mammalian systems, it has been shown that eIF-2 can be contaminated with eIF-2B like preparations. In fact many times it was observed that the eIF-2B co-migrates with eEF-2 preparations during purification (Matts et al., 1983; Reichel et al., 1985; Panniers and Henshaw, 1983).

To determine if the exchange of guanine nucleotides is helped by an eIF-2B like protein associated with wheat germ eEF-2, it becomes important to show that the eIF-2B activity is specifically inhibited upon eIF-2 phosphorylation. Since purified CK-II (Krishna et al., 1994), or NEM-treated lysates were shown here to phosphorylate the p36 and p41-42 subunits of wheat germ eIF-2 (Fig. 21), we have determined here the effect of purified
Fig. 28 Formation of wheat germ binary complex using partially purified (P11) and highly purified (CMS) eIF-2.

Wheat germ binary complex was formed in the presence of 2.5 mM Mg$^{2+}$ as described in 'Materials and Methods' using different concentrations of P11-eIF-2 or CMS-eIF-2 as indicated in the figure. The figure indicates the amount of wheat germ binary complex formed (in pmols).
Fig. 29 Exchange of unlabeled GDP for labeled GDP in wheat germ binary complex.

Labeled binary complex, eIF-2[^3]H]GDP, was prepared as described in 'Materials and Methods'. Dissociation of the preformed binary complex (70 µl, 25 pmols) was studied at 25°C with and without the addition of 40 µM unlabeled GDP. At different time intervals (2.5, 6 and 10 min.), 20 µl aliquots of the reaction mixtures were withdrawn and the reactions were stopped with the addition of 3 ml of cold wash buffer. The reaction mixtures were then filtered through millipore filters (HAWP 0.45 µm), air dried and the radioactivity bound to the filters was measured in a liquid scintillation counter. One pmol of eIF-2[^3]H]GDP gives ~1900 cpm. Pmols of eIF-2[^3]H]GDP dissociated were determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP from eIF-2.
CK-II or NEM-treated lysate on the guanine nucleotide exchange activity associated with wheat germ eIF-2 in vitro

4.3.1 Effect of CK-II phosphorylation on GNE activity associated with wheat germ eIF-2

The experiment was carried out in two different ways: 1) in the presence and absence of the phosphorylating enzyme, CK-II (Fig 30) and 2) in the presence and absence of the phosphate donor, ATP (Fig. 31).

In the first set of experiments, ATP was present in both the reactions. However, CK-II was added to only one of the reactions to determine the effect of phosphorylation of eIF-2 by CK-II on GNE activity. In another set of reactions, CK-II was present in both the reactions, however, ATP was included only in one of them to facilitate phosphorylation. After carrying out the phosphorylations of eIF-2 in the respective experiments, binary complexes were formed and GNE activity was studied as described in 'Materials and Methods'.

Kinetics of the GDP exchange reaction were found similar for phosphorylated and unphosphorylated eIF-2 (Fig 30 and Fig. 31). Presence of ATP however reduced the GDP exchange marginally (Fig 31).

4.3.2 Effect of NEM on the GNE activity of wheat germ eIF-2:

Translating wheat germ lysates were incubated with and without 1 mM NEM and 100 μM ATP, for 15 minutes to activate the endogenous kinase(s). Wheat germ binary complex was formed as described in 'Materials and Methods' and added to the translating lysates to study the GNE activity.

GNE activity was decreased in NEM-treated lysates compared to the non-NEM treated lysates (Fig. 32). This decrease in the GNE activity could be due to either NEM induced phosphorylation of eIF-2 or due to a direct modification of eIF-2 by NEM. Studies by Suzuki el al.,(1990) show that the formation of ternary complex, eIF-2GTP.Met-tRNAi, is inhibited by NEM. This inhibition is shown to be mediated, atleast partially, due to the binding of NEM to the γ-subunit of eIF-2. Four NEM binding sites,
Fig. 30 Effect of phosphorylation of wheat germ eIF-2 by CK-II on the dissociation of eIF-2[3H]GDP. (Control reactions contain ATP and lack CK-II).

The experiment was carried out in 3 steps. In step 1, phosphorylation of wheat germ eIF-2 (1 μg) was carried out at 30°C by CK-II (10 ng) for 5 min. in a 10 ml reaction mixture in the presence of Tris buffer (20 mM Tris-HCl, pH 7.6; 80 mM KCl; and 2.5 mM Mg2+) and 100 μM ATP. The control unphosphorylated reaction mixtures lacked the CK-II protein. In step 2, the phosphorylated and the unphosphorylated reaction mixtures (70 μl) were incubated with [3H]GDP to form eIF-2[3H]GDP binary complex as described in 'Materials and Methods'. In step 3, the dissociation of the preformed binary complex (70 μl, 31.5 pmols) was studied at 25°C with and without the addition of 40 μM unlabeled GDP to the reaction mixtures. At different time intervals (2.5, 6 and 10 min.), 20 μl aliquots of the reaction mixtures were withdrawn and the dissociation of the preformed binary complex monitored as described in 'Materials and Methods'. One pmol of eIF-2[3H]GDP gives ~1900 cpm. Pmols of eIF-2[3H]GDP dissociated were determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP from eIF-2.
Fig. 31 Effect of phosphorylation of wheat germ eIF-2 by CK-II on the dissociation of eIF-2[^3H]GDP: (Control reactions contain CK-II and lack ATP).

In step 1, phosphorylation of wheat germ eIF-2 (1 μg) was carried out at 30°C by CK-II (10 ng) for 5 min. in a 10 μl reaction mixture in the presence of Tris buffer (20 mM Tris·HCl, pH 7.6; 80 mM KCl; and 2.5 mM Mg²⁺) with and without the addition of 100 μM ATP. The control unphosphorylated reaction mixtures contained CK-II protein without ATP. In step 2, the phosphorylated and the unphosphorylated reaction mixtures (70 μl) were incubated with[^3H]GDP to form eIF-2[^3H]GDP binary complex as described in 'Materials and Methods'. In step 3, the dissociation of the preformed binary complex (70 μl, 31.5 pmols) was studied at 25°C with and without the addition of 40 μM unlabeled GDP to the reaction mixtures. At different time intervals (2.5, 6 and 10 min.), 20 μl aliquots of the reaction mixtures were withdrawn and the dissociation of the preformed binary complex was monitored as described in 'Materials and Methods'. One pmol of eIF-2[^3H]GDP gives ~1900 cpm. Pmols of eIF-2[^3H]GDP dissociated were determined as described in the 'Materials and Methods'. The figure indicates the dissociation of labeled GDP from eIF-2.
out of which two highly reactive ones, were identified in the Y-subunit. Binding of NEM to
the y-subunit decreases the binding of guanine nucleotides to the subunit, thereby
preventing the formation of ternary complex. NEM also binds to the a-subunit of eIF-2,
but this binding did not affect the binding of the guanine nucleotides to eIF-2 (Suzuki et al.,
1990).

To eliminate the possibility of direct modification of eIF-2 by NEM, we carried out
the above experiment with a 0-70% ammonium sulfate cut fraction of NEM-treated and
untreated lysates instead of total lysates. In contrast to the above observations, the 0-70%
ammonium sulfate cut fraction of NEM-treated lysates did not decrease the GNE activity
of wheat germ eIF-2 (Fig. 33).

As can be seen from the data, neither conditions (CK-II or NEM induced
phosphorylations of wheat germ eIF-2) inhibit significantly the guanine nucleotide
exchange on wheat germ eIF-2. These findings suggest that the protein synthesis inhibition
caused by CK-II or NEM-treatment are at least not mediated by a reduction in the GNE
activity of wheat germ eIF-2.

The NEM-treated lysates or purified CK-II may not be phosphorylating wheat
germ eIF-2 at a proper site that can effectively inhibit the eIF-2B activity. A recent study
infact emphasizes the importance of Ser-51 phosphorylation and the importance of
adjacent unphosphorylated Ser-48 residue in the wild type human eIF-2(aP) to effectively
sequester the eIF-2B guanine nucleotide exchange activity (Ramaiah et al., 1994). Further,
studies in our laboratory (Krishna, 1996, Ph.D. Thesis) indicate that guanine nucleotide
exchange on wheat germ eIF-2 can also proceed in inhibited heme-deficient, or heme and
poly IC-treated reticulocyte lysates. Under these conditions reticulocyte eIF-2B activity is
impaired due to the phosphorylation of Ser-51 residue in reticulocyte eIF-2α. These
lysates cannot support the guanine nucleotide exchange on reticulocyte eIF-2 but however
can support the GDP exchange on wheat germ eIF-2.

Since significant dissociation of wheat germ eIF-2.[3H]GDP occurs in heme-
deficient and poly IC treated lysates, one can eliminate the possibility of a small
contaminant of eIF-2B or of a p67 like protein in the eIF-2 preparations. A careful
analysis of purified wheat germ eIF-2 preparation did not indicate any major contaminants
The experiment was carried out in three steps. The wheat germ lysates were treated with 100 μM ATP and incubated at 25°C for 10 min. with or without the addition of NEM (1.5 mM) to facilitate the activation of endogenous eIF-2 kinase(s) and eIF-2 phosphorylation. In step 2, the eIF-2-[3H]GDP, binary complex was prepared as described in 'Materials and Methods'. In step 3, The NEM-treated and untreated lysates (7 μl) were then incubated for 5 min. at 25°C with the preformed binary complex (25 pmols) in the presence of 100 μM ATP in a final volume of 77 μl to facilitate the phosphorylation of preformed wheat germ binary complex. Afterwards the dissociation of the preformed binary complex was monitored in 22 μl reaction mixtures at different time intervals with and without the addition of 40 μM unlabeled GDP as described in 'Materials and Methods'. One pmol of bound eIF-2-[3H]GDP gives 2200 cpm. The figure indicates the dissociation of labeled GDP from eIF-2.
Fig. 33 Effect of NEM-treated lysate fraction on wheat germ eIF-2[^3]H]GDP dissociation.

The experiment was carried out in three steps. The wheat germ lysates were treated with 100 μM ATP and incubated at 25°C for 10 min, with or without the addition of NEM (1.5 mM) to facilitate the activation of endogenous eIF-2 kinase(s) and eIF-2 phosphorylation. The lysate proteins were then immediately precipitated by the addition of 2.5 volumes of saturated ammonium sulfate (70% final). In step 2, the eIF-2[^3]H]GDP, binary complex was prepared as described in 'Materials and Methods'. In step 3, The NEM-treated and untreated lysate fractions (7 μl) were then incubated for 5 min. at 25°C with the preformed binary complex (25 pmols) in the presence of 100 μM ATP in a final volume of 77 μl to facilitate the phosphorylation of preformed wheat germ binary complex. Afterwards the dissociation of the preformed binary complex was monitored in 22 μl reaction mixtures at different time intervals with and without the addition of 40 μM unlabeled GDP as described in 'Materials and Methods'. One pmol of bound eIF-2[^3]H]GDP gives ~2200 cpm. The figure indicates the dissociation of labeled GDP from eIF-2.
(Krishna et al., 1994). It is likely that the exchange reaction is mediated by mass reaction (depends upon the concentration of GDP or GTP). These observations are consistent with the recent studies (Shaikin et al, 1992) which suggested that the affinity of wheat germ eIF-2 for GDP is only 10 times higher than that for GTP. In contrast, mammalian eIF-2 has much higher affinity for GDP than for GTP (Walton and Gill, 1975). These findings suggest that eIF-2B analogs may not be required for the exchange of GTP for GDP in wheat germ eIF-2 and the latter appears to be different from mammalian eIF-2 preparations. The findings are thus consistent with the idea that phosphorylation of eIF-2 may not regulate protein synthesis in higher plants (Shaikin et al., 1992). Alternatively, one of the eIF-2 subunits may be serving the function of eIF-2B activity. If this is true, a high level of eIF-2 phosphorylation is required to sequester completely the eIF-2B activity.

The findings presented here suggest that wheat germ eIF-2 behaves different from mammalian eIF-2 since it exchanges guanine nucleotides independent of an eIF-2B like protein. Hence it raises a question regarding the functional significance of eIF-2B like factor, if any, in wheat germ lysates and also the mechanism of inhibition in protein synthesis mediated by eIF-2α phosphorylation in plants.

A recent report however, indicates that phosphorylation of p41-42 subunit in wheat germ lysates can occur by relatively higher concentrations of dsRNA. Presumably these conditions stimulate an eIF-2α kinase like PKR and phosphorylation of wheat germ eIF-2 subunits. These authors have also demonstrated that phosphorylation of the above subunit of wheat germ eIF-2 impairs protein synthesis. However, a) they have not studied the mechanism of protein synthesis under those conditions. It is not known if these conditions can also decrease the lysate eIF-2B like activity, b) the authors also mention that significant variations exist between wheat germ lysate preparations in terms of dsRNA levels required for inhibition, with several showing no response to dsRNA (like the results that we have mentioned). The required response or the lack of response of wheat germ lysates towards dsRNA is presumed to be due to varying levels of a PKR inhibitor present in the lysates. In this regard, the authors cite the role for a glycosylated p67-like protein which can inhibit eIF-2α kinase phosphorylation. As far as we are aware of, the p67 protein cannot inhibit the eIF-2α kinase activity (that is its autophosphorylation) but it can
interfere in eIF-2α phosphorylation of *in vitro* reactions (personal observations of Babu
and Ramaiah; Chen and London).

Also the role of purified p67 in lysates for its affects on protein synthesis, eIF-2α
phosphorylation and on eIF-2B activity during protein synthesis has not been worked out
so far. While we do not rule out the possibility of such a protein involved in the regulation
of eIF-2α phosphorylation and protein synthesis, however we think that this may not be a
reason for various workers in failing to identify an eIF-2α phosphorylation mechanism in
plants that can inhibit protein synthesis. Our findings presented here suggest two most
important points:

- The guanine nucleotide exchange on wheat germ eIF-2 can occur independent of an
eIF-2B like protein.
- The phosphorylation of wheat germ eIF-2 occurs under several conditions but does
  not lead to an inhibition in GNE activity associated with wheat germ eEF-2.

The findings mentioned above suggest that probably wheat germ eIF-2 is able to
exchange guanine nucleotides both by mass exchange and a reaction catalyzed by eIF-2B
like protein. The latter is substantiated by findings of Krishna et al., (1994) which indicate
that a functional reticulocyte eIF-2B activity can enhance the GDP exchange on wheat
germ eIF-2. This may reflect two states of the protein and it may be similar to guanine
nucleotide exchange of eIF-2 in Drosophila embryo or Artemia (Mateu and Sierra, 1987,

Also consistent with these interpretations, ribosomal salt wash did not yield
significant amount of eIF-2. We could purify eIF-2 only from the post ribosomal
supernatant. Recently, it was suggested that functional eIF-2B activity is required to
release eIF-2 from the 60S subunits of 80S initiation complexes in reticulocyte lysates
Otherwise eIF-2 stays bound to the 60S subunits of polysomes (Ramaiah *el at.*, 1992;
Thomas *et al.*, 1985). Since GDP in wheat germ eIF-2 can be readily exchanged either by
mass exchange or because of an intrinsic eIF-2B like activity, it is quite likely that most of
the eIF-2 is released from the ribosomes without any difficulty and is found in the post
ribosomal supernatant.
There is considerable confusion regarding the designation of the various subunits of wheat germ eIF-2. Going by the criteria of the size of the different subunits in eIF-2, the 38 kDa of wheat germ eIF-2 is considered to be the smallest and is equivalent to the smallest subunit in reticulocyte eIF-2 (eIF-2α). However, the reticulocyte eIF-2α kinases phosphorylate the 41-42 kDa of wheat germ eIF-2. This suggests that part of reticulocyte eIF-2α sequences are conserved in the 41-42 kDa of wheat germ eIF-2. Consistent with this notion, the recent observations based on molecular cloning and cDNA sequencing of wheat germ eIF-2 indicate (unpublished observations cited by Langland et al., 1996) that the p41-42 doublet subunit is equivalent to reticulocyte eIF-2α. However the report suggests that 17% sequences in plant eIF-2α around the phosphorylation domain (45-56 region) may be different from human eIF-2α sequence. A modification in an amino acid adjacent to phosphorylation site has been shown to overcome the protein synthesis inhibition and inhibition in eIF-2B activity mediated by human eIF-2α phosphorylation (Murtha-Reil et al., 1993; Ramaiah et al., 1994). Also a study with yeast cells identified several mutants within 40 amino acids of the phosphorylation site that can overcome the inhibitory effect of eIF-2α phosphorylation at residue Ser-51 mediated by GCN2 kinase (Hinnebusch, 1994). Also a mutation in eIF-2B protein can counter the phosphorylation of eEF-2α (Vazquez de Aldana et al., 1993). So it is possible that modification in some of the amino acid sequences in wheat germ eIF-2 may be the reason for its inability to inactivate eIF-2B like activity associated with it, as mentioned above, or of reticulocyte lysates (Krishna, 1996, Ph. D. Thesis).