Part I

Chapter 1

Expression and Purification of the Human Eukaryotic Translation Initiation Factor 2 (eIF2) subunits in Bacteria: Characterization of the subunits.
Introduction:

The human eukaryotic translation initiation factor 2 (eIF2) is a trimer and plays a critical role in the initiation of translation by transferring the Met-tRNAi to the 40S ribosomes in a GTP dependent manner. The native eIF2 consists of α, β and γ subunits in 1:1:1 ratio. Phosphorylation of the conserved Ser51 residue in eIF2α is one of the key mechanisms in the regulation of translation. Although, this mechanism is conserved both in yeast and mammals, the mechanistic details involved in the regulation of protein synthesis through phosphorylation of eIF2α appear to differ. Recent studies from this lab have expressed the human eIF2 subunits in insect cells using the baculoviral expression system to study the intersubunit interactions and observed that the recombinant α and β subunits are phosphorylated in the insect cells during expression and that all three subunits interact with each other in a dot blot and ELISA assays (Suragani et al., 2006 and 2005).

One of the objectives of the present thesis is to express the subunits of human eIF2 in bacteria which are not phosphorylated during expression in order to further understand the intersubunit and interprotein interactions and to evaluate the effect of phosphorylation of the α and β-subunits on these interactions. Accordingly, the first part of the chapter deals with the cloning of the subunits of human eIF2 into bacteria, time-course of their expression, the ability of the α and β-subunits to serve as substrates for bacterial kinases during expression, purification of the subunits by affinity matrix and the phosphorylation of the purified subunits by purified kinases in-vitro. Since eIF2 has a high affinity for GDP in the presence of physiological concentrations of magnesium (Mg$^{2+}$), and the GDP bound eIF2 cannot participate in initiation unless the bound GDP is exchanged for GTP, we have also evaluated the abilities of the subunits alone and the combinations of different subunits to bind and retain labeled GDP.

Results & Discussion:

Cloning of cDNAs of the subunits of human eIF2 into pET32a vector: The cDNAs representing the subunits of human eIF2 and mutants of eIF2α harbored in the baculovirus transfer vector, pFast Bac HT (Suragani et al., 2006), were amplified in the DH5α cells of E. coli. The mutants of eIF2α are as follows: S51A represents a non-phosphorylatable form where the 51 serine residue is replaced by an alanine; S48A, a mutant of eIF2α in
which serine 48 is replaced by alanine and contains the conserved 51 phosphorylation site, and S51D, a phosphomimetic form of eIF2α in which the 51 serine residue is replaced by aspartic acid. Plasmid DNA was isolated and the cDNAs of α-, β-, and mutants of eIF2α were then excised from the parent plasmid using NcoI and Hind III. However, restriction endonucleases NcoI and XhoI were used to release the γ-cDNA. The excised and released cDNAs were then ligated into the pET32a vector linearized by the restriction endonucleases as mentioned above. The flow chart depicting the release of cDNAs from parent vector to cloning them into pET32a vector with a 6x his-tag has been shown in Fig 1.1. The recombinant clones of pET32a vector harboring the subunits of human eIF2 were confirmed by restriction digestion analysis as shown in Fig 1.1A and also by PCR using gene-specific primers as shown in Fig.1.1B. The clones were then transformed into BL21(DE3)pLysS cells and the protein expression was induced in the presence of 1 mM IPTG. Expressed proteins were then purified using Ni-NTA agarose affinity matrix.

**Expression and purification of recombinant subunits of human eIF2:**

BL21(DE3)pLysS cells, transformed with recombinant clones were grown in LB broth containing ampicillin and chloramphenicol. 1 mM IPTG was then added to the cultures and incubated for 3-4 hrs to induce the expression of recombinant proteins. Cells were then harvested and the cell pellet was resuspended in SDS sample buffer. The expression of the recombinant α-, β- and γ- subunits of human eIF2, in response to IPTG was analyzed by a western blot and probed by an anti his-tag antibody. There was negligible or no expression without the addition of IPTG (Fig 1.2).

Time based expression of the recombinant eIF2 subunits indicates that the expression is time-dependent (Figs 1.3 A-C). The expression of the α and β subunits starts at 30 min post induction (Figs 1.3 A and B), and that of the γ subunit starts around 1 hr (1.3C). Optimum expression of all the subunits is observed around 4 hrs post induction. After 4 hrs, the expression of the proteins remained more or less constant. The expression of the subunits in extracts prepared from IPTG-treated cells was detected in coomassie stained gels (Fig 1.3. A, B and C).
Fig 1.1:

The figure depicts the flowchart describing the cloning of the subunits of human eIF2 α-, β- and γ- and the mutants of eIF2α into pET 32a vector. The inserts representing the cDNAs of the eIF2 subunits harbored in the pFAST Bac vector were excised and ligated into the linearised pET32a vector as described in the text.
Fig 1.1A: Restriction analysis of recombinant clones: The excised cDNAs of eIF2α-, β-, γ- and mutants of eIF2α were ligated into pET32a vector and the ligation mixture was transformed into DH5α cells. Antibiotic resistant colonies were selected and recombinant plasmids were isolated as described in ‘Materials and Methods’. The plasmids were subjected to restriction digestion with Nco I and Hind III for recombinant pET32a vector harboring wt and mutants of eIF2α and β- subunits, while Nco I and Xho I were used for pET32a harboring the γ-subunit for confirmation of the recombinant clones. The samples were analyzed by 1% agarose gel electrophoresis. The lanes are as mentioned in the figure.

Fig 1.1B: Recombinant clones confirmation by PCR: The recombinant clones were further confirmed by PCR performed using gene specific primers for eIF2α, β and γ subunits as described earlier (Suragani et al., 2006). The samples were analyzed by 1% agarose gel electrophoresis. Various lanes are as mentioned in the figure.
Fig 1.1A:

Fig 1.1B:
Fig 1.2: Expression of the subunits of human eIF2 in bacteria: The cell pellets were suspended in SDS-PAGE sample buffer and the samples analyzed on 10% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane. The figure represents a western blot probed with anti-histag antibody. Lanes 1 and 2 represent the expression of the \( \gamma \)-subunit, whereas lanes 3, 4 and 5, 6 represent the expression of \( \alpha \)- and \( \beta \)-subunits respectively in the absence and presence of 1 mM IPTG. Lane M represents molecular weight marker.
Fig. 1.3A:

Expression of the \( \alpha \)-subunit of human eIF2.

Fig. 1.3B:

Expression of the \( \beta \)-subunit of human eIF2.
Fig. 1.3C:

Expression of the γ-subunit of human eIF2.

Fig. 1.3A, B and C: Time-course of expression of recombinant eIF2α, β or γ: Transformed BL-21(DE3)-pLysS E.coli cells were cultured at 37°C till an O.D of 0.8 at 600 nm was attained. 1 mM IPTG was then added to cultures to induce protein expression for different time periods ranging from 30 min–4 hrs. Cell pellets from different time points were briefly boiled in SDS-sample buffer and separated by 10% SDS-PAGE. The figures are coomassie stained gels indicating the time-course of expression of α (1.3A), β (1.3B) and γ (1.3C). Various lanes are as follows: UI represents uninduced culture and the other lanes represent the induced cultures for different time periods as depicted in the figure.
Recombinant subunits were purified by Ni-NTA affinity chromatography. The columns were washed with low concentrations (30 and 50 mM) of imidazole and the bound fractions were then eluted by 250 mM imidazole as described in the ‘Materials and Methods’. The purification profile of the recombinant subunits was analyzed by coomassie stained gels (Fig 1.4 A, B and C).

**Phosphorylation of the recombinant α- and β- subunits:**
Since the α- and β- subunits of human eIF2 are phosphorylated *in-vitro* by various purified kinases and are also found phosphorylated during their expression in insect cells (Suragani et al., 2006), I evaluated the ability of bacterially expressed α- and β- subunits of human eIF2 to serve as substrates for different kinases *in-vitro* and also by bacterial extracts (Figs 1.5 A-C). Our observations here indicate that bacterially expressed recombinant human eIF2 α- (wt and S48A mutant) and β- subunits were phosphorylated *in-vitro* by purified kinases as shown in Fig 1.5 A and B respectively. Unlike the baculovirus expressed subunits which are phosphorylated in insect cells, the bacterially expressed subunits are not phosphorylated by any of the bacterial kinases (Fig 1.5C).

**[H3] GDP binding by the recombinant subunits:** The ability of the recombinant α-, β- and γ- subunits of human eIF2, to bind [H3] GDP was analyzed as described in ‘Materials and Methods’. Although, the γ-subunit is implicated in GDP binding and the β-subunit is suggested to aid the γ-subunit in the above function (Nika et al., 2001), it was observed here that all the three subunits bind to labeled GDP. However, the GDP dissociation analysis which is inverse to the retention efficiency in the presence of excess unlabeled GDP suggests that the trimeric complex binds and retains the GDP most efficiently (Fig 1.6).

**Polyclonal antibodies against the recombinant eIF2α- subunit:** The bacterially expressed and purified eIF2α- subunit was used to raise polyclonal antibodies in rabbit according to the standard protocols described in the book ‘Molecular Cloning’ by Sambrook et al. The ability of the antibodies to detect and immunoprecipitate the α-subunit of reticulocyte and HeLa cell lysates indicates that the antibody recognizes specifically the α-subunit of eIF2 (Fig 1.7).
Fig. 1.4A:

Purification profile of recombinant **eIF2α** by Ni-NTA chromatography: The figure represents a coomassie stained gel. Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-5, wash fractions; Mr depicts the molecular weight marker and lanes 6-8, represent the elution fractions.

Fig. 1.4B:

Purification profile of recombinant **eIF2β** by Ni-NTA chromatography: Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-6, wash fractions; Mr depicts the molecular weight marker and lanes 7-9, represent the eluted fractions.
Fig. 1.4C:

Purification profile of recombinant eIF2γ by Ni-NTA chromatography: Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-4, wash fractions; Mr depicts the molecular weight marker and lanes 5-9, represent elution fractions.

Fig. 1.4 A, B and C: Purification of the recombinant subunits by Ni-NTA agarose: The cultures expressing recombinant his-tagged subunits of human eIF2 were harvested and the cell pellet was processed as described in ‘Materials and Methods’. The cell lysate was passed through pre-equilibrated Ni-NTA resin. The column was processed as described in ‘Materials and Methods’ and the bound proteins were eluted with 250 mM Imidazole. The figures represent coomassie stained gels indicating purification of recombinant human eIF2 α, (1.4A), β (1.4B) and γ (1.4C).
Fig 1.5A: **Phosphorylation of recombinant eIF2α (wt and mutants):** The bacterially expressed and purified human eIF2α and its mutants were phosphorylated by purified recombinant PERK *in-vitro* in the presence of $[\gamma^{32}P]$ ATP as described in Materials and Methods. The samples were separated by 10\% SDS-PAGE and transferred to a nitrocellulose membrane and the membrane was exposed to an X-ray film. Panel (a) represents an autoradiogram indicating the phosphorylation status of the proteins in the presence and absence of PERK. Panel (b) represents a western blot indicating the levels of the eIF2α-subunit and its mutants as detected by an anti-eIF2α antibody. Various lanes are as follows: 1) eIF2α (wt), 2) eIF2α (wt) + PERK, 3) S51A, 4) S51A+ PERK, 5) S48A, 6) S48A+ PERK, 7) S51D, 8) S51D+ PERK.

The wt and S48A, but not S51D or S51A mutants of human eIF2 α-subunit were phosphorylated in the presence of PERK.

Fig 1.5B: **Phosphorylation of the recombinant β-subunit:** The purified recombinant β-subunit expressed in bacteria was phosphorylated in the presence of $[\gamma^{32}P]$ ATP by purified kinases like PKA, PKC, CK II and DNA-PK *in-vitro* as per the manufacturer’s instructions and as described in Materials and Methods.

Panel (a) represents the phosphorylation status of recombinant eIF2β as judged by a phosphorimage, and panel (b) is a western blot indicating the levels of the β-subunit in the reactions as judged by a monoclonal anti-eIF2β antibody. Various lanes are as follows: lanes 1, 3, 5, and 7, represent control lanes containing the β-subunit alone; lane 2, eIF2β + CK II; lane 4, eIF2β + PKC; lane 6, eIF2β + DNA-PK; lane 8, eIF2β + PKA.
Fig. 1.5A

Panel (a)

Panel (b)

Fig. 1.5B
Fig. 1.5C: Phosphorylation status of recombinant $\alpha$ and $\beta$-subunits in bacterial extracts and by purified kinases in-vitro:

Extracts of BL21(DE3)pLysS, *E. coli* cells was prepared as described in ‘Materials and Methods’. 5 µg of purified recombinant $\alpha$ or $\beta$ subunits was incubated with bacterial cell extracts containing ~25 µg of total protein in the presence of a phosphorylation buffer and 10$\mu$Ci of [$\gamma$-$^{32}$P]ATP in a total volume of 25 µl at 30$^0$C for 10 min. Purified subunits were also incubated separately without extracts in the presence of a phosphorylation buffer labelled $\gamma$-$^{32}$[P]ATP with 0.5 µg of PERK, an eIF2 $\alpha$ kinase or with purified eIF2$\beta$-subunit specific kinases like PKA, PKC and CKII that are adjusted to the same specific activity at 30$^0$C for 10 min as described in ‘Materials and Methods’. Samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then analyzed by phosphorimage (Panel I) and western blot (Panel II).

Various lanes in both the panels are as follows: 1, Bacterial extract; 2, extract + eIF2$\alpha$; 3, eIF2$\alpha$ + PERK; 4, $\alpha$ + BSA; Mr, Molecular weight Markers; 5, extract; 6, extract + eIF2$\beta$; 7, eIF2$\beta$ + PKA; 8, eIF2$\beta$ + PKC; 9, eIF2$\beta$+ CKII; 10, eIF2$\beta$ + BSA.
Fig 1.5C

Panel I

Panel II
Fig. 1.6: [H³] GDP binding by the recombinant eIF2 subunits: The binding of [H³] GDP and the retention efficiency in the presence of excess unlabeled GDP of the individual subunits, complexes containing two of the subunits and the trimeric complex was analyzed as described in ‘Materials and Methods’. The dark bars represent the binding efficiency and the grey bars represent the retaining efficiency of the bound GDP in the presence of unlabelled GDP. The maximum binding and retention efficiency was found for the trimeric eIF2 and was taken as the 100% count (binding cpm = 1498; retention of bound [H³] GDP in the presence of excess unlabelled GDP cpm = 934).
Fig 1.7: Immunoprecipitation of $\alpha$-subunit of eIF2 by antibodies raised against recombinant human eIF2$\alpha$: Antibodies raised against recombinant $\alpha$-subunit of human eIF2 were used to immunoprecipitate the eIF2$\alpha$-subunit from HeLa and rabbit reticulocyte extracts. The immunoprecipitated samples were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The figure is a western blot probed with a commercial anti-eIF2$\alpha$ antibody. Various lanes are: lane 1, recombinant eIF2$\alpha$; lane 2, eIF2$\alpha$ from HeLa cell extracts; lane 3, eIF2$\alpha$ from rabbit reticulocyte extracts; Mr depicts molecular weight marker.
Chapter 2

Intersubunit and Interprotein Interactions of \( \alpha \)- and \( \beta \)-Subunits of Human eIF2: Effect of Phosphorylation.
Introduction:
The initial step of the translation initiation is the transfer of the Met-tRNA\textsubscript{i} to the 40S ribosomes, which is mediated by the eukaryotic initiation factor 2 (eIF2). It also plays an important role in the recognition of the initiator AUG codon on the mRNA, GDP/GTP binding and hydrolysis etc., These multifaceted actions of eIF2 are accomplished by the coordinated interactions within the subunits and also with other proteins. Much effort has gone into determining the interactions among the components of the initiation machinery and a lot of them are yet to be deciphered. Most of the information now available on the intersubunit and interprotein interactions of eIF2 has been derived from studies on yeast and based on these studies it was proposed that the $\gamma$-subunit forms the central core in the structure of eIF2 interacting with $\alpha$- and $\beta$-subunits on either side, and that there is no interaction between the $\alpha$- and $\beta$-subunits (Marintchev and Wagner, 2004). In contrast to these above observations, our recent studies with the baculovirus expressed $\alpha$- and $\beta$-subunits of human eIF2, indicate that they interact with each other in addition to interacting with the $\gamma$-subunit in ELISA and dot blot assays (Suragani et al., 2005).
Further, eIF2 also interacts with other initiation factors like eIF2B, eIF5, etc., and also with other proteins such as PP1, Nck1, CK II, etc.,. Studies from yeast have indicated that the non-productive interaction between eIF2 and eIF2B, that occurs as a consequence of eIF2$\alpha$ phosphorylation inhibits the GDP/GTP exchange activity of eIF2B and is mainly mediated by the $\alpha$-subunit of eIF2 and the regulatory subcomplex comprising the $\alpha$, $\beta$ and $\delta$ subunits (Pavitt et al., 1998; Krishnamoorthy et al., 2001), but not the catalytic subcomplex constituted of $\gamma$- and $\epsilon$-subunits of eIF2B. But, an earlier observation (Kimball et al., 1998) and also recent observations from our lab (Suragani et al., 2005) have shown that the $\beta$-rather than $\alpha$-subunit of mammalian eIF2 interacts with eIF2B. These observations suggest that the interaction patterns of mammalian eIF2 subunits are different from that of the yeast. Although, earlier studies have pointed that $\alpha$ subunit of eIF2 acts as a substrate for caspases (Satoh et al., 1999, Marissen et al., 2000), no such reports exist for the $\beta$-subunit or the effect of phosphorylation of the $\alpha$- or $\beta$-subunits on their cleavage by caspases. Persistent eIF2$\alpha$ phosphorylation is implicated in promoting apoptosis under certain conditions that is mediated by cytochrome c (Aparna et al., 2003; Sahdev et al., 2003).
Consistent with these observations, a recent report has shown the localization of phosphorylated eIF2α with cytochrome c in ischemic neuronal cells (Page et al., 2003). Since baculovirus expressed human eIF2α and β- subunits are phosphorylated during expression, as shown in our earlier studies (Suragani et al., 2006), I expressed the human eIF2 subunits in bacteria to primarily obtain them in an unphosphorylated status and to evaluate the effect of phosphorylation of the α- and β-subunits on the intersubunit interaction and also on their interaction with other proteins using pull down and far western analyses. Our findings here indicate that the interaction between α- and β- subunits is enhanced by the phosphorylation of the α- but not β-subunit of eIF2. The non-productive interaction that occurs between phosphorylated α- subunit in the eIF2 complex and eIF2B holoprotein is mediated by the β-subunit. Interestingly, it was observed here, that the phosphorylation of eIF2α or β- subunits resist caspase 3 action. Recombinant bacterially expressed eIF2β subunit is found to interact with Nck1, a co-factor of eIF2α phosphatase and eIF5, a GTPase activating protein of reticulocyte and HeLa cell extracts and the interaction with Nck1, but not with eIF5 is modified by the phosphorylation of the β-subunit. Interestingly, we also observed that phosphorylated eIF2α interacts efficiently with cytochrome c than the unphosphorylated form.

Results:

**Phosphorylation of eIF2α enhances its interaction with eIF2β and eIF2B:** Previously, we have shown that baculovirus-expressed human eIF2 subunits interact with each other in ELISA and dot blot assays (Suragani et al., 2005). Bacterially expressed subunits of eIF2 are also found to interact with each other to form αβ, βγ and αγ complexes both in far western analysis (Fig 2.1A) and pull down experiments (2.1B).

In a far western analysis, antibodies against the subunits of eIF2 have been used to detect the interaction between the recombinant subunits separated on a membrane and the subunits of reticulocyte lysate eIF2 (Fig. 2.1A). In panel (a), the recombinant α-, β- and γ-subunits of eIF2, separated on a membrane were detected by an anti his-tag antibody to indicate the levels of these subunits used in the interaction studies. Lanes 2 and 3 of panel (b) indicate the interaction between the recombinant β and γ-subunits with the α-subunit of
lysate eIF2 as detected by an anti-eIF2α antibody which also recognizes the uninteracting recombinant α-subunit as shown in lane 1. Panel (e) indicates the interactions between the recombinant α- and γ-subunits with the β-subunit of lysate eIF2 as detected by an anti β-antibody (lanes 1 and 3). The antibody also recognizes the uninteracting β-subunit (lane 2). Panels (d) and (e), represent the controls where the membrane containing different recombinant subunits were incubated with anti eIF2α- or β- antibodies respectively to determine the specificity of the antibodies.

Complementing these observations, we observed purified subunits interact with each other to form similar complexes in a pull down assay as described in the legend to Fig 2. 1B. In panel (i), his-tagged γ-subunit is incubated with the Ni-NTA-agarose resin, and the resin was then washed before incubating it with his-tag cleaved recombinant α-subunit to determine the interaction between γ- and α-subunits. Unbound or wash fractions showed very little of the γ- or α-subunits (lanes 1-4 of Fig. 2.1B). The α-subunit co-elutes with the γ-subunit (lanes, 5 and 6; upper portion containing γ and the lower portion indicating the α-subunit). Similarly, we observed that β-subunit co-elutes with the γ-subunit and the α-subunit co-elutes with the β-subunit (Fig. 2.1 B panels ii and iii). These findings reiterate our earlier conclusions (Suragani et al., 2005) that unlike yeast, human eIF2α and β-subunits interact with each other in addition to their interactions with the γ-subunit.

**Phosphorylation of the α-subunit enhances its interaction with the β-subunit:** Unlike baculovirus expressed eIF2 subunits (Suragani et al., 2006), bacterially expressed subunits of human eIF2 are not phosphorylated by any of the bacterial kinases (Fig. 1. 5C). However, they are phosphorylated *in-vitro* by their respective kinases (Fig. 1.5 A&B). Hence we could evaluate here the effect of phosphorylation on α-β interaction (Fig. 2. 2A). To determine the importance of phosphorylation of the α-subunit on the interaction between α and β-subunits, recombinant eIF2α was phosphorylated *in-vitro* by a purified recombinant ER-resident eIF2α kinase and bound to Ni-NTA resin prior to incubating with the his-tag cleaved β-subunit (Fig. 2.2A). Immunoblot analysis of the
**Fig 2.1A:** Interaction between subunits; Far western analysis: Interaction between purified recombinant eIF2 subunits with the subunits of rabbit reticulocyte lysate eIF2, were determined by far western analysis. In this assay, 5 µg of purified his-tagged recombinant α, β and γ- subunits were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with recombinant subunits or with rabbit reticulocyte lysates. The membranes were processed as described in ‘Materials and Methods’. The lanes of panel (a) represent the levels of recombinant eIF2α, β and γ loaded in the respective lanes as determined by anti his-tag antibody. Panel (b) represents the interaction between recombinant β and reticulocyte lysate α (in lane 2) or between recombinant γ and lysate α (lane 3) as judged by an anti-eIF2α antibody. Lane 1 represents recombinant eIF2α. The lanes of panel (c) represent the interaction between recombinant α and reticulocyte lysate β (in lane 1) or between recombinant γ and lysate β (lane 3) as judged by anti-eIF2β antibody. Lane 2 represents recombinant eIF2β. Panels (d) and (e) represent the controls, where the different subunits on the membrane are incubated with anti eIF2α- or β-antibodies respectively. The antibodies recognize the specific subunits only.
Fig 2.1B: Intersubunit interactions by pull down assays: Pull down assays as described in Materials and Methods, were carried out to study the interactions among the purified recombinant eIF2 subunits. In panels (i) and (ii), recombinant purified γ-subunit or in panel (iii), purified β-subunit were bound to the Ni-NTA resin. The resin bound proteins were incubated with enterokinase treated, his-tag cleaved recombinant α- or β-subunit in panels (i) and (ii) respectively, or with α-subunit in panel (iii) to monitor the interactions between the recombinant γ-α, γ-β and β-α respectively. The unbound, wash and bound/eluted fractions were collected and separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane. The membranes were probed with anti his-tag antibody for the γ-subunit and anti eIF2α- or β- antibodies for the specific subunits respectively as shown in the figure. The figure is a western blot. Various lanes are as follows: In all panels, lanes 1 and 2 represent unbound fractions, lanes 3 and 4 represent wash fractions, and lanes 5 and 6 are eluted fractions.
Fig 2.1B:
unbound, wash and eluted fractions of this pull down assay indicate that β-subunit interacts and co-elutes with the α-subunit (lane 7, panels i and ii) and this interaction is enhanced (lane 8, panel i) when eIF2α is phosphorylated (lane 8, panel iii, and iv, the bar diagram). However, we have not observed any enhanced interaction between the α- and β-subunits when β-subunit is phosphorylated (panel v).

The above enhanced interaction between α and β-subunits upon phosphorylation of eIF2α was further confirmed by mutants of eIF2α such as S51A (the nonphosphorylatable form), S51D (a phosphomimetic form) and S48A (a mutant eIF2α that is phosphorylated on its serine 51 residue) (Fig. 2. 2B). The observations indicate that the phosphorylated wt eIF2α and phosphomimetic form S51D interacts more efficiently with the recombinant his-tag cleaved β-subunit of eIF2 than the nonphosphorylatable S51A (lanes 14 and 16 versus lane 15). Also we observed the mutant phosphorylated or unphosphorylated S48A eIF2α cannot interact so efficiently with the β-subunit as wt phosphorylated eIF2α (lanes 17 and 18 versus lane 14).

**Phosphorylated eIF2α enhances its interaction with eIF2B and eIF2β of reticulocyte lysates:** To understand the physiological significance of the above interaction, we studied the interaction of the recombinant α-subunit with the eIF2B and the β-subunit of eIF2 of the lysate. An earlier study in yeast has shown that phosphorylated eIF2α interacts with the regulatory complex of eIF2B in a non-productive manner that inhibits the GDP/GTP exchange activity of eIF2B (Krishnamoorthy et al., 2001; Pavitt et al., 1998). In contrast to this observation, a previous study from this laboratory has shown that baculovirus expressed and phosphorylated human eIF2α-subunit interacts with purified eIF2B only in the presence of β-subunit of eIF2 in an ELISA study (Suragani et al., 2005). Here we have evaluated the interaction of bacterially expressed, purified and phosphorylated human eIF2α interaction with the eIF2B and eIF2β-proteins of the rabbit reticulocyte lysate in a pull down assay (Fig. 3. 1). Analysis of the various unbound and bound fractions using anti-eIF2BE, anti-eIF2β and α-antibodies (panels I- III respectively) indicate that phosphorylated eIF2α-subunit interacts efficiently with the lysate eIF2B protein as
**Fig.2.2A. Effect of phosphorylation of the α-subunit on its interaction with the recombinant β-subunit:** Phosphorylation of the α-subunit was carried as described in ‘Materials and Methods’. Pulldown assays with 5 μg of unphosphorylated and phosphorylated α-subunit of eIF2 and 5 μg of enterokinase treated, his-tag cleaved β-subunit were carried out as described in the legend to Fig 2.1C. Unbound and bound fractions were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane. The western blots are probed with anti eIF2 α- or β-antibodies to detect the specific subunits as in Panels (i) and (ii) respectively. Phosphorylation status of the α-subunit of different fractions was assessed by an Amersham typhoon phosphorimager as in panel (iii). Various lanes in all the panels are as follows: lanes 1 and 2, unbound fractions, lanes 3-6 wash fractions and lanes 7 and 8 eluted fractions. Panel (iv) represents bar diagram indicating the relative levels of the β-subunit in unbound (bars 1 and 2) and in eluted fractions (bars 3 and 4) in the presence of unphosphorylated and phosphorylated α-subunit respectively. Panel (v) represents the interaction of recombinant β-subunit phosphorylated with different kinases with the his-tag cleaved α-subunit as detected by anti-eIF2α antibody in the upper portion and anti-eIF2β antibody in the lower portion. Various lanes are as follows: lanes 1-4, represent the unbound fractions; lanes 5-8, wash fractions and lanes 9-12, represent the elution fractions of a pull down assay.
Fig 2.2A
Fig 2.2B Interaction of unphosphorylated and phosphorylated recombinant eIF2α (wt and mutants) with the β-subunit: Interactions between the unphosphorylated and phosphorylated wt and mutants (S51A, S51D and S48A) of eIF2α with his-tag cleaved recombinant β-subunit was analyzed by a pull down assay. The figure is a western blot probed with an anti-eIF2β antibody. Panel (i) indicates the levels of β-subunit co-eluted with the wt and mutants of eIF2α (lanes 13-18), and panel (ii) indicates levels of eIF2α as detected by anti-eIF2α antibody. The lanes in the figure are as follows: Lanes 1 and 2, represent the unbound fractions of unphosphorylated and phosphorylated eIF2α; 3 and 4, unbound fractions of S51A and S51D; 5 and 6, unbound fractions of S48A in the presence and absence of kinase respectively. Lanes 7-12 represent the corresponding wash fractions and 13-18, the corresponding elution/bound fractions of the pull down assay.
measured by the intensity of the ε-subunit and also with the β-subunit of lysate eIF2 (Fig 3.1, Panel I). Bar diagram indicates the relative levels as indicated (Panel IV). The diminished interaction between the β-subunit of eIF2 and phosphorylated S48A mutant of eIF2α, and the enhanced interaction between the β-subunit and S51D, a phosphomimetic form of eIF2α support our previous and present observations that phosphorylation of Ser51 in eIF2α and the maintenance of the Ser48 residue in eIF2α aids in a complex formation between eIF2 and eIF2B holoproteins (Ramaiah et al., 1994; Sudhakar et al., 1999 and 2000) and that this interaction is mediated by the β-subunit of eIF2. Hence we suggest that the interaction between eIF2α- and β-subunits is physiologically relevant and regulates the productive and non-productive interaction between eIF2 and eIF2B holoproteins, and thereby the GDP/GTP exchange activity of eIF2B. However, the mechanism by which phosphorylation of α-subunit causes the β-subunit to switch from productive to non-productive interaction is not known.

Based on the crystal structural data of yeast and human eIF2α, it has been suggested that the interaction between phosphorylated eIF2α and the regulatory sub-complex of eIF2B can occur due to better surface complementarity and/or more favorable electrostatic interactions (Marintchev and Wagner, 2004). As there is no direct interaction between eIF2α and eIF2B in mammals (Suragani et al., 2005; Kimball et al., 1998), it is likely that phosphorylation of eIF2α induces a conformational change in the eIF2 complex so that β-subunit of eIF2 comes in close proximity presumably to the regulatory sub-complex or more precisely to the δ-subunit of eIF2B. A more detailed study involving the mutations of the subunits is required to determine the possible regions involved in the interaction between α and β-subunits. However, it is likely that the N-terminal region of human β-subunit that is not homologous to eIF5 may be involved in the interaction with the α-subunit of mammalian eIF2.

**β-subunit interacts with Nck1 and eIF5:** The β-subunit in eIF2 aids the γ-subunit in all its functions (Hinnebusch, 2005) and is also reported to interact with several initiation factors, with mRNA, Nck1, a cofactor of an eIF2α phosphatase, CKII and type1 phosphatase (Kapp and Lorsch, 2004; Laurino et al., 1998; Gaspar et al., 1994; Llorens et -
**Fig 3.1: Interaction of recombinant eIF2α with eIF2β and eIF2Bε of reticulocyte lysates: Effect of α phosphorylation:** Recombinant eIF2α was phosphorylated by PERK in the presence of 100 µM unlabelled ATP as described in Materials and Methods. Both unphosphorylated and phosphorylated eIF2α were bound to Ni-NTA resin. Pull down assay in the presence of lysate was carried out as described in Materials and Methods. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. Panels I and II, indicate the levels of eIF2B and the eIF2β of the lysates co-eluted with unphosphorylated and phosphorylated recombinant eIF2α as detected by an anti-eIF2Bε and anti-eIF2β antibodies. Panel III, indicates the levels of eIF2α eluted under similar conditions. Various lanes are as follows: lane 1, input; lane 2, unbound fraction; lanes 3 and 5, wash fractions and lanes 4 and 6, eluted fractions.

Panel IV, represents a bar diagram indicating the relative levels of eIF2Bε (dark bars) and eIF2β (grey bars) from reticulocyte lysate eluted in the presence of unphosphorylated and phosphorylated α-subunit.
Fig 3.1:

Panel I

Panel II

Panel III

Panel IV

**Input Unbound Wash Elution (α)**

**Wash (α)**

**Elution (α)**

**Elution (αp)**

**Mr**

**eIF2Bε**

**eIF2β**

**eIF2α**

**Panel IV**

![Graph showing eIF2β/2Bε Binding](image)

**Y-axis:** eIF2β/2Bε Binding

**X-axis:**
- Input
- α Elution
- α (p) Elution

Legend:
- **eIF2Bε**
- **eIF2β**
Here we have evaluated the interaction of bacterially expressed recombinant β-subunit with eIF5, a GTPase activating protein, and Nck1 of reticulocyte and HeLa cell extracts by far western and pull down analysis (Figs. 3.2A and B). In far western analysis, the three subunits of human eIF2 are separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Fig. 3.2A panel i). The membranes were incubated with rabbit reticulocyte lysates (panels ii and iii) and probed with anti- Nck1 or eIF5 antibodies to detect the interaction of these subunits with Nck1 (ii) or eIF5 (iii) respectively. Our observations here, indicate that bacterially expressed human eIF2 β-, but not α and γ-subunits interacts with Nck1 and eIF5 (ii and iii) respectively. However, we also observed a faint interaction between the recombinant eIF2γ-subunit and eIF5 (panel iii).

Consistent with these above observations, the recombinant β-subunit was able to interact with Nck1 and eIF5 of the reticulocyte and HeLa cell extracts in a pull down assay (Fig 3.2B). Panel I indicates the interaction between eIF2β and Nck1, where the reticulocyte or HeLa cell lysate specific Nck1, coelutes with eIF2β (lanes 4 and 8 of the upper portion representing the reticulocyte and HeLa cell Nck1 respectively) as detected by an anti Nck1 antibody, while the lower portion indicates eIF2β as detected by an anti eIF2β antibody. Similarly, panel II indicates the interaction of the recombinant eIF2β-subunit with eIF5 of the extracts.

Further, the effect of phosphorylation of eIF2β on its interaction with Nck1 and eIF5 of reticulocyte lysate was also studied here. The recombinant eIF2β-subunit was able to interact with Nck1 of reticulocyte lysate in a pull down assay as analyzed by an anti-Nck1 antibody (Fig. 3.2C, panel i, lane 9) as also stated above (Fig 3.2B). This interaction was enhanced when the β-subunit was phosphorylated by PKA and PKC (lanes 10 and 11) and decreased when it was phosphorylated by CKII (lane 12). However, the amount of the β-subunit eluted under similar conditions was almost same (Fig. 3.2C, panel ii). Phosphorylation status of β-subunit for the corresponding lanes is shown in the phosphorimage (panel iii).
Fig 3.2A: Interaction of recombinant eIF2 subunits with Nck1 and eIF5: Far western assay was carried out as described in Materials and Methods to determine the interaction between different subunits of eIF2 and Nck1 or eIF5 of lysates. The figure is an immunoblot, where panel (i) indicate the levels of recombinant subunits used in the assay as detected by an anti his-tag antibody. Panels (ii) and (iii) indicate the interaction between the recombinant eIF2 subunits with Nck1 and eIF5 of reticulocyte lysate as detected by an anti Nck1 or eIF5 antibodies.
**Fig 3.2B: Interaction of the β-subunit with Nck1 and eIF5- Pull down assay:**

Recombinant eIF2β-subunit was bound to Ni-NTA resin and pull down assay in the presence of lysate was carried out as described in ‘Materials and Methods’. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. Panels I and II depict the interaction of the β-subunit with Nck1 and eIF5 respectively of reticulocyte and HeLa cell lysates. The upper portion of both panels indicates Nck1 or eIF5 as detected by anti- Nck1 or eIF5 antibodies, co-eluted with the recombinant β-subunit as shown in the lower portion. Various lanes in both panels are as follows: lanes 1 and 5 unbound; lanes 2, 3, 6 and 7 represent wash fractions and lanes 4 and 8 are elution fractions.
Fig 3.2C: Phosphorylation of the β-subunit- Interaction with Nck1 and eIF5: The recombinant subunit was phosphorylated in the presence of [γ\(^{32}\)P] ATP with PKA, PKC and CKII as described in ‘Materials and Methods’. Pull down assay with the unphosphorylated and phosphorylated subunits in the presence of lysates was carried out as described in ‘Materials and Methods’. Panel A represents the interaction between unphosphorylated and phosphorylated eIF2β by different kinases with Nck1 of reticulocyte lysate. The upper portion (i) of the western blot represents the levels of Nck1, as analyzed by an anti-Nck1 antibody co-eluted with β-subunit indicated in the lower portion (ii) as judged by an anti-eIF2β antibody. The various lanes are as follows: lane 1, unbound β-subunit without any kinase; lanes 2-4 represent unbound β-subunit phosphorylated by PKA, PKC and CKII respectively; lanes 5-8 represent the wash fractions corresponding to lanes 1-4, and lanes 9-12 represent the eluted fractions of Nck1 or eIF2β corresponding to lanes 1-4. Panel (iii) corresponds to the phosphorimage indicating the phosphorylation status of the β-subunit by different kinases in different fractions as mentioned above.

Panel B represents the interaction between unphosphorylated and phosphorylated eIF2β with eIF5 of reticulocyte lysate. Top portion (iii) is a western blot representing the levels of reticulocyte lysate eIF5 co-eluted with unphosphorylated (lane 5) and phosphorylated β-subunit (lane 6) as analyzed by an anti-eIF5 antibody. Panel (iv) indicates the phosphorylation status of eIF2β by PKA as analyzed by phosphorimage. Various lanes are as follows: lanes 1 and 2 unbound; lanes 3 and 4, wash fractions and lanes 5 and 6 represent eluted/bound fractions of the unphosphorylated and phosphorylated β-subunit respectively.
Fig 3.2C:
However, the levels of eIF5 co-eluted with unphosphorylated (panel iv, lane 5) and PKA phosphorylated β-subunit (lane 6) was almost similar suggesting that the phosphorylation of the β-subunit (panel v) does not affect its interaction with eIF5. The fact that Nck1, a cofactor of eIF2α phosphatase, interacts with the β-subunit and phosphorylation of the β-subunit modifies its interaction with Nck1 also suggests that probably the phosphorylation of eIF2α is regulated through changes in the β-subunit phosphorylation and its interaction with Nck1.

**Caspase 3 interacts and processes the recombinant α- and β-subunits of eIF2:** Dot blot analysis indicates that caspase 3 interacts with recombinant eIF2α and β-subunits (Fig 3.3). Panel A, indicates the interaction between caspase 3 spotted on the membrane and recombinant eIF2α expressed both in insect cells and bacteria. CPK and BSA serve as controls. Similarly recombinant eIF2β also interacts with caspase 3 as indicated in panel II.

**Phosphorylation of α- and β-subunits resists caspase-3 action:** Previous studies have shown that caspase-3 processes readily unphosphorylated eIF2α (Satoh et al., 1999; Marissen et al., 2000). Phosphorylation of eIF2α is a cause and consequence of caspase activation. Phosphorylated eIF2α is required to maintain the adaptive response and to induce caspase activation and apoptosis as has been suggested (Scheuner et al., 2006; Aparna et al., 2003; Harding et al., 2000). However no such information is available on the β-subunit or on the effect of phosphorylation of these subunits on the caspase action. Hence we have studied the ability of the bacterially expressed, phosphorylated and unphosphorylated human eIF2α and β-subunits to serve as substrates for caspase-3 (Fig 3.4A). Bacterially expressed human eIF2α was cleaved by caspase-3 in a time dependent manner and the cleavage was inhibited by Ac-DEVD CHO, a caspase-3 inhibitor (Fig 3.4A, Panels I and II) as expected. Caspase-3 was also found to process the bacterially expressed β-subunit in a time-dependent manner and the caspase inhibitor mitigates caspase-3 action (Fig 3.4A, Panels III and IV).
Fig 3.3: Interaction of recombinant α- and β-subunits with caspase 3: The interaction of the recombinant α- and β-subunits with caspase 3 was analyzed by a dot blot assay. Caspase 3 was spotted on a nitrocellulose membrane, air dried and blocked with 3% non-fat milk powder in TBST for 60 min. The membranes were then incubated with insect cell or bacterially expressed α-subunit (Panel A) or β-subunit (Panel B) as indicated in the figure. CPK and BSA were used as control proteins. Panels A and B, indicate the interaction between caspase 3 spotted on the membrane and eIF2α or eIF2β, as detected by an anti eIF2α- or β- antibodies.
Fig 3.4A. Caspase 3 cleaves recombinant α- and β-subunits of eIF2: Panels I and II represent the processing and time-course of the cleavage of recombinant eIF2α subunit by caspase 3 respectively. The caspase 3 cleavage of the α-subunits is mitigated in the presence of Ac-DEVD-CHO, a specific inhibitor of caspase 3 (lanes 3 vs 2 of Panel I). The time-course of the cleavage indicates that maximum cleavage of the α-subunit is observed at around 8 hrs (Panel II).

Panels III and IV represent the processing and time-course of the cleavage of recombinant β-subunit of eIF2 by caspase 3 respectively. Caspase 3 processes the β-subunit efficiently and the cleavage is inhibited in the presence of Ac-DEVD-CHO, a specific inhibitor of caspase 3 (lanes 6 vs 5 of Panel III). Maximum cleavage of the β-subunit is observed at around 6 hrs (Panel IV). The subunits are not accessible as substrates for caspase 6 (Panel V).
Fig 3.4A.

Cleavage +/- Ac-DEVD CHO

Panel I

Panel II

Panel III

Panel IV

Panel V

eIF2β + Caspase 6

Kinetics of Cleavage

Time in hrs: 0 4 6 8

Time in hrs: 0 1 2 4 6
Phosphorylation of bacterially expressed eIF2α was carried out in the presence of recombinant mouse PERK and labeled ATP prior to caspase treatment (Fig 3.4B, Panel A, lane 4) and also after caspase treatment (lane 2). Both processed and unprocessed eIF2α subunit serve as substrates for kinase. However, phosphorylated eIF2α resists caspase-3 processing (Fig 3.4B, lane 4 vs. 2). Analysis of phosphorylation of bacterially expressed recombinant human β-subunit reveals that it is phosphorylated by PKA (Fig 3.4B, lower part of panel B, lane 5), PKC (lane 6) and CKII (lane 7). However, PKA is found to phosphorylate more efficiently than PKC or CKII in-vitro (5 vs. 6 and 7). As has been observed with the α-subunit, the β-subunit is efficiently processed by caspase-3 prior to its phosphorylation than after phosphorylation by any of the three kinases tested here i.e., PKA, PKC and CKII (Fig. 3.4B, upper portion, lanes 12, 13 and 14 vs. lanes 8, 9 and 10). Recombinant α- and β-subunits of human eIF2 serve as substrates for caspase 3 (Fig 3.4A) but phosphorylation of these subunits makes them resistant to caspase 3 action (Fig 3.4B). However, these subunits are not accessible as substrates for caspase 6 (Fig 3.4A, panel V).

Since β-subunit interacts with eIF2α, eIF2B, eIF5 and Nck1, aids the γ-subunit in many of the eIF2 functions, regulates the productive and non-productive interactions between eIF2 and 2B complexes and is a substrate for kinases and caspase (s), these support the idea that the β-subunit of mammalian eIF2 plays a critical role in the function and regulation of eIF2 activity.

**Cytochrome c interacts with eIF2α:** Since eIF2α interacts with caspases (Marissen et al., 2000), its phosphorylation is implicated in apoptosis (Scheuner et al., 2006; Aparna et al., 2003) and a recent study has shown the that phosphorylated eIF2α and cytochrome c are co-localized in ischemic neuron'al cells (Page et al., 2003), we have studied here, the interaction between recombinant human eIF2α and purified horse cytochrome c using co-immunoprecipitation, pull down assays and the equilibrium constant for the binding of eIF2α and cytochrome c was also calculated (Figs 3.5 A-C).

Immunoprecipitation assays were carried out as described in ‘Materials and Methods’ and the immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti- eIF2α or cytochrome c
Fig 3.4B: Effect of phosphorylation of α- and β-subunits on their cleavage by caspase 3: As the recombinant α- and β-subunits are phosphorylated by their respective kinases (Figs 1.5 A and B) and also serve as substrates for caspase 3 (Fig 4.2A), the effect of phosphorylation of the subunits by respective kinases on their cleavage by caspase 3 was analyzed here. Panel A represents the effect of phosphorylation of the α-subunit on its cleavage by caspase 3. Phosphorylation of the α-subunit by PERK was carried out in the presence of [γ³² P] ATP before and after caspase 3 processing as described in ‘Materials and Methods’. The figure represents a phosphorimage indicating the phosphorylation status of the α-subunit before and after caspase 3 treatment. Various lanes are as follows: lane1, eIF2α-subunit + PERK; lane 2, eIF2α + caspase 3 + PERK; lane 3, eIF2α + PERK; lane 4, eIF2α + PERK + caspase 3.

Panel B represents the cleavage of unphosphorylated and phosphorylated β-subunit by caspase 3. Phosphorylation of the β-subunit by PKA, PKC and CKII was carried out in the presence of [γ³² P] ATP before and after caspase 3 processing as described in ‘Materials and Methods’. The upper portion is a coomassie stained gel indicating the caspase 3 cleavage of the phosphorylated (lanes 8, 9 and 10) and unphosphorylated β-subunit (lanes 12, 13 and 14) respectively. Lower portion represents the corresponding autoradiogram of the phosphorylation of β-subunit by different kinases before and after caspase 3 treatment. The various lanes are as follows: lane 1, + eIF2β; lane 2, + PKA; lane 3, + PKC; lane 4, + CKII; lane 5, eIF2β + PKA; lane 6, eIF2β + PKC; lane 7, eIF2β + CKII; lane 8, PKA phosphorylated eIF2β + caspase 3; lane 9, PKC phosphorylated eIF2β + caspase 3; lane 10, CKII phosphorylated eIF2β + caspase 3; Mr represents marker lane; lane 11, eIF2β + CKII; lane 12, caspase 3 treated β + PKA; lane 13, caspase 3 treated β+ PKC; lane 14, caspase 3 treated β + CKII.
Fig 3.4B.

Panel A

Panel B

1  2  3  4  5  6  7  8  9  10 Mr  11  12  13  14

97
66
43
29
20

← eIF2β
← Processed β
← fragments
antibodies to determine the interaction between these two proteins (Fig 3.5A, panels I and II). Complementing these observations with purified cytochrome c, we have also carried out another experiment as described in ‘Materials and Materials’ to demonstrate the interaction between the recombinant α-subunit of eIF2 and cytochrome c of HeLa cell extracts (Fig 3.5A panel III). Results of these experiments suggest that eIF2α interacts with cytochrome c. Determination of the equilibrium constant for the binding of eIF2α and cytochrome c at 415 nm resulted in the KSS ~2.7 µM⁻¹, which indicates a significant binding between the two proteins (Fig 3.5B). Further the effect of phosphorylation of eIF2α was also analyzed by a pull down assay (Fig 3.5C). The results indicate that phosphorylated eIF2α interacts efficiently with cytochrome c than the unphosphorylated form (Fig 3.5C Panel I and panel II representing a bar diagram). We have also analyzed the ability of cytochrome c bound eIF2 to serve as a substrate for caspase 3 (Fig 3.6 Panel A). The results indicate that eIF2α interaction with cytochrome c does not alter the efficiency of eIF2α processing by caspase 3, although eIF2α can interact with both cytochrome c and caspase 3 at the same time (3.6 Panel B). However, the physiological significance of this interaction is not clear and requires further studies.
Fig 3.5A: Interaction between eIF2α and Cytochrome c: Co-immunoprecipitation studies with either anti- eIF2α or cytochrome c antibodies were carried out as described in Materials and Methods. Panels I and II represent the interaction between the two purified proteins. Panel I shows the interaction where the complex was precipitated with anti-eIF2α antibody, fractions were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane, which was probed with anti-cytochrome c antibody to detect the interacting protein. Likewise in panel II, the complex precipitated by anti-cytochrome c antibody and the membrane probed with anti-eIF2α antibody to detect the interacting proteins. Panel III represents the interaction of the recombinant α-subunit with the cytochrome c of HeLa cells. The complex precipitated with anti-cytochrome c antibody and the membrane was probed with anti-eIF2α antibody to detect the interaction. Various lanes are as follows: 1, Unbound; 2 and 3, Wash fractions; and 4, Eluted fractions.
Fig 3.5B: Interaction between eIF2α and Cytochrome c – Determination of the binding constant: The equilibrium constant for the binding of eIF2α with Cytochrome c was determined as described in ‘Materials and Methods’. Panel (i) represents the changes in the visible absorption spectra of the cytochrome c in the presence of various concentrations of eIF2α. Panel (ii) represents the analysis of the absorbance values as a function of eIF2α concentration. The equilibrium association constant is calculated from the value of pK_{ass}, which is obtained from the X intercept of the straight line. Equilibrium binding constant as calculated using equations as described in ‘Materials and Methods’ is found to be \( \sim 2.7 \ \mu\text{M}^{-1} \).
Fig 3.5B:

Panel (i)

Panel (ii)

\[ \log[\text{eIF2-}\alpha_{\text{free}}, (\mu\text{M})] \]

\[ \log[\Delta A/\Delta A^\infty - A] \]
Fig 3.5C: Phosphorylation of eIF2α- Effect on eIF2α and Cytochrome c interaction:
Recombinant eIF2α was phosphorylated by PERK in the presence of 100 µM unlabelled ATP as described in ‘Materials and Methods’. Pull down assay with unphosphorylated and phosphorylated eIF2α and cytochrome c was carried out as described in ‘Materials and Methods’. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. The upper portion of panel I represent the levels of Cyt c co-eluted with unphosphorylated (lane 5) and phosphorylated eIF2α (lane 6). Bottom portion of panel I depicts the levels of eIF2α eluted under these conditions. Various lanes are lanes 1 and 2, unbound fractions of unphosphorylated and phosphorylated eIF2α respectively; lanes 3 and 4, corresponding wash fractions; lanes 5 and 6 are corresponding elution fractions. Panel II is a bar diagram representing the quantification of the corresponding fractions.
**Fig 3.6: eIF2α - Cytochrome c interaction: Effect on caspase 3 cleavage of the α-subunit:** The figure is a western blot. Panel A represents the cleavage of recombinant α-subunit of human eIF2 by caspase 3 in the absence (lane 3) and presence of cytochrome c (lane 4). Caspase 3 cleavage of recombinant α-subunit in the presence of cytochrome c was carried out as described in ‘Materials and Methods’. Panel B represents the interaction between recombinant eIF2α, cytochrome c and caspase 3 by a pull down assay carried out with three proteins as described in ‘Materials and Methods’. Various lanes in Panel A are as follows: 1, eIF2α; 2, eIF2α + Cyt c; 3, eIF2α + Caspase 3; 4, eIF2α + Cyt c + Caspase 3 and Mr represent Marker. Lanes in the top, middle and bottom portions of panel B are lane1, unbound fraction; lanes 2 and 3, wash fractions and lane 4 represent the elution fraction.