Synthesis of A Fluorescent mRNA Cap Analogue and A Study of Cap Structure Recognition, Guanosine Nucleotide Binding and Exchange, and Helicase Activity in Wheat Germ Protein Synthesis Initiation

By

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The City University of New York

Abstract

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For the study of the cap binding reaction, one approach is to use fluorescence spectroscopy. A ribose modified fluorescent cap analogue, Ant-m^7GTP, was designed and synthesized for this purpose. This fluorescent cap analogue was found to have a high quantum yield, resistance to photobleaching, and avoided overlapping of excitation and emission wavelength with those of proteins. The binding of Ant-m^7GTP with wheat germ initiation factors eIF-4F and eIF-(iso)4F was measured. The fluorescent cap analog was found to react with cap binding protein with similar affinity with m^7GpppG. The microenvironment of Ant-m^7GTP when bound to protein was tested by fluorescence quenching experiments. The second portion is devoted to study of the recycling of wheat germ eIF-2

in protein synthesis initiation. A direct fluorescence spectroscopic study on the binding of wheat germ eIF-2 with GDP and GTP was performed. Results indicated that dissociation constants for wheat germ eIF-2•GTP and eIF-2•GDP were more similar than for mammalian eIF-2. A substitution experiment was performed and it was found that wheat germ eIF-2 bound GDP can be readily exchanged with free GTP at a relatively low GTP/GDP concentration ratio. This suggests the possibility that in the wheat germ system, guanosine nucleotide exchange may be regulated by controlling the GTP/GDP concentration ratio and may not require an analog of the mammalian guanosine nucleotide exchange factor eIF-2B. In the third major portion, the interaction of two wheat germ eukaryotic protein synthesis initiation factors, eIF-(iso)4F and eIF-4A, with mRNA has been examined for helicase activity. It was found that eIF-4A and eIF-(iso)4F could unwind the double stranded RNA. ATP and Mg^{2+} were required for this reaction. The reaction was cap-dependent and required a single stranded region. These studies combined with binding affinity studies suggest that the cap-binding subunit of eIF-(iso)4F forms a complex with mRNA. Subsequently, eIF-4A is bound. Hydrolysis of ATP provides energy for the protein complex to unwind the double-stranded region.

Abbreviations

Ac	acetate
BME	β-mercaptoethanol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
eIF	eukaryotic initiation factor
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
kDa	kilodlaton
m^7G	7-methylguanosine
PEG	polyethyleneglycol
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
STI	soybean trypsin inhibitor
Tris	tris(hydroxymethyl)aminomethane
DMF	N,N-dimethylformamide
Ant-m^7GTP	anthraniloyl 7-methylguanosine triphosphate

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Chapter 1 Introduction

1.1 Overview of protein synthesis initiation

Eukaryotic protein synthesis is divided into three phases: initiation; elongation; and termination. The reactions in each phase are promoted by soluble protein factors that transiently interact with the ribosome, mRNA, and aminoacyl-tRNAs. During the initiation phase, the various binding reactions are promoted by at least 10 initiation factors (abbreviated eIF). The initiation pathway can be identified four major steps:

Step 1. ribosome dissociation into 40S and 60 S subunits

Two initiation factors eIF-3 and eIF-4C shift the equilibrium between 80S ribosome and dissociated subunits towards dissociation by binding to the 40S subunit and preventing their association with 60S subunit. Another factor, eIF-6, binds to the 60S subunit and prevents subunit association.

Step 2. Met-tRNA$_i$ binding to the 40S ribosomal subunit to form a 40S preinitiation complex

eIF-2, in a binary complex with GTP, binds Met-tRNA$_i$ to form a ternary complex. The ternary complex binds to the 40S ribosomal subunit to form a 40S preinitiation complex.

Step 3. mRNA binding to the 40S preinitiation complex to form a 40S initiation complex

The 40S preinitiation complex either binds to the 5'-terminus of the mRNA, then scans linearly down the mRNA until it recognizes the initiator codon or binds to an internal region of the mRNA and starts the scanning process. In either case, formation of the 40S initiation complex is promoted by ATP hydrolysis and by a number of initiation factors. Following recognition of the cap structure by the initiation factors (eIF-4F, eIF-4E) and melting out of secondary structure near the 5'-terminus (eIF-4A, eIF-4B and eIF-4F, in mammalian systems), the 40S preinitiation complex binds to the cap-proximal region of the mRNA. The 40S ribosomal subunit then begins the scanning process in search of the initiation codon, AUG.

Step 4. Junction of the 40S initiation complex with the 60S subunit to form an 80S initiation complex and initiation factors recycling

The 60S ribosomal subunit binds to the 40S subunit carrying mRNA and Met-tRNA$_i$ positioned at the initiation codon. The reaction requires the function of eIF-5 and the hydrolysis of the GTP molecule bound to eIF-2. The GTPase reaction results in the ejection of eIF-2•GDP. The eIF-2•GDP binary complex must exchange the GDP for GTP in order to catalyze another round of initiation. This reaction requires catalysis by eIF-2B in mammalian systems.

As mentioned above, a variety of initiation factors, including eIF-1, eIF-2, eIF-2B, eIF-3, eIF-4A, eIF-4B, eIF-4C, eIF-4D, eIF-4F, eIF-(iso)4F, eIF-5, eIF-6 etc, are involved in the initiation phase. A summary of the function and characterization of these initiation factors is given below.

1.2 Overview of protein synthesis initiation factors

eIF-1

Eukaryotic initiation factor type 1 (eIF-1) is one of the smallest and least well studied protein synthesis initiation factors (1-3). By gel filtration and sodium dodecyl sulfate (SDS) gel electrophoresis, eIF-1 appears to be a single polypeptide of molecular weight 15,000 Da. The reason that its function is unclear is because eIF-1 only slightly stimulates polypeptide synthesis at a number of steps. It did not form a stable complex with 40S, 60S or 80S ribosomes and thus, like all other translation factors, would appear to cycle on and off the ribosome. eIF-1 has not been purified from wheat germ or other plants.

eIF-2

The primary function of eIF-2 is to bind the initiation tRNA, Met-$tRNA_i$, in a GTP dependent manner. This is important both for providing

an aminoacyl-tRNA in the P site of the ribosome and for identifying the initiation AUG codon. eIF-2 has been found to contain three ploypeptides with molecular masses of 35 kDa, 38 kDa, and 52 kDa and named α, β and γ respectively. eIF-2 has also been purified from wheat germ with three distinct subunits with molecular weight 38 kDa, 42 kDa and 55 kDa (4-8).

eIF-2B

As a product of initiation, a complex of eIF-2 and GDP is released. In mammalian systems, in as much as eIF-2 has a 100 fold preference for GDP over the substrate GTP, a guanosine nucleotide exchange factor designated GEF or eIF-2B is required for the recycling of mammalian eIF-2 (9-15). The mammalian eIF-2B binds to eIF-2•GDP, releases the GDP and allows eIF-2 to bind GTP and Met-tRNA$_i$ again. The phosphorylation of the α subunit of mammalian eIF-2 causes eIF-2 to bind eIF-2B more tightly, effectively sequesters the eIF-2B and allows the pool of inactive eIF-2-GDP to build up. The lack of functional eIF-2•GTP•Met-tRNA$_i$ prevents the initiation process and protein synthesis is shut down. Up to now, a mammalian eIF-2B like guanosine nucleotide exchange factor has not been found in wheat germ or other plant systems. Phosphorylation of wheat germ eIF-2 did not repress the protein synthesis activity. At this time it is not clear whether or not the activity of eIF-2 in higher plants is also regulated in a similar manner.

Co-eIF-2

Co-eIF-2 was found stimulate the formation of eIF-2•GDP and eIF-2•GTP•Met-tRNA$_i$ complex. Two Co-eIF-2 were purified from wheat germ, Co-eIF-2a (15,000 Da) and Co-eIF-2b (83,000 Da) (16-18). Both factors are heat stable and their activities are not synergistic, suggesting that they act independently. They were found not to act as guanosine nucleotide exchange factors and their relationship to other initiation factors is not known.

eIF-3

The major effect of eIF-3 in initiation is to bind 40S ribosomal subunits in the absence of other initiation factors and facilitate the dissociation of 80S ribosomes. eIF-3 also enhances the binding of ternary complex, eIF-2•GTP•Met-tRNA$_i$, to the 40S ribosome and is required for the subsequent binding of mRNA to the 40S ribosome. eIF-3 is the largest of the initiation factors, with an overall molecular mass of 600 to 650 kDa (1-3, 19-21). The mammalian protein contains 8 different subunits, whereas wheat germ eIF-3 contains 10 different subunits. Wheat germ eIF-3 binds mRNA in the absence of 40S ribosomes. The binding of eIF-3 to mRNA is inhibited by any single-stranded nucleic acid (RNA or DNA) and the binding is not affected by mononucleotides (NTPs, m^7GTP, m^7GpppG or GDP).

eIF-4A

The role of eIF-4A is to work in concert with eIF-4F and eIF-4B to catalyze the ATP dependent movement and/or unwinding of secondary structures in the 5' of mRNAs prior to binding of the 40S preinitiation ribosome complex and facilitate the binding of the 40S preinitiation ribosome complex to mRNA. eIF-4A is characterized as a single-stranded RNA-dependent ATPase, an activity characteristic of RNA helicases or unwinding proteins. eIF-4A functions as a single subunit of about 45 kDa (1-3, 21-26). eIF-4A was also purified from wheat germ and like mammalian eIF-4A, is a single subunit of about 45 kDa. Both plant and mammalian eIF-4A have been isolated in a complex with eIF-4F. Mammalian eIF-4A has been shown to cycle in and out of the eIF-4F complex during the initiation process. Wheat germ eIF-4A binds ATP and catalyzes the hydrolysis of ATP. Wheat germ eIF-4A does not bind mRNA or mRNA analogues.

eIF-4B

The most dramatic role of eIF-4B in initiation is to act in concert with eIF-4F and eIF-4A, to enhance the ATPase activity and the ATP-dependent unwinding of secondary structures in the 5' of mRNAs prior to binding of the 40S preinitiation ribosome complex. eIF-4B is generally characterized by gel electrophoresis from mammalian sources as an 80 kDa polypeptide (1-3, 27). A factor from wheat germ that corresponded in molecular weight to mammalian eIF-4B (~80,000 kDa)

was isolated and was incorrectly identified as eIF-4B in early reports (28,29). This factor has been correctly identified as an isoenzyme form of eIF-4F and termed eIF-(iso)4F. A factor was subsequently isolated from wheat germ that had similar enzymatic properties to mammalian eIF-4B. This factor was then correctly named eIF-4B when eIF-(iso)4F was identified. Wheat germ eIF-4B is a single subunit with a molecular weight of 59,000 Da as measured by SDS gel electrophoresis (30,31).

eIF-4C

The effect of eIF-4C in initiation of protein synthesis is to stimulate the dissociation of 80S ribosomes and prevent the reassociation of 40S and 60S subunits: AUG-directed Met-puromycin synthesis, AUG-directed binding of Met-tRNA$_i$ to 40S and 80S ribosomes. eIF-4C is a single subunit, low molecular weight initiation factor of only 17,000 Da. It has been characterized in both mammalian and wheat germ systems (1-3, 21, 32-34). In the wheat germ system, eIF-4C appears to be the only heat-stable initiation factor, maintaining more than 85% of its activity after being heated to 90 °C for 5 min.

eIF-4D

The specific function of eIF-4D is largely unknown in protein synthesis although it was thought to participate in the formation of the first peptide bond (1, 32). No stimulation of polypeptide synthesis in vitro was

ever observed. Like eIF-4C and eIF-1, eIF-4D is a low molecular weight protein, about 16,000 Da.

eIF-4F

The major effect of eIF-4F is recognizing the 5' m^7G cap structure of RNA (35-40) and processing helicase activity in concert with other initiation factors (41-43), eIF-4A and eIF-4B. eIF-4F purified from mammal and wheat germ cells contains different numbers of subunits. Mammalian eIF-4F contains three subunits with molecular weight 26 kDa, 45 kDa and 220 kDa, respectively (44). Whereas wheat germ eIF-4F lacks the eIF-4A like, 45kDa subunit (28, 29). The small subunit, occasionally referred to as eIF-4E, appears to be uniquely responsible for recognition of the m^7G cap structure at the 5' end of eukaryotic mRNAs. The 45 kDa subunit is eIF-4A and is the core factor responsible for helicase reaction.

eIF-(iso)4F

eIF-(iso)4F is uniquely found in wheat germ. It contains two subunits with molecular weights of 28 kDa and 82 kDa (28, 29). It was identified as wheat germ eIF-4B when first isolated due to its molecular weight. The smaller subunit was found to bind the m^7G cap structure in a manner similar to the eIF-4F smaller subunit 28 kDa (eIF-4E) (45-48). eIF-(iso)4F was found to be able to substitute eIF-4F in protein synthesis

initiation (45-49). It is a isoenzyme of eIF-4F and therefore named eIF-(iso)4F.

eIF-5

eIF-5 is required for the hydrolysis of GTP bound to the eIF-2•Met-tRNA$_i$ complex. Hydrolysis of GTP is necessary for the release of eIF-2•GDP, allowing the joining of the 60S subunit and forming the 80S ribosome complex. eIF-5 purified from mammalian cells is a single polypeptide chain of 125 kDa (1,2,50,51). eIF-5 has only been partially purified from wheat germ. Relatively, little is known about the wheat germ protein.

eIF-6

eIF-6 appears to provide ribosome anti-reassociation activity, preventing the association of 40S and 60S ribosomal subunits by binding to the 60S ribosome (52-54). This protein appears functional as a single subunit of about 25 kDa. As was true for eIF-5, there has been relatively little work done on eIF-6 since it was first purified and characterized.

Chapter 2 Outline the Problems and Approaches

2.1 7-methylguanosine(m7G) cap structure and fluorescent cap analogue

The recognition of mRNA by components of the translation machinery is crucial in the control of protein synthesis. In the mRNA recognition step of protein synthesis initiation, the 5' terminal 7-methylguanosine (m^7G) cap structure and several cap specific binding proteins play an important role. A number of initiation factors have been shown to bind the m^7G cap structure, including eIF-4E, eIF-4F and an isoenzyme of eIF-4F unique to wheat germ, eIF-(iso)4F. It has been shown that eIF-4E or its similar components in eIF-4F and eIF-(iso)4F was responsible for direct cap binding reactions. These cap specific reactions have been studied in several systems (for reviews, see reference 1-5).

One approach to study the mechanism of the cap recognition reaction in protein synthesis initiation is the use of fluorescence spectroscopy. A previous report (6) has used a fluorescent derivative of poly(A), polyetheno A, as a competitive ligand to study the cap binding reaction. Protein fluorescence has also been used to study these reactions (7-12). Another approach is to directly use a fluorescent cap structure to study the cap binding reactions. Use of a fluorescent cap derivative itself has several advantages: in general, fluorescent derivatives have a greater intensity than intrinsic protein fluorescence; and monitoring cap binding in the presence of several proteins is more feasible with a fluorescent cap derivative. Although the m^7G cap itself is fluorescent (6, 13-16), its fluorescence intensity is very low, making it difficult to use for detailed, quantitative studies of cap binding reactions. In addition, its excitation and emission

wavelength range overlap with that of proteins and therefore interpretation of protein interactions is not straightforward. A fluorescent cap structure with an excitation and emission range suitable for protein binding studies has not been reported. We therefore have made effort to synthesize a fluorescent cap structure for cap recognition study.

Fluorescent m^7G cap analogue

It has been shown (17-21) that only 7-methylguanosine, ribose and the phosphoryl moiety are necessary for recognition by cap binding proteins. m^7GDP and m^7GTP demonstrate binding properties very similar to dinucleotide caps and were used as cap analogues for protein binding and cap recognition studies. A derivative of m^7GTP should therefore serve as a suitable substrate for cap binding proteins.

Protein recognition of the cap structure is sensitive to alterations in base and phosphoryl moieties, but relatively insensitive to alteration in the ribose moiety (17-20). The cis-diol structure was not essential for cap recognition (22-24) and therefore, is a desirable target for modification.

For nucleotide specific enzymes which are sensitive to alteration in the base and phosphoryl moiety of the nucleotide, ribose modified nucleotides are specially useful and widely used. Several fluorescent groups, 2,4,6-trinitrophenyl (TNP), dansyl-β-alanine, fluorescamine, fluorescein, rhodamine, anthraniloyl (Ant) and methylanthraniloyl (Mant), have been

successfully linked to the ribose moiety (25-30). These alterations on the ribose ring were found to be well tolerated by nucleotide specific enzymes.

A desirable fluorescent cap analogue will have a high quantum yield, remain stable to photobleaching, and have absorption and emission maxima distinct from those of protein and nucleic acids. In addition, a simple synthesis and purification is desirable. An anthraniloyl group linked to the ribose of m^7GTP is a good candidate for such a probe. Dansyl and fluorescamine derivatives of nucleic acid bases have been used (27,28), however the synthesis and purification were very complicated. Similarity, fluorescein and rhodamine groups are not ideal because they are larger than the original nucleotides. The synthesis of such derivatives requires breaking of the ribose ring and changing it to a six membered ring (29). Open ring side products are also produced. 2,4,6-trinitrophenyl (TNP) derivatives have a low quantum yield and are unstable to photobleaching (25,28). In contrast, Ant and Mant derivatives do not have the above disadvantages. Ant-ATP and Ant-GTP have been synthesized and successfully used in several enzyme reactions (30,31). Modification of the ribose ring had no effect on the enzyme binding properties of these analogues. These results suggested the possibility of synthesizing a fluorescent m^7GTP cap analogue labeled on the ribose moiety with an anthraniloyl group.

Here we present the synthesis of the fluorescent derivative of m^7GTP, anthraniloyl m^7GTP (Ant-m^7GTP) (Fig.2.1), and its spectroscopic and reaction properties with wheat germ cap binding proteins.

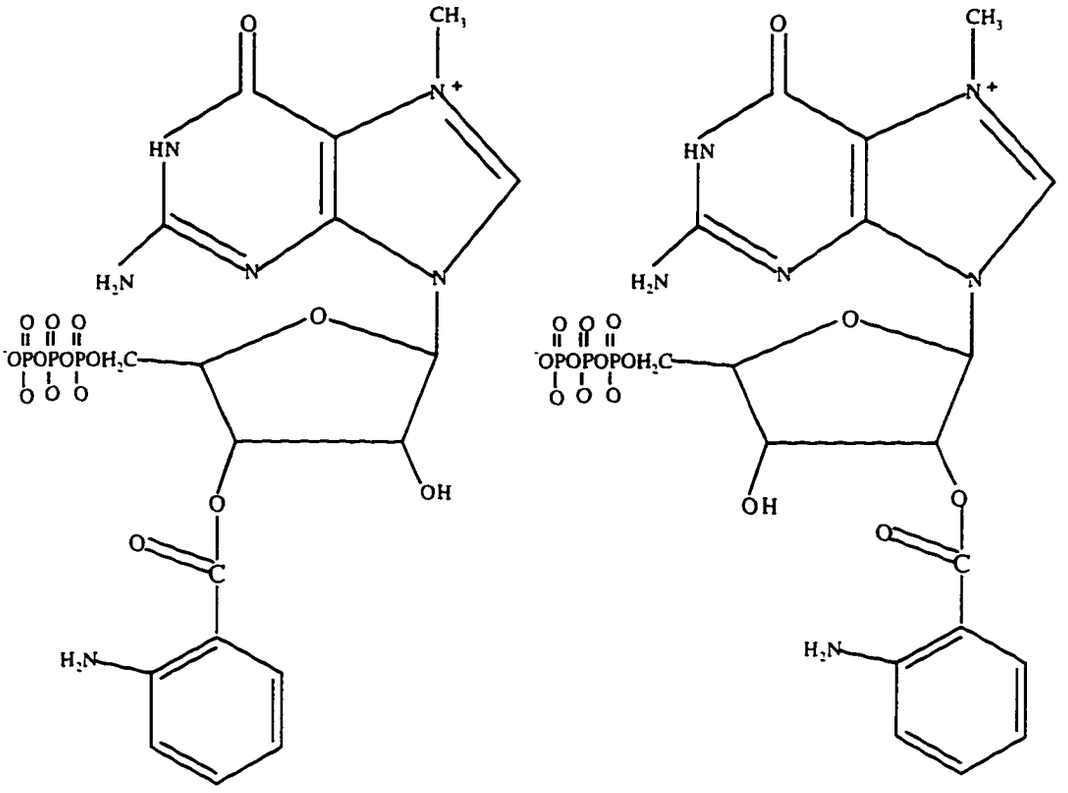

Fig. 2.1 Structure of anthraniloyl(Ant) derivative of m^7GTP

2.2 Effect of wheat germ eIF-2 in initiation and fluorescent guanosine nucleotides

In one of the first stages in the initiation of protein synthesis, eukaryotic initiation factor eIF-2 is involved in the binding of Met-tRNA to the ribosomal 40S subunit and subsequent binding to mRNA as part of the protein synthesis initiation process. For review of the performance of eIF-2 from several systems, see Ref. 1,2,32.

An important step in eIF-2 binding Met-tRNA is the formation of eIF-2•GTP binary complex. This complex subsequently binds Met-tRNA and transfers it to the 40S ribosomal subunit. After GTP hydrolysis and Met-tRNA binding to the 40S subunit, the binary complex leaves the ribosomal complex as eIF-2•GDP. eIF-2•GDP complex is inactive in Met-tRNA and ribosome binding. Recycling of eIF-2 requires exchange of GDP for GTP. In most mammalian systems, for the large difference in dissociation constant of eIF-2 binding between GTP and GDP, which for GDP being two orders of magnitude higher than that for GTP (33-36), the recycling step is completed enzymatically with the assistance of another initiation factor eIF-2B. For this reason, eIF-2B is also called guanosine nucleotide exchange factor (GEF) (37-43).

In the mammalian system, it was found that the phosphorylation of eIF-2 totally destroyed its activity and repressed the rate of overall protein synthesis (37, 44-50) by increasing the binding affinity of eIF-2 to eIF-2B

and effectively sequestering eIF-2B. Therefore eIF-2B is unavailable to mediate guanine nucleotide exchange.

The initiation factor eIF-2 was also purified from wheat germ by several different procedures (51-55). Whereas up to now, mammalian eIF-2B like guanosine nucleotide exchange factor has not been found in wheat germ although some Co-eIF-2, factors which increase protein synthesis but do not affect nucleotide exchange, have been isolated (53,56,57). It's unclear which factors are involved in the guanosine nucleotide exchange in the eIF-2 complex in the wheat germ system. In contrast to the mammalian system, phosphorylation of wheat germ eIF-2 did not inactivate its function in protein synthesis (54). We will the guanosine nucleotide binding and exchange in wheat germ system.

Fluorescence labeled nucleotides

In eIF-2 nucleotide binding and exchange study, ^{32}P labeled GTP, GDP and filter binding assays were normally used. The disadvantages of filter binding assays are big error in measurement, especially for weak binding reactions, the inability to trace fast reaction and radioactive hazard. One approach to study the mechanism of the biological reaction of eIF-2 is to use fluorescence labeled GTP and GDP analogs.

eIF-2 was found to be sensitive to alterations on the phosphoryl and base moiety of guanosine nucleotide, but modifications in the ribose moiety were well tolerated (58). Ribose modified nucleotide analogs are especially

useful and widely used for studies of nucleotide requiring enzymes. Several fluorescence probes, dansyl-β-alanine (27), fluorescein, rhodamine, (29), fluorescamine (28), anthraniloyl (Ant), methylanthraniloyl (Mant) (30,31), and 2,4,6-trinitrophenyl (TNP) 25,26) have been successfully conjugated with GTP and GDP ribose rings and some have been used in mammalian eIF-2 studies (27-29).

In this report, we present the results of a direct fluorescence study of wheat germ eIF-2 binding GTP and GDP using the fluorescent analogs, Ant-GTP and Ant-GDP. A previous study (30) showed that this modifying of ribose ring in GTP and GDP has no influence on its protein binding properties.

2.3 Unwinding and helicase reaction in protein synthesis initiation

An early step in eukaryotic protein synthesis translation is the 40S ribosomal subunit binding to mRNA and then scanning this mRNA (1, 2). For this purpose, mRNA to be translated has to be single stranded. In mammalian systems, three initiation factors have been implicated in this process, eIF-4A, eIF-4B and eIF-4F (59-61). The first step of the unwinding reaction is the binding of eIF-4F to the cap of mRNA through its 28 kD cap binding subunit (3,59) Subsequently, other factors are involved in the unwinding of the double stranded regions.

In the wheat germ system, eIF-4A was found functionally equivalent to mammalian eIF-4A and able to substitute for mammalian eIF-4A in translation (51,62,63). eIF-4F was also found functionally and physically similar with mammalian eIF-4F except that it lacks an eIF-4A like subunit (51,64). An isoenzyme, eIF-(iso)4F, of wheat germ eIF-4F was also found (51,64). Its 28 kD subunit specifically binds to the cap of mRNA and in this respect is analogous to the 26 kD subunit of wheat germ eIF-4F (65-68). Fluorescence studies of eIF-(iso)4F and eIF-4A showed that eIF-(iso)4F formed a complex with eIF-4A and the complex bound to the cap of mRNA (69), analogous to wheat germ eIF-4F and mammalian eIF-4F. Other investigations showed that eIF-(iso)4F could catalyze the RNA dependent ATPase activity of eIF-4A (66,70). Furthermore, wheat germ eIF-(iso)4F was found to be able to substitute for eIF-4F in translation (70), and the presence of both eIF-4F and eIF-(iso)4F was unnecessary (64,67). Functionally eIF-(iso)4F is very similar to eIF-4F even though these two proteins are antigenically distinct proteins (64). Therefore, a logical assumption is that the function of eIF-(iso)4F in combination with eIF-4A may be involved in cap binding and double stranded mRNA unwinding to facilitate 40S ribosome subunit binding and scanning. Our research will try to elucidate the effect of wheat germ eIF-(iso)4F in protein synthesis initiation.

Helicase assay by electrophoresis

Prompted by the considerations that eIF-(iso)4F may be involved in cap binding and double stranded mRNA unwinding, we developed a

helicase assay similar to those used in other studies using gel electrophoresis (71). Gel electrophoresis is the most common way to study the helicase reactions. A partially double stranded oligonucleotide was used for studying the unwinding of a double stranded region by eIF-(iso)4F in concert with other factors. For very low concentration of oligonucleotides used, a silver staining method was employed for detecting the oligonucleotides bands on gel. The silver staining method was reported to detect trace amounts of protein. A test was done for detection of oligonucleotide by silver staining and indicated that it was also a sensitive method for oligonucleotide detection without radioactive hazard. Therefore, gel electrophoresis combined with silver staining was used for study of this helicase reaction. The details of the helicase reaction of wheat germ eukaryotic initiation factors eIF-4A and eIF-(iso)4F was demonstrated using this assay.

Chapter 3 Instrumentation

The fluorescence instrument used in this study was a SPEX-τ 2 spectrofluorometer consisting of six major components: lamp, excitation monochromator, modulator compartment, sample compartment, emission monochromator and the detector. Figure 3.1 shows a schematic diagram of the Fluorolog τ2 spectrofluorometer with the six components. The broad range of light generated by the lamp was passed through the monochromator, a single wavelength of light was selected and sent to the sample chamber. The fluorescence signal was collected at 90 degrees to the incoming light. The fluorescence intensity as a function of emission wavelength was collected and processed by a computer.

3.1 Lamp

A 450W ozone-free xenon arc lamp has been used to provide continuous light output from 250 nm to 700 nm. The advantage of the xenon lamp is that it provides a broad continuum of light for sample excitation.

The lamp emitted light as a result of the recombination of electrons with ionized Xe atoms. These ions were generated by collisions of the Xe atoms with the electrons which flow across the arc. The broad continuous light output was from the ground state atoms, while the sharp lines around 467 nm were from excited atoms.

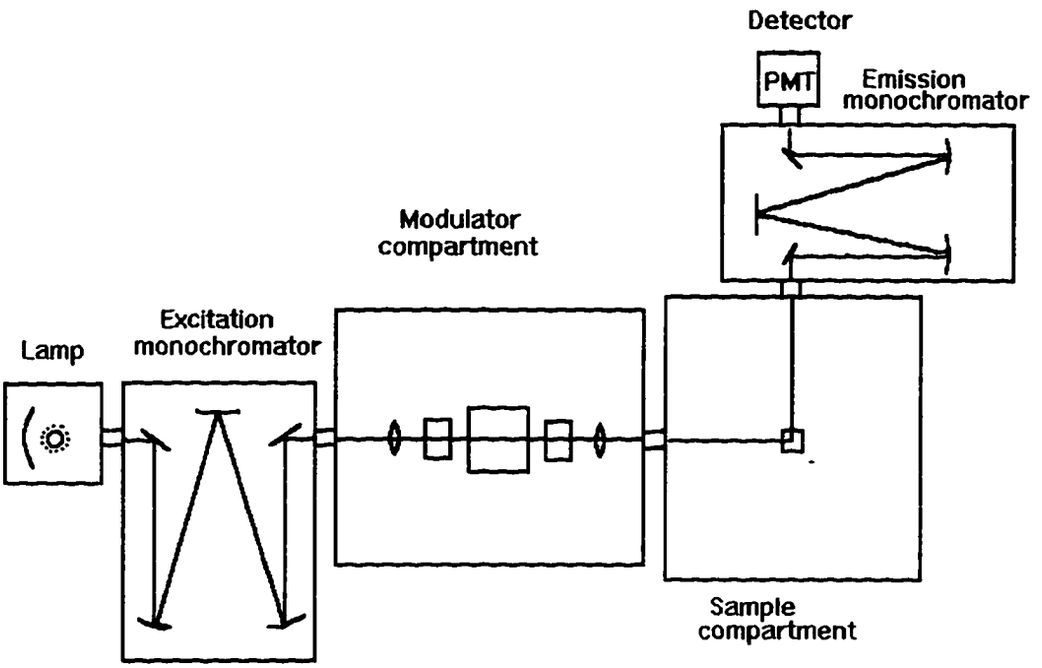

Fig. 3.1 Schematic graph of SPEX fluorometer tau II

3.2 Excitation and emission monochromator

An excitation monochromator was used to select a single excitation wavelength. The Fluorolog tau II was equipped with a modified Czerny-Turner single-grating monochromator in the excitation position to disperse the light from 200 nm to 900 nm. The single grating excitation monochromator (optimized for UV performance) filtered a narrow wavelength of white light. The monochromator was motorized to allow automatic scanning of wavelength. Two slits were used to control bandpass and the intensity of light passing through the sample chamber.

The emission monochromator is also a Czerny-Turner single-grating monochromator the same as the excitation monochromator.

3.3 Modulator compartment

The modulator compartment in the SPEX Fluorolog τ 2 system modulated the light for life time measurement. It also contained a beam spliter to provide two beams of light, one for sample (s) and one for reference (r) to allow the measurement of s/r mode. Variation in the intensity of the arc lamp by voltage fluctuations was corrected by the division of the signal of the sample by that of the reference. Approximately 8% of the excitation light was sent to the reference and the rest to the sample.

3.4 Sample compartment

The sampling module includes an automated four position sample changer. Mode switches allowed manual or automatic control of the sample by the computer. A programmable shutter was also in the sample chamber. The shutter had three modes of operation: Manual, Automatic and Minimum. Manual allowed the operator to control the shutter; automatic allowed opening the shutter during detection and closing the shutter after the scan. Minimum controlled the shutter opening time to be a minimum while engaging the automatic mode, ideal for some protein samples that photobleach.

3.5 Detector

The emission monochromator was connected to an emission signal detector which was a Hamamatsu R928-P photomultiplier mounted in a room temperature housing. Only a narrow range of fluorescence wavelength passed through the emission monochromator at a time to allow the signal detector to record the fluorescence intensity at a certain wavelength. This detector was operated in the photon counting mode when making steady-state measurements. The photomultiplier amplified and transferred the photon signal into an electronic signal which was recorded and stored in the computer.

The reference detector used the same kind of photomultiplier and rhodamine-B as a reference. The use of the rhodamine-B extended the wavelength range to 600 nm. The reference acted as a quantum counter. It measures the fluorescence emission which was proportional to the photon spectral irradiance of the excited source regardless of wavelength range.

Chapter 4 Experiments and Methodology

4.1 Materials

Wheat germ was purchased from Shiloh Farms, Inc. (Sulphur Spring, AR). Chromatographic resin Sephadex G-15, G-25, anion exchange DE-52, m^7GTP-Sepharose, and m^7GTP, isatoic anhydride were from Sigma Chemical Co. (St. Louis, MO). Phosphoryl cellulose P-11 was from Whatman (Hillsboro, OR). T7 RNA polymerase, T7 primer and m^7GpppG, ATP, CTP, GTP, UTP, and GDP were from Pharmacia Biotech (Piscataway, NJ). Silica gel and cellulose TLC plates were from Whatman and J. T. Baker (Phillipsburg, NJ), respectively. OPC (Oligonucleotide Purification Cartridge) cartridge was from Applied Biosystems. Other reagents were of reagent, biochemical or molecular biology research or higher grade. All materials used for RNA work were RNase free or sterilized for RNA work.

4.2 Preparation of chromatographic columns

Buffers

Buffer B contained 20 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES-KOH), pH 7.6, 0.1 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT), 10% glycerol, and KCl as indicated.

Buffer E consisted of 20 mM HEPES-KOH, pH 7.6, 1 mM $Mg(Ac)_2$,

2 mM $CaCl_2$, 6 mM β-mercaptoethanol (BME), 120 mM KCl, 0.1 mg/ml of soybean trypsin inhibitor (STI), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (from a 50 mM solution of PMSF in isopropanol prepared just prior to use).

Preparation of DEAE-Cellulose column

DE-52 resin was soaked in 25 volumes of 0.5 N NaOH for 5 min and rinsed until the pH was lower than 11. The resin was then soaked in 25 volumes of 0.5 N HCl for 5 min and rinsed until the pH was higher than 4. The resin was then brought to the desired pH value (pH 7.6) by the addition of 10 fold concentration of buffer B and loaded onto the column (2.5 cm x 40 cm). The column was monitored by an on-line UV absorbance detector at 280 nm. The column was equilibrated with buffer B-40. The pH of the fractions was tested. The equilibration was finished when the pH reached 7.6 and absorbance went to the baseline.

Preparation of m^7GTP-Sepharose column

1 ml of m^7GTP-Sepharose resin was poured into a small column and washed with B-120. This column can be regenerated after using and reused.

The m^7GTP-Sepharose column was regenerated by washing at room temperature with 2 ml 100 mM Tris•HCl (pH 8.5) solution containing 500 mM KCl and then washed with 20 ml buffer B-120.

Preparation of Phosphocellulose (P11) column

The phosphocellulose powder was treated as for DE-52 resin. The powder was soaked in 25 volumes of 0.5 N NaOH for 5 min and rinsed until the pH was lower than 11. The resin was then soaked in 25 volumes of 0.5 N HCl for 5 min and rinsed until the pH was higher than 4. The resin was then brought to the desired pH value (pH 7.6) by the addition of Buffer B and poured onto the column (1 cm x 20 cm). The column was monitored by an on-line UV absorbance detector at 280 nm. The column was equilibrated with Buffer B-100. The pH of the fractions was tested. The equilibration was finished when the pH reached 7.6 and absorbance returned to the baseline.

4.3 Experiments and data processing methods

Spectral measurements

Absorption spectra were measured with a Cary-3 double beam UV spectrophotometer. Fluorescence measurements were performed on a SPEX tau II fluorometer with an excitation wavelength of 332 nm. Fluorescence

emission was measured from 400 nm to 460 nm using 1 cm quartz cuvette. Slit widths on both excitation and emission monochromators were 2 nm. Buffer background was subtracted where necessary. The absorption of the sample was not allowed to exceed 0.01 at the excitation wavelength to obviate the need to correct for the inner filter effect. Photo stability of fluorescent analogs was tested and photobleaching was not found under our experimental conditions. The quantum yield of Ant-m^7GTP was measured by the method of Parker and Rees (1) using quinine sulfate in 0.1 N H_2SO_4 as a standard (quantum yield 0.70) (2). The quantum yield was calculated according to the following equation:

$$Q_{unknown} = 0.70 \bullet \frac{F_{unknown}}{F_{quinine}} \bullet \frac{A_{quinin}}{A_{unknown}}$$

where $Q_{unknown}$ is the quantum yield of the unknown fluorophore; F is the fluorescence intensity; and A is the absorbance. NMR spectra were acquired using a Varien Unity Plus NMR system operating at 500 mHz for proton observation.

Cap binding assay of wheat germ initiation factors

The cap binding properties of eIF-4A, eIF-4F and eIF-(iso)4F were typically measured in 300 µl solutions containing 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl and Ant-m^7GTP and initiation factors as indicated. Samples were excited at 332 nm and emission intensity was

measured from 400 nm to 460 nm. Samples were allowed to incubate for 5 min to attain equilibrium binding before spectra were measured.

Calculation of cap binding equilibrium constant

Calculation of equilibrium constant: Competitive substitution reactions were used to measure the relative binding affinity of Ant-m^7GTP and m^7GTP with eIF-4F and eIF-(iso)4F using a single reciprocal plot, $1/\Delta F$ vs m^7GpppG. ΔF was calculated from the difference in fluorescence intensity between before addition of protein (F_o) and after protein and m^7GpppG addition (F_a), where $\Delta F = F_a - F_o$. The intercept on the baseline is the 50% inhibition m^7GpppG concentration. 50% inhibition indicates the same concentration of m^7GpppG and Ant-m^7GTP bound to the protein. The ratio of the concentrations of m^7GpppG and Ant-m^7GTP at 50% inhibition gives the ratio of binding constants of the two ligand. The equilibrium binding constant of Ant-m^7GTP with eIF-4F and eIF-(iso)4F was calculated using the binding constant ratio and the value of binding constant for m^7GpppG (3).

Assay of the reaction of wheat germ eIF-2 with Ant-GTP and Ant-GDP

The eIF-2 and Ant-GTP, Ant-GDP binding assay were typically performed at 10 °C in solution(300 µl) containing 20 mM HEPES (pH 7.6), 100 mM KCl, 2.4 mM DTT and wheat germ eIF-2, Ant-GTP, Ant-GDP as indicated. Fluorescence quenching experiments were done by

adding quencher solutions to above reaction solution. Samples were allowed to incubate for 5 min to attain equilibrium binding before spectra were measured, except for the time course experiments which fluorescence was measured at indicated time.

Calculation of dissociation constants of eIF-2•Ant-GDP and eIF-2•Ant-GTP

The dissociation constants and number of binding site on wheat germ eIF-2 for GTP and GDP were calculated as follows.

For the reaction of protein (P) with ligand (L)

$$L + P \rightarrow PL$$

The ratio of F/F_o was used to calculate bound ligand from the equation:

$$L_{bound} = L_{total} \cdot f_{bound} = L_{total} \cdot \left(\frac{F}{F_0} - 1\right) / (Q - 1)$$

Here, L_{total} is the total ligand; F_o is the fluorescence intensity of the probe, Ant-GDP or Ant-GTP, in the absence of protein; F is the fluorescence intensity determined for each addition in the presence of protein. Q is the maximal fluorescence enhancement or called enhancement factor $Q=F_{max} /F_o'$, F_o' was the fluorescence intensity of fluorescent analogues in the absence of protein. F_{max} is obtained by titration of a fixed

amount of Ant-GDP or Ant-GTP with increasing amounts of the protein, P. The double reciprocal plot of 1/F vs. 1/P, was extrapolated to infinite protein indicated by zero point on 1/P axis. The intercept on 1/F axis equal to F_{max}.

The data were then analyzed in terms of the Scatchard method (4,5).

$$\nu = n - K_d \cdot \nu / [L]$$

$$\nu = L_{bound} / P_{total}$$

Where P_{total} is the total protein; [L] is the free probe concentration which was obtained from the difference of the total Ant-GDP or Ant-GTP and calculated bound Ant-GDP or Ant-GTP; n is the number of binding sites on protein, wheat germ eIF-2; K_d is the dissociation constant of eIF-2•Ant-GDP or eIF-2•GTP complex.

Plotting ν versus ν/ [L] gives a slope which is the negative of K_d and a Y-axis intercept which is n.

Helicase assay

For the RNA helicase assay, typical reaction mixture (10 µl) contained 20 mM Tris-HCl (pH 7.6), 70 mM KCl, 1~2 mM DTT, 2 mM ATP, 1 mM $MgAc_2$, 5~10 units of RNasin (RNA guard), 2~4 ng dsRNA substrate and initiation factors as indicated. After 15~20 min incubation at

37 °C, the reaction mixtures were chilled on ice and reactions were terminated by adding 2 ml of loading solution containing 50% glycerol, 2% SDS, 20 mM EDTA, 0.1% bromphenol blue. The samples were loaded onto 15% nondenaturing polyacrylamide gel and electrophoresed in TBE (tris/borate/EDTA) buffer to analyze the degree of unwinding of the duplex RNA. In all experiments, control lanes with duplex and unhybridized RNAs were carried out for comparison. The gel was silver stained according to method of Merril (6-8) with modification: The gel was soaked in fixing solution (50% methanol/12% acetic acid) for 30 min, successively in potassium dichromate solution for 5 min, rinsed with water for 2 min to remove potassium dichromate, soaked in 0.012 N silver nitrate solution for 30 min., washed with water 2 times and developed by 0.5 ml/L formalin aldehyde solution and stopped staining by 1% acetic acid. Silver stained gel was scanned by a soft laser-scanning densitometer (Zenith) or Scanlyst scanner (Ambis). The unwinding efficiency was calculated according to the percentage of double stranded and unwound single stranded RNAs relative to total input duplex RNA.

Chapter 5 Protein Purification

Among these initiation factors, the study was focused on the effect of wheat germ eIF-2, eIF-4A, eIF-4B, eIF-4F and eIF-(iso)4F in protein synthesis initiation as discussed in Chapter 2 using the methodology described in Chapter 3. All these proteins are purified in the laboratory according to the protocols (1-6) with some modifications. The detailed purification procedures for each of them are described below.

5.1 General procedure

All procedures were carried out at 0-4 °C in a cold box or ice-water bath unless indicated otherwise. All purified proteins were stored in small aliquots in a -150 °C freezer. Protein fractions were collected and analyzed on 10% SDS polyacrylamide gel electrophoresis (7) and stained with coomassie blue. Concentrations of proteins were measured on a Cary-3 UV spectrophotometer by the method of Bradford (8) using a Bio-Rad protein assay reagent.

Wheat germ extract

Wheat germ (200g) was reduced to a fine powder by grinding in a Waring blender at medium speed at 15 sec intervals. The powdered wheat germ was mixed thoroughly with 340 ml of Buffer E and centrifuged for 20 minutes at 15,000 g in a DuPont centrifuge. The fatty layer on the top was removed; the supernatant passed through a Sephadex G-25 column. The

effluent from the column (about 450 ml) was centrifuged for 20 min at 30,000 g; the entire supernatant was centrifuged for 3.5 hr at 170,000 g in a DuPont centrifuge. The pellet contained the ribosomal fraction. The entire supernatant was designated 120 mM KCl postribosomal supernatant. This 120 mM KCl supernatant was stored in a -150 °C freezer or further fractionated with ammonium sulfate for initiation factor purification.

Ammonium sulfate fractionation

If stored in the freezer, the frozen 120 mM KCl supernatant was slowly thawed in a 4 °C cold box prior to use. The supernatant was brought to 40% saturation by the gradual addition of ammonium sulfate (22.6 g/100 ml). After stirring for 30 min, the precipitate was collected by centrifugation at 15,000 g for 15 min. The supernatant was then brought to 70% of saturation by the addition of 18.2 g/ml of ammonium sulfate, and the precipitate was collected as described above. Each of the precipitates was suspended in about 50 ml of buffer B-40 and was dialyzed against buffer B-40 overnight.

5.2 Chromatography of the 40% ammonium sulfate fraction on DEAE-Cellulose (DE-52) column

The 40% ammonium sulfate fraction was applied to the DE-52 column and washed with B-40 until the absorbance returned to the baseline. The

column was then washed with buffer B-80. The peak fractions were collected for final purification of eIF-(iso)4F and eIF-4B. After the collection of B-80 peak, the column was washed with buffer B-120. The peak fractions were collected and used for final purification of eIF-4F. Finally, the column was washed with B-500 until the absorbance returned to the baseline.

The fractions from each peak were pooled separately and brought to 80% of saturation by the addition of 51.6 g / 100 ml of ammonium sulfate. Precipitates were collected by centrifugation at 12,500 rpm in the DuPont centrifuge for 25 min. The precipitates were suspended in 10 ml of B-120 and dialyzed overnight against B-120. The samples were then ready to apply to m^7GTP Sepharose column for the final purification of eIF-4F and eIF-(iso)4F.

5.3 Purification of wheat germ eIF-(iso)4F and eIF-4F

The above dialyzed samples were loaded onto the m^7GTP Sepharose affinity column. The m^7GTP was an affinity column. eIF-4F and eIF-(iso)4F were adsorbed on the column. The column was washed with 20 to 30 ml of Buffer B-120. Flow through solution from eIF-(iso)4F purification was collected and left for purification of eIF-4B. eIF-(iso)4F and eIF-4F retained on the column were eluted with Buffer B-120 containing 70 μM m^7GTP. Fractions were collected and pooled, dialyzed against B-120, and concentrated to about 0.5 ml by dialyzing against Buffer

B-120 containing 15% PEG. The samples were checked on SDS denaturing polyacrylamide gel electrophoresis and stained with coomassie blue. Two bands, 28 kDa and 82 kDa, were observed for eIF-(iso)4F and two bands, 26 kDa and 220 kDa, for eIF-4F.

5.4 Purification of eIF-4B

Concentrating eIF-(iso)4F flow through solution

5 to 10 batches of the m^7GTP-Sepharose flow-through from eIF-(iso)4F purification were pooled with a total volume of 50 to 100 ml. The pooled eIF-4B was then concentrated with 80% ammonium sulfate precipitation (51.6 g/100 ml) and centrifuged at 15,000 g in a DuPont centrifuge (12,500 rpm) for 25 minutes. The precipitate was resuspended in 3-5 ml of B-100 and dialyzed overnight against B-100.

Purification of eIF-4B on P-11 column

When the absorbance returned to the baseline, 3-5 ml samples from the previous step were loaded onto the column. The column was washed with buffer B-100 buffer until the absorbance returned to the baseline to remove all of the non-bound proteins. Wheat germ eIF-4B was retained on the column. The column was then developed with a 120 ml linear 100 - 400 mM KCl gradient in Buffer B. Each fraction was tested by SDS

polyacrylamide gel electrophoresis. The fractions containing the 59 kDa band on gels were pooled and dialyzed against 33% ammonium sulfate in buffer B-100 for 5 hours. The precipitated protein was collected by centrifugation at 15,000 g for 25 minutes, suspended in 0.4 ml buffer B-100 and dialyzed overnight against B-100.

5.5 Chromatography of the 70% ammonium sulfate fraction on the DEAE-cellulose column

The 70% saturated ammonium sulfate fraction was applied to the same DE-52 column used for the 40% saturated ammonium sulfate fraction and was washed with Buffer B-40 until the absorbance returned to the baseline. The column was developed with a 1000 ml linear 40 to 150 mM KCl gradient in Buffer B, and then washed with buffer B-150, following by buffer B-250. Fractions of 10 ml were collected and assayed by SDS polyacrylamide gel electrophoresis. Normally gradient fractions from the 60 to 90 mM KCl contained eIF-2 and fractions at 150 mM KCl contained eIF-4A.

5.6 Purification of wheat germ eIF-2

The eIF-2 fractions from the DE-52 column were pooled, concentrated and then applied to a 20 ml phosphocellulose P-11 column (preparation procedure was described in section 5.2) equilibrated with

buffer B-100, washed with B-100, followed by B-250 and eluted with B-500. Fractions were collected and analyzed on 10% SDS polyacrylamide gel electrophoresis (7) and stained with coomassie blue. The fractions containing pure eIF-2, which indicated by three bands on SDS acrylamide gel electrophoresis as reported (1,9-12), were pooled and concentrated by dialysis against 12% PEG in buffer B-100. Purified wheat germ eIF-2 was analyzed by SDS polyacrylamide gel electrophoresis and showed 3 bands as reported previously (1,9-12). The purified protein was made 50% in glycerol and stored in aliquots at -150 °C until use.

5.7 Purification of wheat germ eIF-4A

The fractions from the DE-52 column containing eIF-4A were pooled and precipitated with 80% saturated ammonium sulfate. The precipitated protein was suspended in 4-5 ml of buffer B-100 and dialyzed against buffer B-100. The sample was then applied to a Sephadex G-75 column (1.5 cm x 100 cm) (preparation procedure was the same as for the G-25 column) equilibrated with buffer B-100. The column was washed with B-100 and fractions were collected. Each fraction was analyzed by SDS polyacrylamide gel electrophoresis. The fractions containing a 45 kDa band and low amounts of contaminant protein were pooled and concentrated to about 0.5 ml by dialysis against 15% PEG in buffer B-100.

Chapter 6 Synthesis of A Fluorescent Cap Analogue, Ant-m^{7}GTP, and Fluorescent Guanosine Nucleotide Analogues, Ant-GTP and Ant-GDP

6.1 Synthesis of a fluorescent m^7GTP analogue, Ant-m^7GTP

A 10 mg sample of m^7GTP was dissolved in 0.5 ml of water and the pH adjusted to 9.5 with 2N NaOH. To this solution, 5 mg of isatoic anhydride was added with continuous stirring. The pH of the solution was maintained at 9.6 by titration with NaOH and the reaction continued for 3 hours at 37 °C. After completion of the reaction, the pH of the solution was adjusted to 7.0 with 1 N HCl. The solution was then loaded onto a Sephadex G-15 column (1 cm x 138 cm, packed in deionized water) and developed with deionized water at a flow rate of about 6 ml/hour. Fractions of 1 ml were collected and speed vacuum concentrated. Each peak fraction was checked on a silica gel TLC plate which was developed in solvent I (1-propanol/NH_4OH/H_2O, 6:3:1, v/v/v, containing 0.5 g/l of EDTA) and on a cellulose TLC plate which was developed in solvent II (2-propanol/H_2O/HCl, 65:18.4:16.6, v/v/v). The product showed a single brilliant blue spot under a 360 nm ultraviolet lamp (Spectroline). A spectrum of each fraction obtained was also tested on UV spectrophotometer. The product UV spectrum showed two maxima at 254 and 332 nm, and a shoulder at around 280 nm. The fluorescent cap analogue was eluted from the Sephadex column after unreacted m^7GTP as shown in Fig. 6.1. The unreacted m^7GTP fractions showed one maximum at 254 nm and one shoulder at 280 nm, no absorbance maximum at 332 nm and was observed. No fluorescence was observed on TLC plates for the unreacted m^7GTP under UV illumination. The product fractions showed two maxima as described above, and one fluorescence spot on TLC plates. The unreacted isatoic anhydride and side product, anthraniloyl acid,

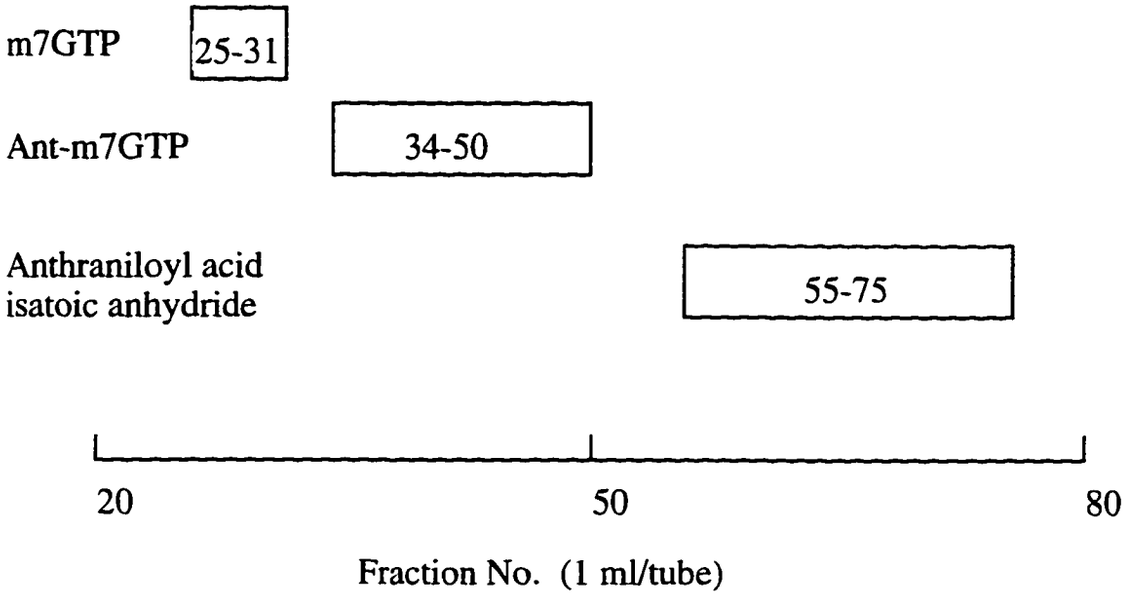

Fig. 6.1 Graphic presentation of chromatographic separation of Ant-m^7GTP

Reaction product was applied on a 138 cm x 1 cm column of Sephadex G-15 packed in water. The flow rate was about 6 ml/h.

fractions showed two maxima at 210 nm and 300 nm and a fluorescent spot on TLC plates which ran much faster than the product.

The product fractions which showed a single brilliant blue spot on both TLC plates were pooled and speed vacuum concentrated to dryness. The residue was dissolved in a minimum amount of water. A large excess of cold ethanol was added and the solution was left at -20 °C for 30 min. The precipitate was collected by centrifugation at 10,000 rpm. The precipitate was dissolved, reprecipitated with ethanol, and dried using a speed vacuum. The purity of the product was analyzed by TLC on silica gel and cellulose plates again and was chromatographically pure, as indicated by a single brilliant blue spot under UV illumination. The product was free of fluorescent by-products and starting materials which run much faster than the product on TLC plates. The structure of the fluorescent cap analogue and the R_f values of both TLC plates are shown in Fig.2.1 and Table 6.1, respectively. The concentration of the product was measured by UV absorbance at 332 nm with $\varepsilon = 4600\ M^{-1}\ cm^{-1}$, divided into aliquots, and stored at -20 °C.

The proton assignments for the one dimensional ^1H NMR spectra are as follows. 3' product: δ(ppm) 7.16-8.18 (benzyl H in Ant group), 6.15 (ribosyl H-1), 5.65 (ribosyl H-3), 5.00 (ribosyl H-2). 2' product: 6.33 (ribosyl H-1), 5.82 (ribosyl H-2), 4.80 (ribosyl H-3). The assignments of relationship are from 2 dimensional ^1H NMR spectra as follows. In 3' product: δ(ppm) 6.15 couples to 5.00, 5.00 couples to 5.65. In 2' product, 6.33 couples to 5.82, 5.82 couples to 4.80. The ratio of the 3' product to 2'

product was obtained by integrating the 2' or 3' H and found to be 65% 3' product to 35% 2' product.

6.2 Synthesis of fluorescent guanosine nucleotides, Ant-GTP and Ant-GDP

50 mg of GTP or GDP was dissolved in 2 ml of H_2O and adjusted to pH 9.5 with 2 N NaOH. To this solution, with continuous stirring, 19 mg of isatoic anhydride was added. The pH of the solution was kept at 9.5 by

Table 6.1 Yield and R_f value of anthraniloyl m^7GTP derivatives
Silica gel plate was developed in solvent I. Cellulose plate was developed in solvent II. (See Materials and Methods)

	R_f		yield
	silica gel	cellulose	
Ant-m^7GTP	0.10	0.30	20%
anthraniloyl acid	0.63	0.81	

titration with 2 N NaOH for 3 hours at 37 °C. After completion of the reaction, the pH of the solution was adjusted to 7.0 with 1 N HCl. The solution was then applied to a 138 cm x 0.5 cm Sephadex G-15 column packed in water. The column was developed with water at 10 ml/h. Ant-GTP and Ant-GDP were eluted after unreacted GTP and GDP. Fractions of 1 ml were collected. Peak fractions of UV absorption were tested on silica gel TLC plate which was developed with solvent I (1-propanol/NH_4OH/H_2O, 16:3:1, v/v/v, containing 0.5 g/l of EDTA) and on cellulose TLC plate developed with solvent II (2-propanol/H_2O/HCl, 65:18.5:16.5, v/v/v). Products showed single brilliant blue spot under illumination by a 360 nm UV lamp (Spectroline). The spectra of fractions were also taken on UV spectrophotometer. The products spectra showed 2 maxima at 254 nm and 332 nm and a shoulder at around 280 nm.

The fractions showing only a single spot on both TLC plates were pooled and evaporated to dryness using a speed vacuum. The residues were dissolved in a minimal amount of water. A large excess of cold ethanol was added and the samples were left at -20 °C for 30 min. The precipitates were collected by centrifugation at 10,000 rpm. This process was repeated. The precipitates were dried using a speed vacuum. The purified products were tested on silica gel and cellulose TLC plates again as indicated above and showed a single bright spot (1000 fold more concentrated than the original fraction) without any fluorescent by-products or starting materials which run much faster than products on both TLC plates. Concentrations of the products were measured by UV absorbance at 332 nm with ε=4600 $M^{-1}cm^{-1}$. The fluorescent products Ant-GTP and Ant-GDP were stored at -20 °C.

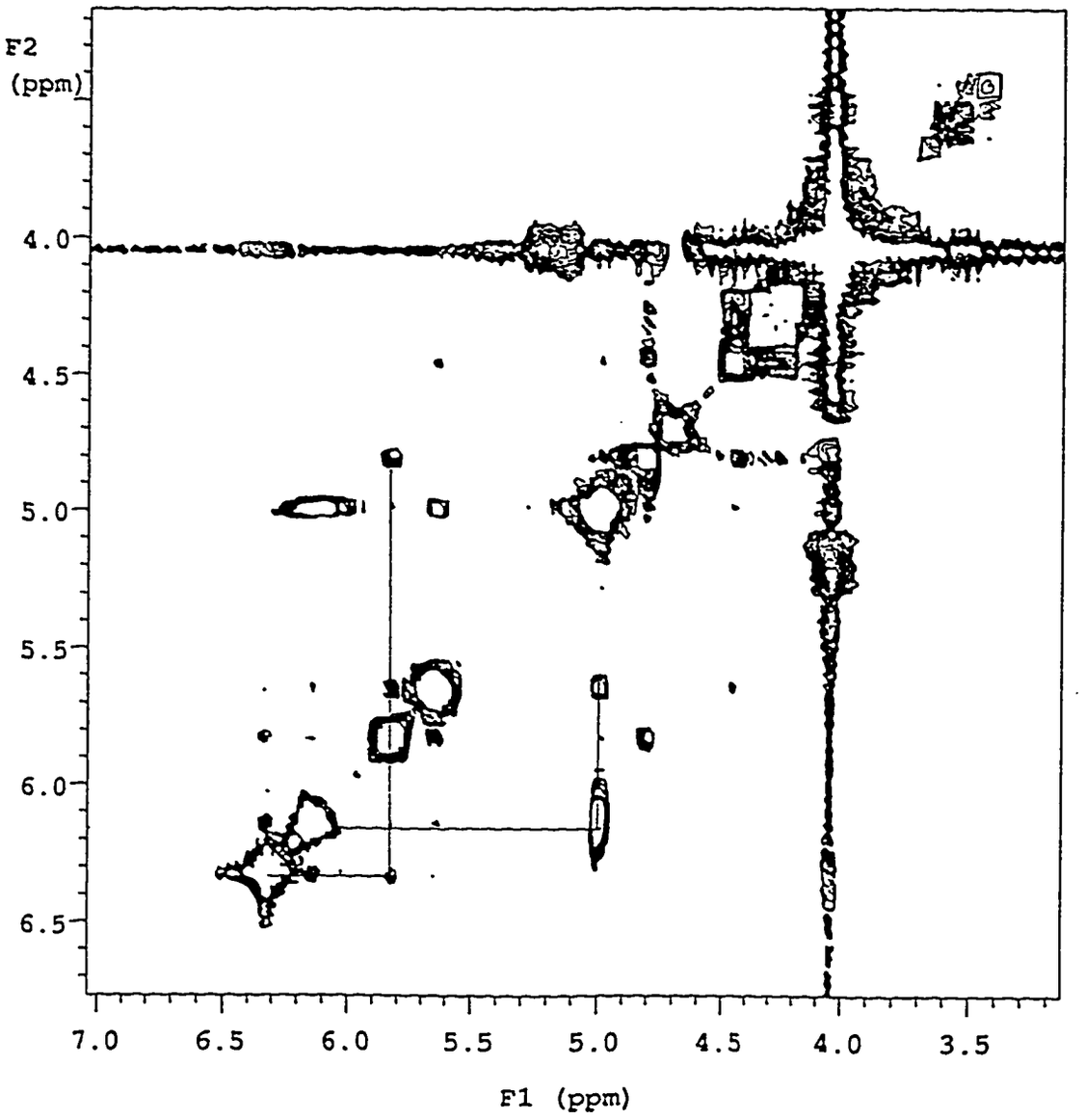

Fig. 6.2 ^{1}H NMR spectrum of anthraniloyl (Ant) derivative of $m^{7}GTP$

Chapter 7 Transcription of Oligonucleotides and Preparation of Double Stranded mRNA

7.1 Preparation of DNA template for RNA transcription

Deprotection

DNA was synthesized on an Applied Biosystems DNA synthesizer. The DNA sequence contained a T7 promoter region and a region complementary to the desired RNA sequence. The crude DNA was deprotected by incubation in NH_4OH (1:1) at 55 °C for 18 hours. Then the deprotected DNA was purified by solid phase extraction column, Oligonucleotide Purification Column (OPC) (1).

Preparation of OPC column

The Applied Biosystems OPC cartridge was connected with a polypropylene syringe. The cartridge was flushed with 5 ml HPLC grade acetonitrile, followed by 5 ml 2.0 M triethylamine acetate.

Purification of DNA

The deprotected DNA solution was diluted with equal volume of deionized water. This solution was placed in the syringe and slowly pushed through the cartridge at 1 to 2 drops per second. The eluted fraction was saved and placed in a syringe and pushed through the cartridge again. DNA

was retained on the column. The cartridge was slowly washed with 3 x 5 ml 1.5 M ammonium hydroxide and followed by 2 x 5 ml deionized water. 5 ml of 2% trifluoroacetic acid solution was used to detritylate the OPC bound DNA. About 1 ml was gently pushed through the cartridge. After 5 min waiting, the remaining TFA solution was flushed through the cartridge. The cartridge was then washed with 2 x 5 ml deionized water. For sequences longer than 40 bases, 1 x 5 ml 1.5 M ammonium hydroxide, followed by 2 x 5 ml deionized water, was then gently pushed through the cartridge. The purified and detritylated DNA was eluted by slowly washing the cartridge with 1 ml of the 20% acetonitrile solution. OPC purified DNA was stored at -150 °C in aliquots. The concentration of the DNA was determined by the UV absorbance at 260 nm by a Cary-3 spectrophotometer.

7.2 Transcription of oligonucleotide

The purified DNA templates were annealed to a 17 base T7 primer complementary to the T7 promoter region in annealing solution containing 10 mM Tris•HCl (pH 7) at 65 °C for 5 min and slowly cooled to 30 °C. These partially double stranded DNA templates were used for transcription.

The transcription reactions were run according to the procedure of Milligan (2) in 40 mM Tris-HCl (pH 8.1 at 37 °C), 1 mM spermidine, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 0.01% (v/v) Triton X-100 and 80 mg/ml polyethylene glycol (8000MW), 20 mM $MgCl_2$, 3.5 mM NTPs, DNA template and T7 RNA polymerase. Reaction mixtures were

incubated at 37 °C for 2 to 3 hours. To obtain 5' capped oligonucleotide, m^7GpppG was included in the transcription mixture at 3 mM and reduce GTP concentration was reduced to 0.5 mM.

After transcription, mRNAs were purified according to Darzynkiewicz et al (3) by DNase 1 digestion, phenol/chloroform extraction, chloroform extraction, ethanol precipitation and then dialysis. Products were analyzed by polyacrylamide gel electrophoresis and stained by the silver staining method (4-6). Purified oligonucleotides showed a clear single band by gel electrophoresis.

7.3 Preparation of double stranded mRNA analogues

Partially double stranded RNAs were prepared by mixing equimolar amounts of each transcript in annealing buffer (40 mM HEPES, pH 7.0, 2 mM EDTA, 500 mM NaCl), heated to 85-90 °C for 3-5 min, switched to 60 °C allowed to slowly cool to 37 °C and incubated overnight at 30~37 °C. The sample was dialyzed to remove salts, etc. The products were tested by polyacrylamide gel electrophoresis.

Oligonucleotides used for helicase assay

RNA A and RNA B were transcribed from DNA templates, annealed and formed a partial double strand RNA flanked by a 5' single stranded tail of 20 nucleotides with m^7GpppG cap at the 5' end and the 3' end

flush double stranded. A representation of the structure of the dsRNA is shown below. This dsRNA was used for helicase assays.

3'-GCGACGCGACGGGG 5' A

5'-m^{7}GpppGGGACCAUGGAACAACAUUAUCGCUGCGCUGCCCC 3' B

Fig. 7.1 Sequence of double stranded RNA substrate used in helicase assays

Chapter 8 Characterization of Fluorescent 7-methylguanosine Analogue and Fluorescence Spectroscopic Study of its Reaction with Wheat Germ Cap Binding Proteins

8.1 Characterization of fluorescent cap analogue, anthraniloyl-m^7GTP

General properties

Like Ant-GTP and Ant-ATP, the analogue is quite stable at neutral pH and could be stored for long periods at -20 °C without detectable degradation. The stability of Ant-m^7GTP in acidic and basic solution was tested. Ant-m^7GTP was found to degrade in strong basic solution, 0.1 N NaOH, as indicated by two fluorescent spots corresponding to Ant-m^7GTP and anthraniloyl acid on a silica gel TLC plate developed in solvent I. Longer incubation times or higher concentrations of NaOH (0.5 N) resulted in complete hydrolysis with only one fluorescent spot corresponding to anthraniloyl acid detected on TLC plate. In contrast, Ant-m^7GTP was resistant to degradation in HCl (0.5 N) solution even following overnight incubation. These properties are similar to those observed for Ant-GTP and Ant-ATP (1).

Absorption and fluorescent properties

The absorption spectrum of Ant-m^7GTP exhibits two maxima at 254 nm and 332 nm and a broad shoulder at around 280 nm (Fig. 8.1). The broad band at 332 nm is associated with the anthraniloyl group and the broad shoulder at 280 nm corresponds to the 7-methyl guanosine moiety.

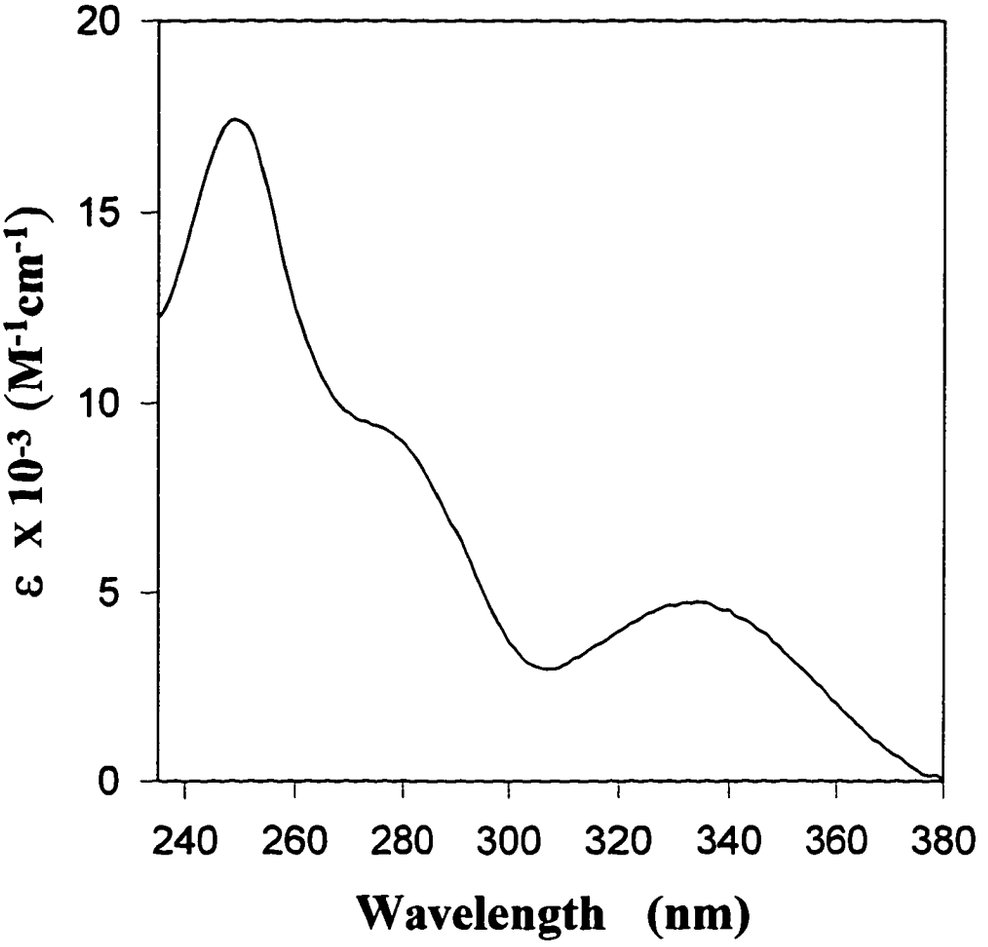

Fig. 8.1 UV absorption spectrum of Ant-m^{7}GTP

Spectrum was measured in 20 mM HEPES (pH 7.6). Temperature was 22 ^oC.

Upon excitation at 332 nm, Ant-m^{7}GTP (aqueous solution, pH 7.6) fluoresced strongly with an emission maximum at 423 nm (Fig. 8.2, curve 1). No photobleaching or emission maxima shift was observed by exposing the sample to light in the room for 10 hours or during spectrometric measurements (data not shown).

Solvent, ionic strength and pH effects on fluorescence intensity of Ant-m^{7}GTP

To be used as a protein environmental probe, the fluorophore must be sensitive to some indicator of local environment. The potential usefulness of this analogue as a fluorescent probe of hydrophobic microenvironments is indicated by the fact that the position of the emission maximum and quantum yield vary significantly with solvent polarity. Fig. 8.2 and Fig. 8.3 shows the effect of solvent polarity on the fluorescence of Ant-m^{7}GTP. The fluorescence intensity and emission maxima vary significantly at constant pH and buffer concentration with varying fractions of ethanol and DMF. Fluorescence quantum yields increased about 4-5 fold in going from water to 80% ethanol or 80% DMF. At the same time, the emission maxima shifted to the blue by 10 nm, for ethanol, and 20 nm for DMF, respectively. (Table 8.1). A concentration up to 500 mM KCl had no influence on the fluorescence of Ant-m^{7}GTP. Ant-m^{7}GTP fluorescence reaches a maximum at approximately pH 7 (Fig. 8.4). As the pH was either increased or decreased, a gradual decrease in fluorescence intensity was observed. No shift in emission maxima occurred with variation of pH,

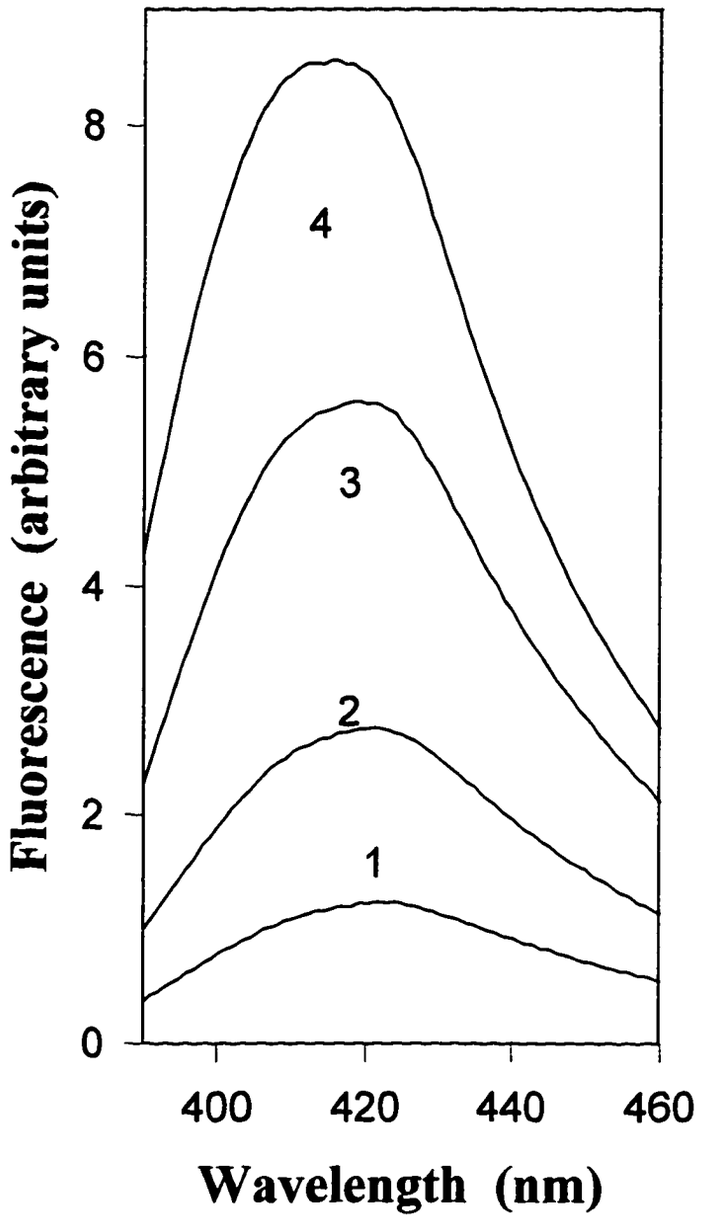

Fig. 8.2 Fluorescence emission spectra of Ant-m^7GTP in ethanol/water
All sample (1.5 μM Ant-m^7GTP) contained 20 mM HEPES (pH 7.6). Excitation was 332 nm. Figure shows the spectra in water/ethanol at the ratio indicated: 1. aqueous solution (0% ethanol) 2. 20% (v/v) ethanol 3. 40% (v/v) ethanol and 4. 80% (v/v) ethanol

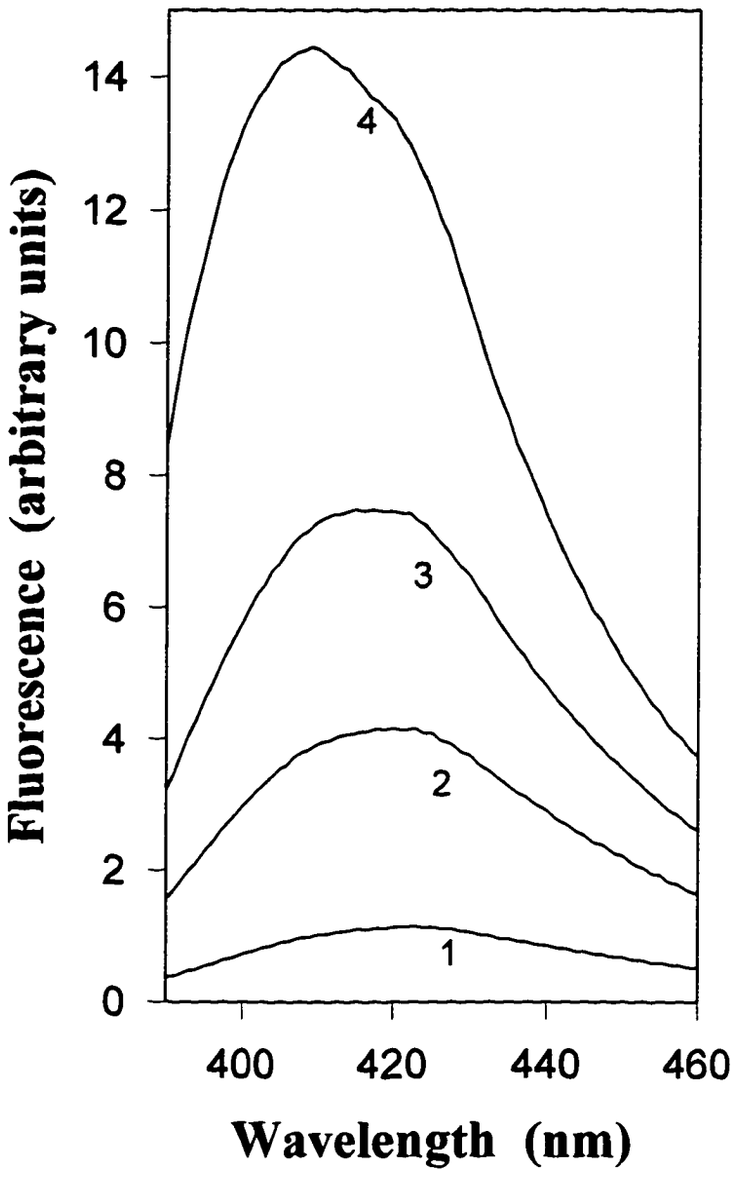

Fig. 8.3 Fluorescence emission spectra of Ant-m^{7}GTP in DMF/water

All sample (1.5 μM Ant-m^{7}GTP) contained 20 mM HEPES (pH 7.6). Excitation was 332 nm. Figure shows the spectra in water/DMF solution: 1. aqueous solution (0% DMF) 2. 20% (v/v) DMF 3. 40% (v/v) DMF and 4. 80% (v/v) DMF

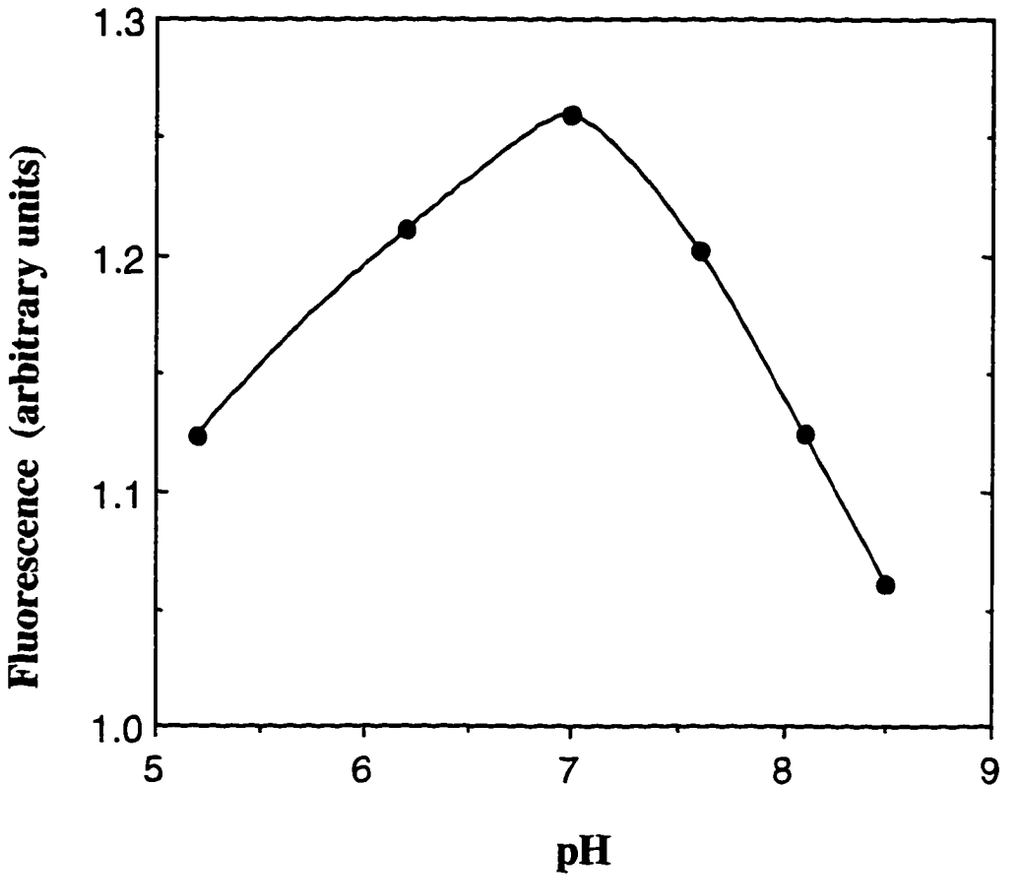

Fig. 8.4 pH dependence of Ant-m^{7}GTP fluorescence emission

The concentration of Ant-m^{7}GTP was 1.5 μM. Excitation was 332 nm. Solution was 20 mM HEPES, pH was adjusted by addition of HCl and KOH.

Table 8.1 Fluorescence properties of Ant-m^{7}GTP in different solvent All results were measured in 20 mM HEPES (pH 7.6) and solvent as indicated. Excitation was 332 nm.

solvent	emission maximum (nm)	quantum yield
aqueous	423	0.17
80% ethanol	415	0.68
80% DMF	410	0.82

suggesting that only one species was the origin of the absorption and the fluorescence.

8.2 Reaction of Ant-m^{7}GTP analogue with wheat germ cap binding proteins

Reaction of wheat germ initiation factors with Ant-m^{7}GTP cap analogue

When Ant-m^{7}GTP reacted with wheat germ initiation factor eIF-4F or eIF-(iso)4F, significant fluorescence enhancement, accompanied by an emission maximum shift, was observed (Fig. 8.5, Fig. 8.6). Whereas the non cap binding protein, eIF-4A, did not enhance the fluorescence intensity of Ant-m^{7}GTP (data not shown). These results show that the fluorescent cap analogue can be recognized by specific cap binding proteins, eIF-4F and eIF-(iso)4F.

Excess (21 fold) unmodified m^{7}GTP can successfully compete with Ant-m^{7}GTP for the cap binding site (Fig. 8.5, Fig. 8.6). The decrease in fluorescence intensity almost to that of free Ant-m^{7}GTP and corresponding shift of emission maximum upon addition of m^{7}GTP indicated that Ant-m^{7}GTP was replaced by m^{7}GTP. In contrast, neither GTP nor ATP could compete with Ant-m^{7}GTP even at much higher concentrations (132 fold excess). In the case of competitive inhibition, Lineweaver-Burk plots meet at the same Y-axis intercept (2) as shown in Fig. 8.7 and Fig. 8.8.

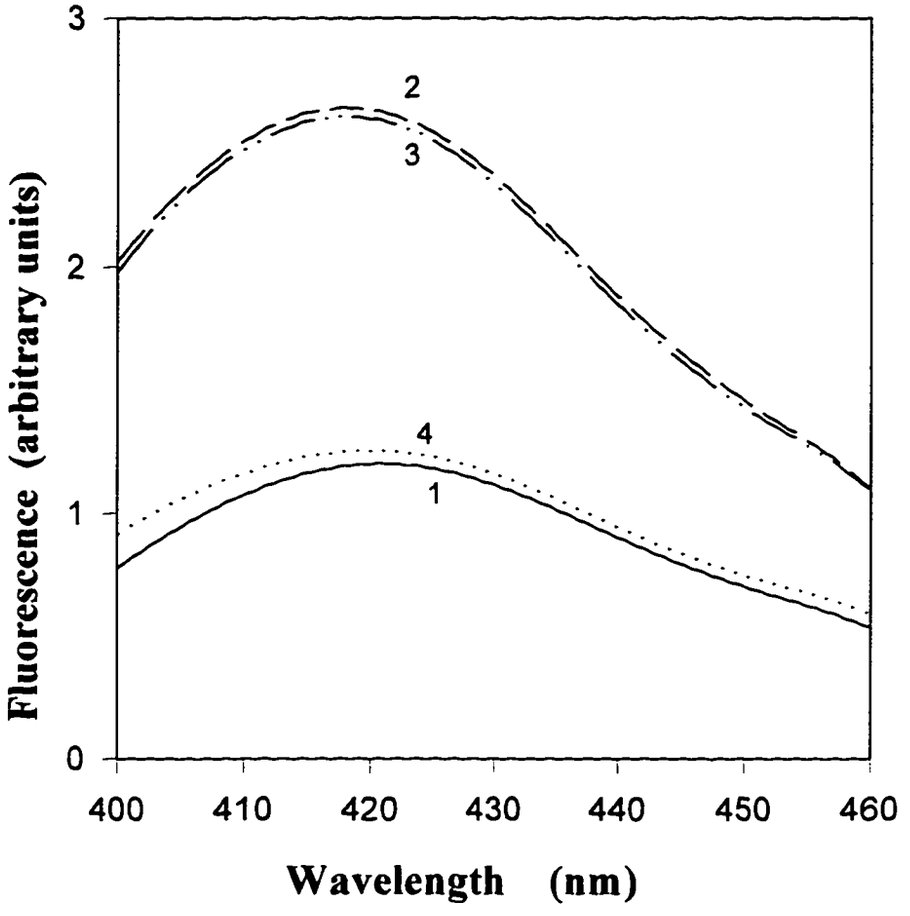

Fig. 8.5 Reaction of wheat germ cap binding proteins eIF-(iso)4F with Ant-m^7GTP

1. Spectrum of 1.5 μM Ant-m^7GTP in buffer containing 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 20 °C and excitation was 332 nm. 2. addition of 0.5 μM eIF-(iso)4F 3. addition of 200 μM ATP or GTP 4. addition of 33 μM m^7GTP

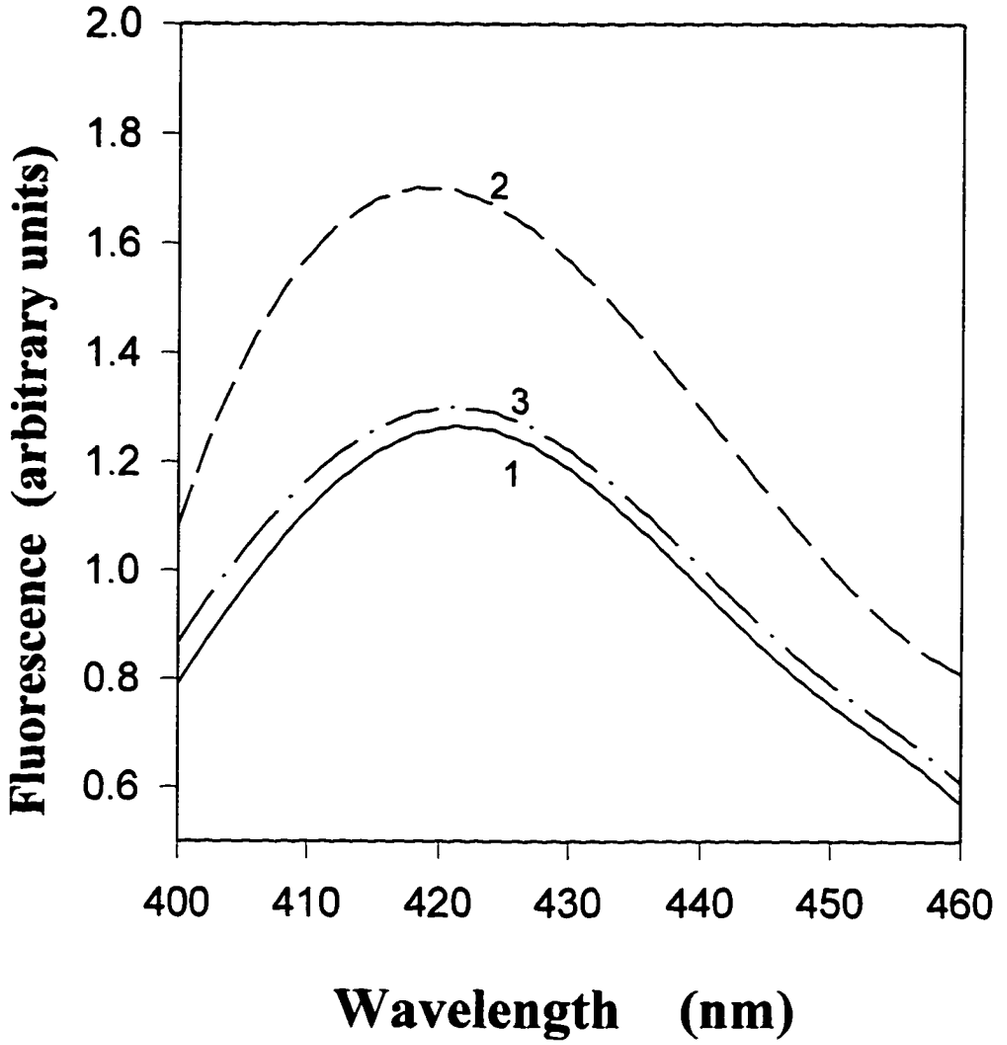

Fig. 8.6 Reaction of wheat germ cap binding proteins eIF-4F with Ant-m^7GTP

1. Spectrum of 1.5 μM Ant-m^7GTP in buffer containing 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 20 °C and excitation was 332 nm; 2. addition of 0.5 μM eIF-4F; 3. addition of 33 μM m^7GTP

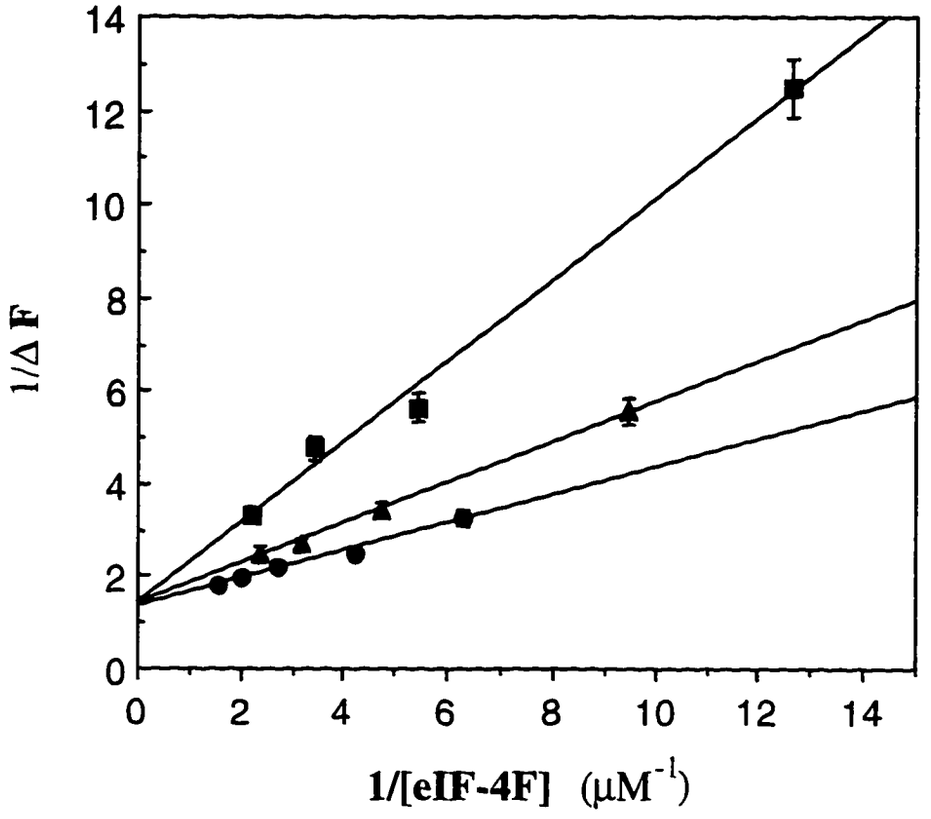

Fig. 8.7 Lineweaver-Burk plot for the competition of Ant-m^7GTP and m^7GTP in binding wheat germ cap binding protein eIF-4F

Spectrum was measured in buffer containing 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 23 °C with 1.5 μM Ant-m^7GTP and m^7GTP as indicated. The samples were excited at 332 nm. The data are indicated by circles: 0 μM m^7GTP; triangles: 1.0 μM m^7GTP; and squares: 3.3 μM m^7GTP.

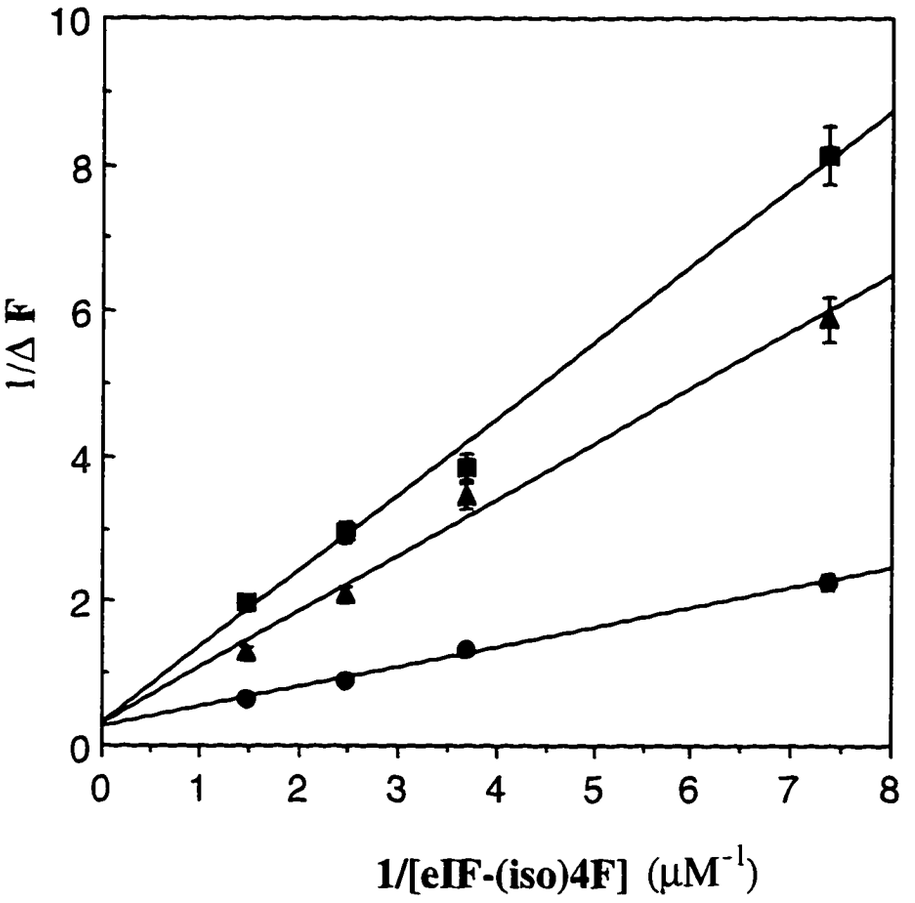

Fig. 8.8 Lineweaver-Burk plot for the competition of Ant-m^7GTP and m^7GTP in binding wheat germ cap binding protein eIF-4F

Spectrum was measured in buffer containing 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 23 °C with 1.5 μM Ant-m^7GTP and m^7GTP as indicated. The data are indicated by circles: 0 μM m^7GTP ; triangles: 3.3 μM m^7GTP; and squares: 6.6 μM m^7GTP.

Lineweaver-Burk plots for m^7GTP competition with Ant-m^7GTP for eIF-4F or eIF-(iso)4F binding meet at the same Y-axis intercept within experimental error. These data suggest that the fluorescent analogue Ant-m^7GTP bound to the cap binding site on these proteins and that modification of the ribose ring had no effect on the binding specificity. This is consistent with previous observations (3-6) that the m^7G base moiety and the first phosphoryl, but not the ribose ring, were important for cap recognition.

Measurement of equilibrium protein binding constant of Ant-m^7GTP

The competition and substitution reactions of unmodified cap were performed at constant Ant-m^7GTP concentration. A plot of $1/\Delta F$ vs [m^7GpppG] (Fig. 8.9) showed 50% substitution m^7GpppG concentration was 2.1 ± 0.1 μM and 1.9 ± 0.1 μM which indicates that Ant-m^7GTP bound eIF-4F only 1.4 ± 0.07 fold and eIF-(iso)4F 1.3 ± 0.07 fold tighter than m^7GpppG (3). Therefore the equilibrium binding constants for Ant-m7GTP with eIF-4F and eIF-(iso)4F were $(1.9 \pm 0.1) \times 10^5\ M^{-1}$ and $(0.8 \pm 0.04) \times 10^5\ M^{-1}$, respectively.

Fluorescence quenching experiments

To determine the microenvironment of Ant-m^7GTP when bound to cap binding proteins, quenching experiments were performed using potassium iodide or acrylamide (Fig. 8.10, Fig. 8.11). Iodide is negatively

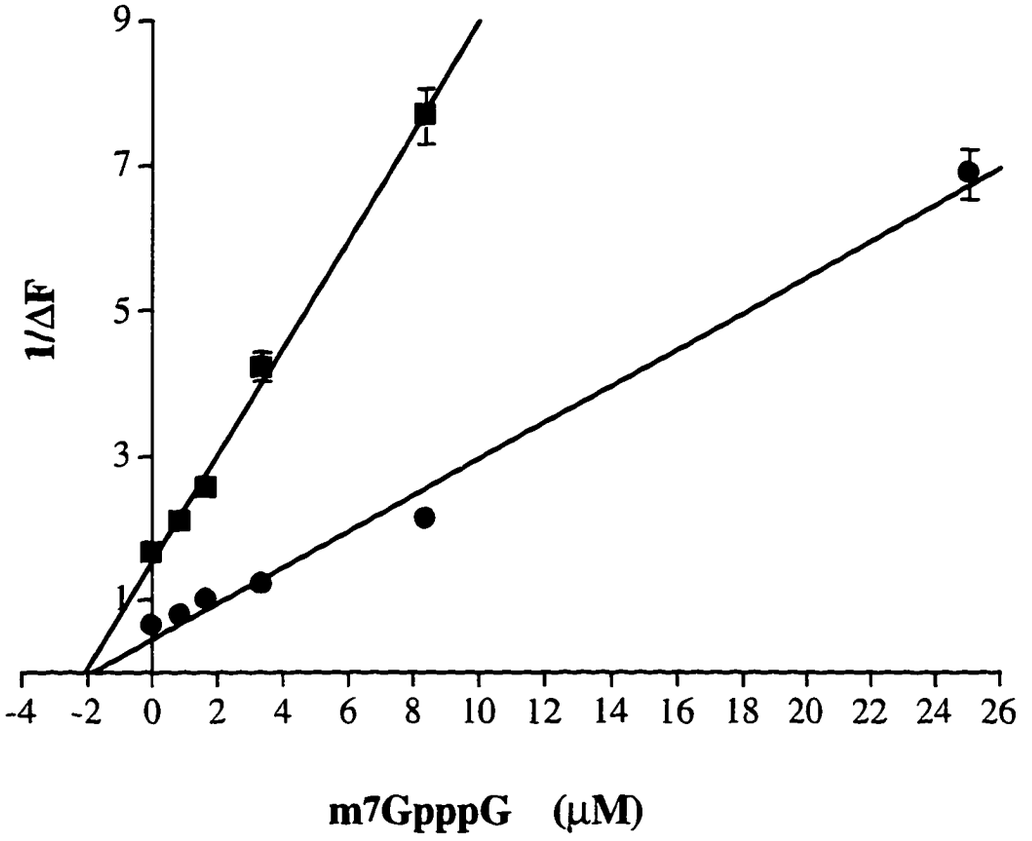

Fig. 8.9 Competition of cap binding reaction presented as single reciprocal plot

Experimental conditions: 20 mM HEPES(pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM Mg^{2+} at 23 °C with 1.5 μM Ant-m^7GTP and 0.5 μM eIF-4F or 0.9 μM eIF-(iso)4F. m^7GpppG was added to substitute for Ant-m^7GTP. The samples were excited at 332 nm. circles: eIF-(iso)4F; squares: eIF-4F.

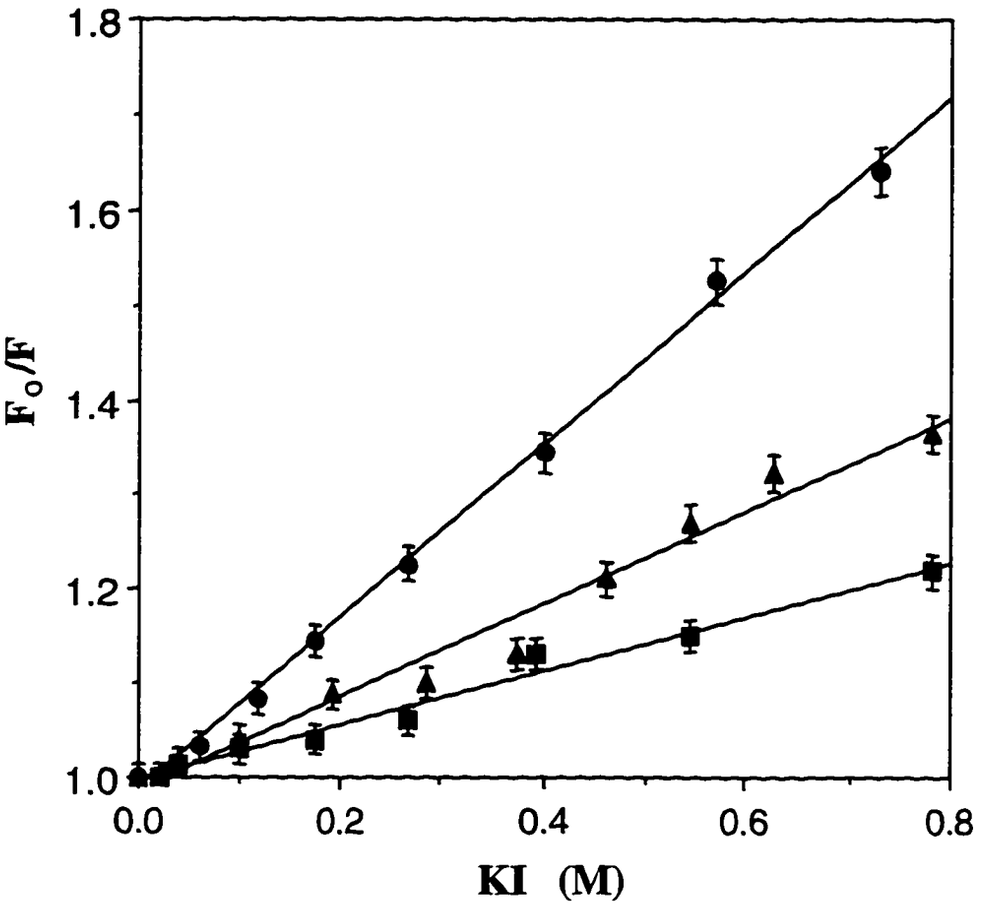

Fig. 8.10 Stern-Volmer plot bound Ant-m^7GTP of fluorescence quenching by KI

Experimental conditions: 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 20 °C with 1.5 μM Ant-m^7GTP and 0.5 μM eIF-4F or 1.5 μM eIF-(iso)4F. The samples were excited at 332 nm. circles: Ant-m^7GTP; squares: eIF-4F:Ant-m^7GTP; triangles: eIF-(iso)4F:Ant-m^7GTP.

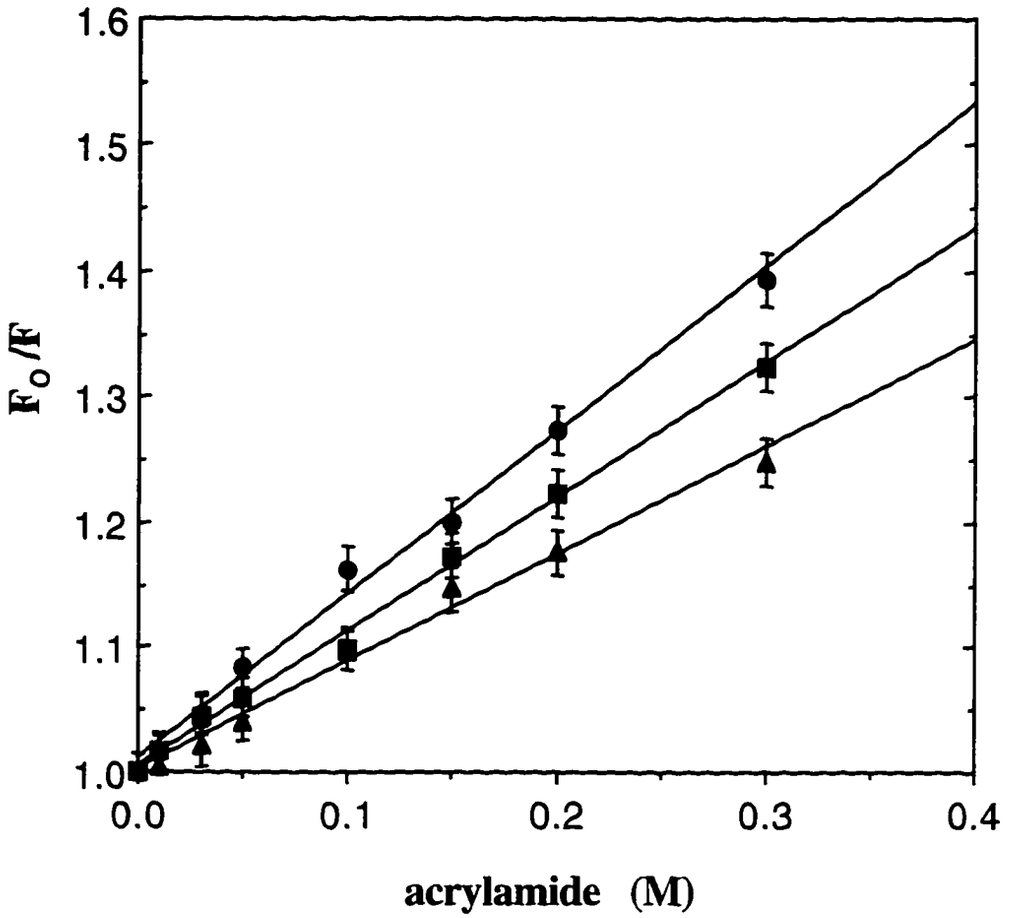

Fig. 8.11 Stern-Volmer plot bound Ant-m^7GTP of fluorescence quenching by KI

Experimental conditions: 20 mM HEPES (pH 7.6), 1 mM DTT, 100 mM KCl, 2 mM $MgCl_2$ at 20 °C with 1.5 μM Ant-m^7GTP and 0.5 μM eIF-4F or 1.5 μM eIF-(iso)4F. The samples were excited at 332 nm. circles: Ant-m^7GTP; squares: eIF-4F:Ant-m^7GTP; triangles: eIF-(iso)4F:Ant-m^7GTP.

charged and generally does not penetrate the nonpolar interior of the protein and is expected to selectively quench surface located fluorophores. The iodide anion is also expected to sense the electrostatic nature of the surrounding of the fluorophore to some degree. It was found that iodide quenching of the complex of Ant-m^{7}GTP and eIF-4F or eIF-(iso)4F was lower than that of free Ant-m^{7}GTP, indicating that the fluorophore was not completely solvent exposed. In contrast, acrylamide is a neutral quencher and can permeate the protein matrix. Acrylamide quenched the fluorescence of the Ant-m^{7}GTP protein complex more efficiently than iodide.

8.3 Discussion and conclusion

The synthesis, purification and characterization of a fluorescent cap analogue, Ant-m^{7}GTP are presented. The synthesis of this cap analogue was relatively simple, requiring only one step. It was not difficult to purify the analog to chromatographic purity and all procedures could be completed within one day. The characterization of the fluorophore showed that the fluorescence quantum yield was high and the fluorescence excitation and emission range did not overlap that of the protein. TNP modified nucleotides also avoid the overlap of excitation and emission with protein fluorescence, but their quantum yields are very low and photobleaching readily occurs (7). The fluorescence of Ant-m^{7}GTP was pH and solvent dependent, but ionic strength independent. The fluorescence reached a maximum at around pH 7, the physiological pH range. Organic solvents, ethanol and DMF, enhanced the fluorescence as well as shifted the emission maxima indicating an environmentally sensitive fluorophore.

These properties were similar to Ant-GTP and were characteristic of the Ant and Mant derivative family of fluorophores (8,9). The Ant-m^{7}GTP's stability and resistance to photobleaching made it a good probe for biochemical and biophysical studies.

The reactions of this cap analogue with several wheat germ initiation factors eIF-4A, 4F and (iso)4F was tested. Ant-m^{7}GTP was recognized by the specific cap binding proteins eIF-4F and (iso)4F, but not the non cap binding protein eIF-4A. Competition and substitution reactions showed that the fluorophore competed with unmodified m^{7}GTP for the cap binding site of eIF-4F and eIF-(iso)4F.

Like most ribose moiety altered nucleotide analogues, the alteration on the ribose moiety of m^{7}GTP did not perturb the binding properties, consistent with other reports (3-6, 10-12). The relative equilibrium binding constant ratio of Ant-m^{7}GTP•m^{7}GpppG was found to be 1.3-1.4, approximately the same as the normal cap structure. Wheat germ eIF-4F and eIF-(iso)4F cap binding properties were found to be very similar. In addition, their cap binding reactions are additive, not catalytic.

For the first time, a fluorescent cap analogue, Ant-m^{7}GTP, suitable for cap binding protein study was synthesized. This provides a direct method to study the cap binding proteins and the cap binding effects in protein synthesis initiation. This fluorescent cap analogue, Ant-m^{7}GTP, was shown to have similar binding properties to the normal cap structure and should prove useful in elucidating the binding of proteins to cap structures.

Chapter 9 Fluorescence Spectroscopic Study of the Guanosine Nucleotide Binding and Exchange of Wheat Germ Protein Synthesis Initiation Factor eIF-2

9.1 Guanosine nucleotide binding to wheat germ eIF-2

Reaction of Ant-GDP with wheat germ eIF-2

It was observed that when Ant-GDP reacted with eIF-2, its fluorescence intensity significantly increased. A 3 nm emission wavelength blue shift was also observed (Fig. 9.1).

To this reaction solution, 200 μM unmodified GDP, which was a 500 fold excess of Ant-GDP, was added. It was found that GDP can successfully compete with Ant-GDP for the binding site on wheat germ eIF-2. The decrease in fluorescence intensity almost to the level of free Ant-GDP and shift of emission maximum back to the same as that of free Ant-GDP indicate that Ant-GDP was displaced by unmodified GDP (Fig. 9.1).

Time course of the binding of Ant-GDP with wheat germ eIF-2

Time course experiment was done by fast scanning from 420-425 nm to trace its fast fluorescence change. The binding of Ant-GDP to eIF-2 was found to be completed within 2 min (Fig. 9.2). This is consistent with Lax's report with filter binding assays (1).

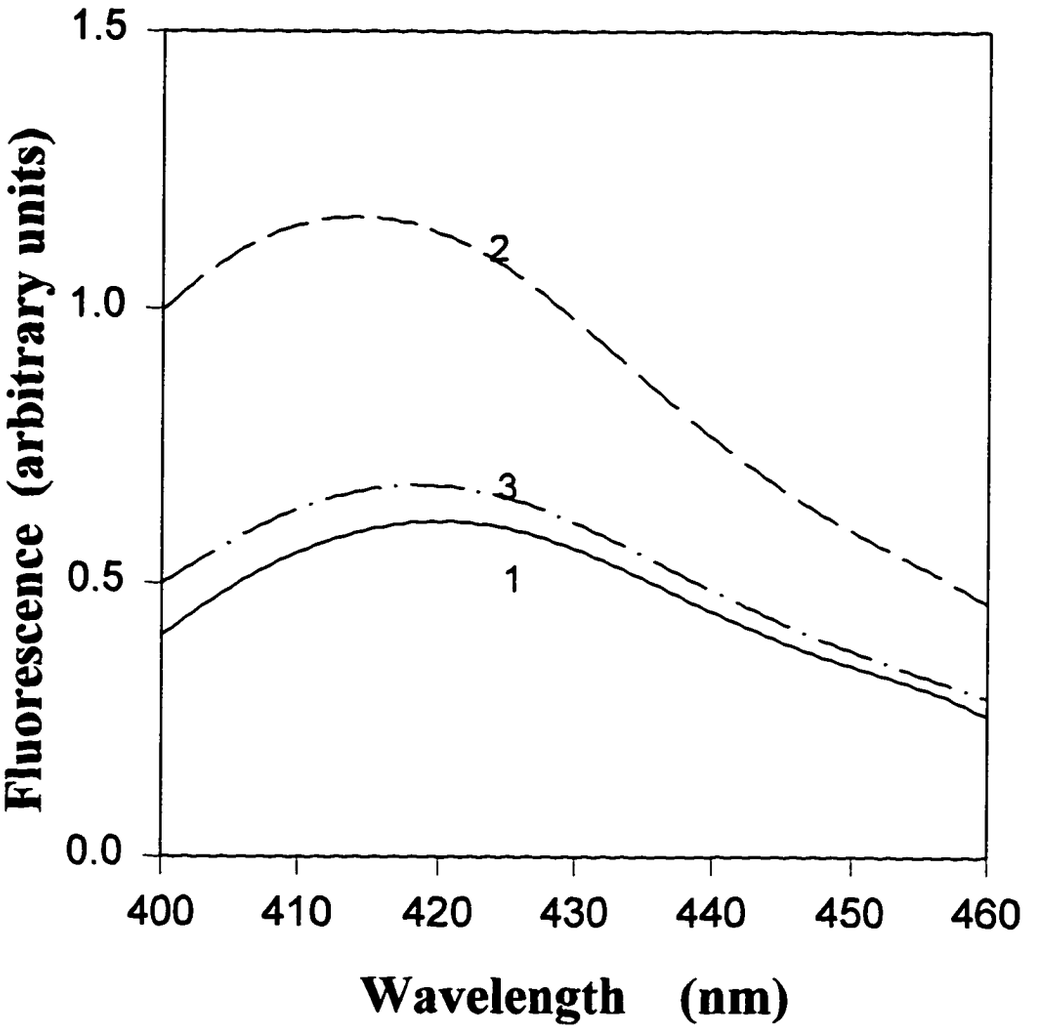

Fig. 9.1 Reaction of wheat germ eIF-2 with Ant-GDP

1. Spectrum of 0.4 μM Ant-GDP in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl at 10 °C and was excited at 332 nm. 2. spectrum after addition of 0.4 μM eIF-2 3. spectrum after addition of 200 μM GDP

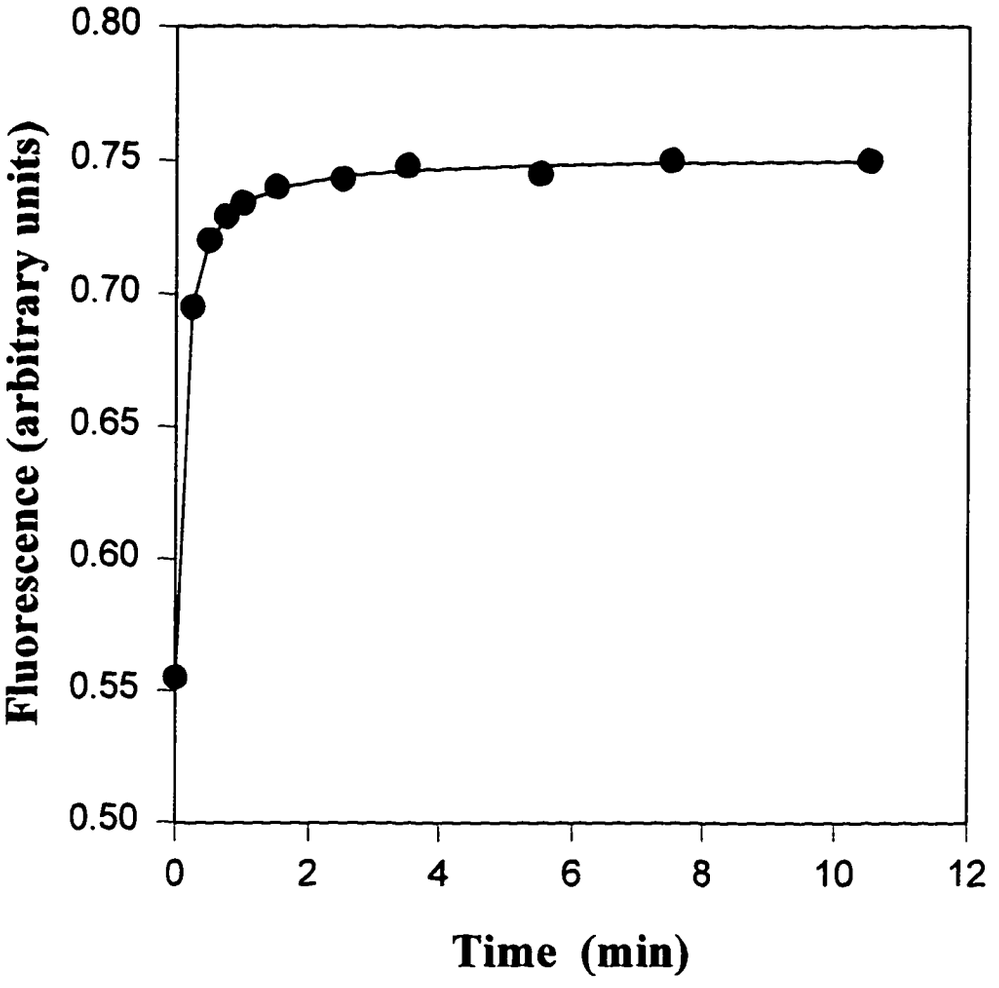

Fig. 9.2 Time course of reaction of wheat germ eIF-2 with Ant-GDP Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl with 0.4 μM Ant-GDP and 0.2 μM eIF-2 at 10 ^{o}C. All samples were excited at 332 nm and emission fluorescence spectra were measured from 420-425 nm.

Relative binding affinity

The relative binding affinity was tested as in Section 8.2 for Ant-m^7GTP. It was found that Ant-GDP bound eIF-2 1.2 fold tighter than GDP (Fig. 9.3). The derivation did not change bind affinity much.

Dissociation constant of binding of Ant-GDP and wheat germ eIF-2

The maximum fluorescence enhancement factor was obtained, as introduced in Section 4.3, by titration Ant-GDP with eIF-2 and extrapolated from double reciprocal plot as shown in Fig. 9.4. The maximal fluorescence enhancement factor was found to be 3.8.

Fig. 9.5 shows a typical Ant-GDP fluorescence titration in the presence and absence of eIF-2. The ratios of these data (F/F_o) were used to calculate f_{bound} and ν. The dissociation constant for eIF-2•Ant-GDP complex and the number of GDP binding sites on eIF-2 were calculated by Scatchard analysis, as shown in Fig. 9.6.

The K_d obtained was approximately 75 nM and 58 nM in the absence and presence of 1 mM Mg^{2+}, respectively, and binding site number were 0.6 to 0.9. This dissociation constant found here showed lower binding than mammalian eIF-2 (30 nM) but was consistent with result of Lax (1) for wheat germ eIF-2 (50 nM).

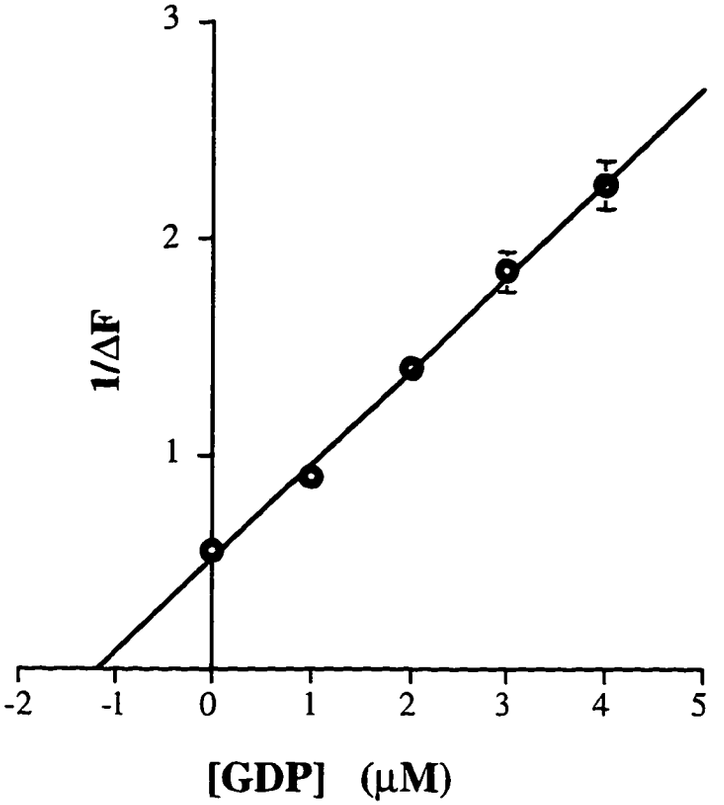

Fig. 9.3 Competition of GDP and Ant-GDP in eIF-2 binding reaction presented as single reciprocal plot

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl with 1 μM Ant-GDP and 1 μM eIF-2 at 10 °C. All samples were excited at 332 nm and emission fluorescence spectra were measured from 420-425 nm.

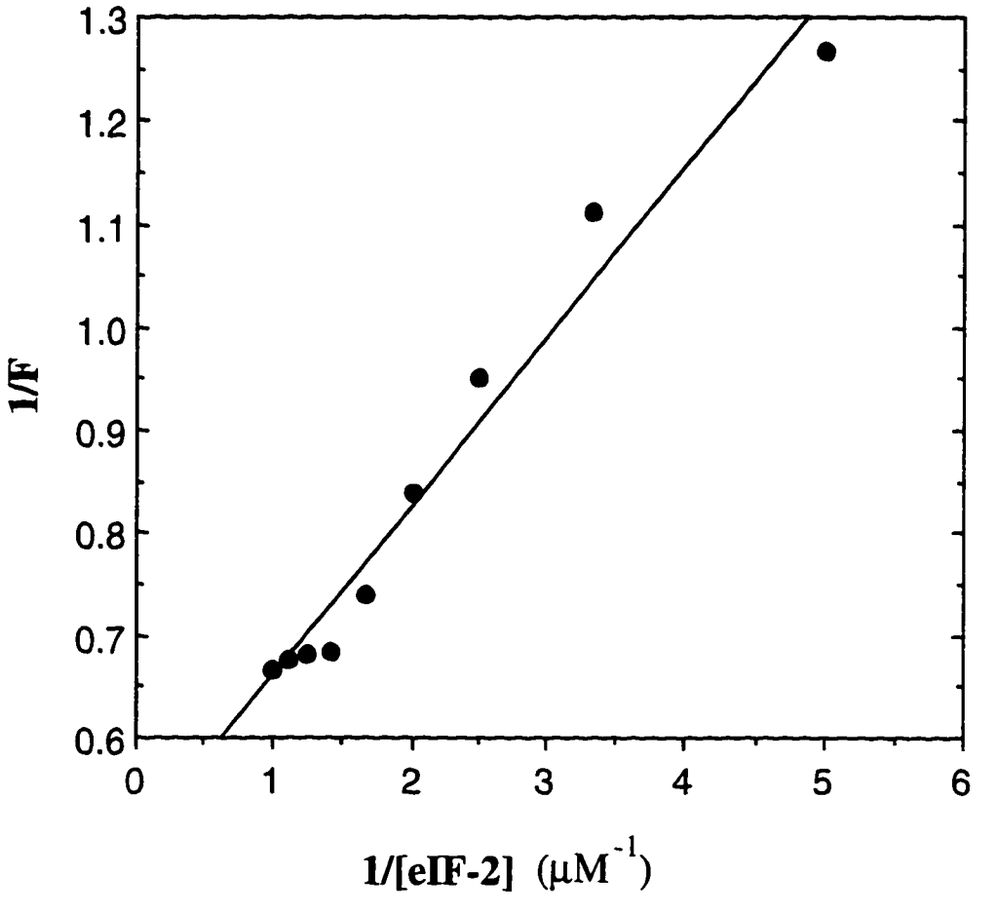

Fig. 9.4 Double reciprocal plot of reaction of Ant-GDP with wheat germ eIF-2

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl with 0.4 μM Ant-GDP at 10 °C and was excited at 332 nm.

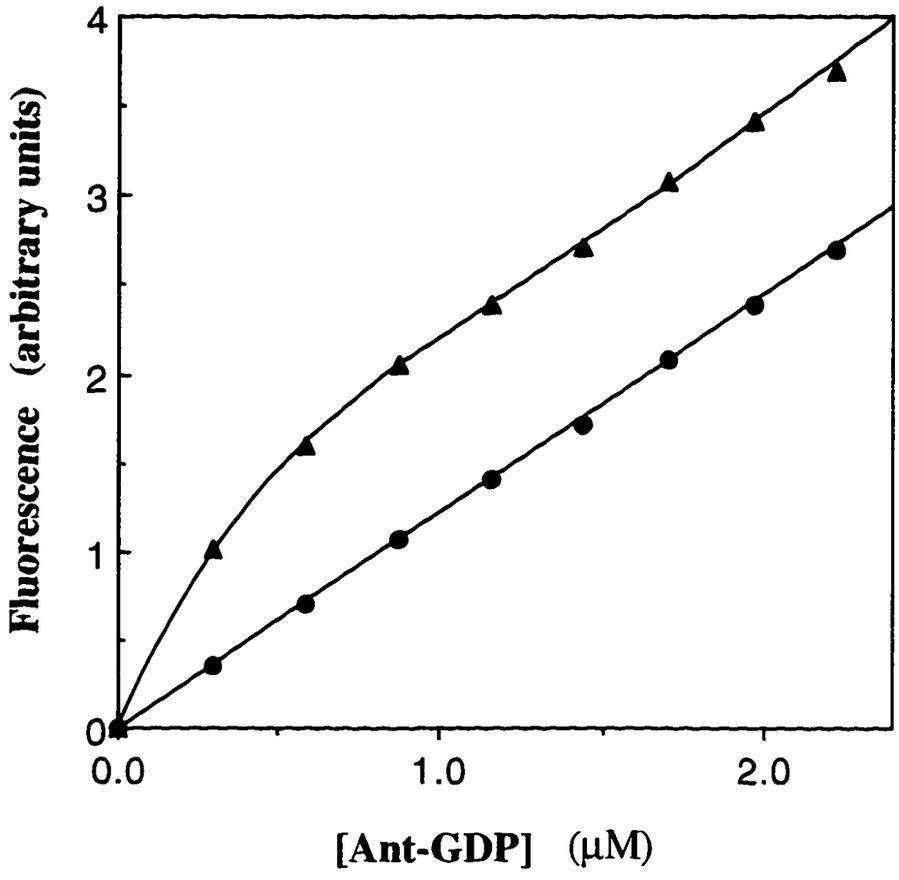

Fig. 9.5 Titration of wheat germ eIF-2 with Ant-GDP

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl at 10 °C and was excited at 332 nm. Circles: fluorescence of Ant-GDP in the absence of eIF-2; triangles: fluorescence of Ant-GDP in the presence of 0.4 μM eIF-2

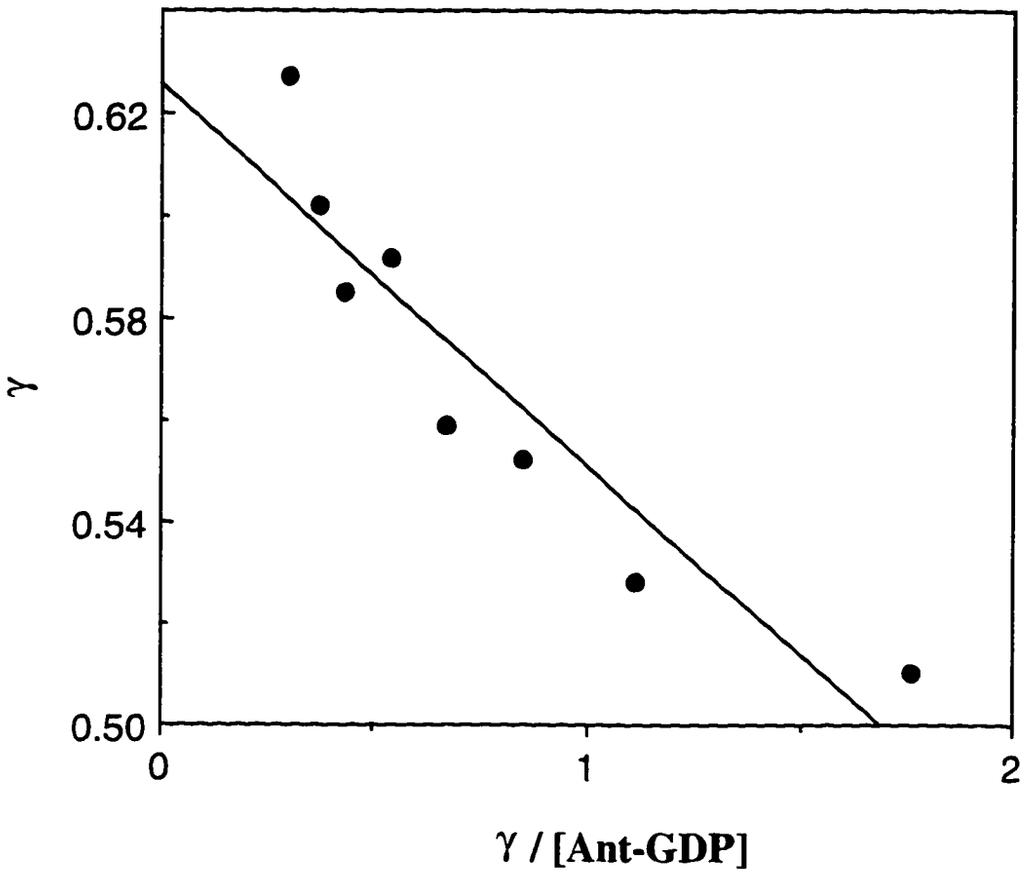

Fig. 9.6 Scatchard plot of reaction of wheat germ eIF-2 with Ant-GDP

Binding of Ant-GTP with wheat germ eIF-2

Binding of Ant-GTP with wheat germ eIF-2 was measured in the same way as Ant-GDP binding, as described above. Relative binding affinity was 1.2. The dissociation constant was found to be approximately 420 nM and 580 nM in the absence and presence of 1 mM Mg^{2+}, respectively, and binding site numbers were 0.7 to 0.9. This dissociation constant showed that the binding affinity of GTP to wheat germ eIF-2 was a little tighter than mammalian eIF-2 (2500 nM) (2). Spremulli (3) reported that wheat germ eIF-2 contained a certain potion tightly bound GTP. This may be caused by the difference between wheat germ and mammalian eIF-2. The most important is that the eIF-2·GTP/eIF-2·GDP dissociation constant ratio is around 10 (Table 9.1). This result is very close to Shaikhin's report (4) although dissociation constants measured were different which might be due to experimental difference, protein purity etc. It indicated that wheat germ performed differently from mammalian eIF-2 which eIF-2·GDP/eIF-2·GTP dissociation constant ratio was 100 fold or more.

Competitive inhibition does not change ΔF_{max}, since infinity concentration of reactant can displace inhibitor from the protein. So, for competitive inhibitor, Lineweaver-Burk plots ($1/\Delta F$ vs $1/P$) using different inhibitor concentration meet at the same Y-axis intercept (5). Lineweaver-Burk plots for GTP competition with Ant-GDP for wheat germ eIF-2 meet at the same Y-axis intercept within experimental error (Fig. 9.7). This provides additional evidence that GTP and Ant-GDP compete each other for the protein binding site. This is clear evidence showed that

Table 9.1 Comparison of guanosine nucleotides binding to wheat germ and mammalian eIF-2

	K_d		K_dGTP/K_dGDP	
	eIF-2•GTP	**eIF-2•GDP**	**wheat germ**	**mammalian**
absence of Mg^{2+}	**420 nM**	**75 nM**	**5.6**	
presence of Mg^{2+}	**580 nM**	**58 nM**	**10**	**200**

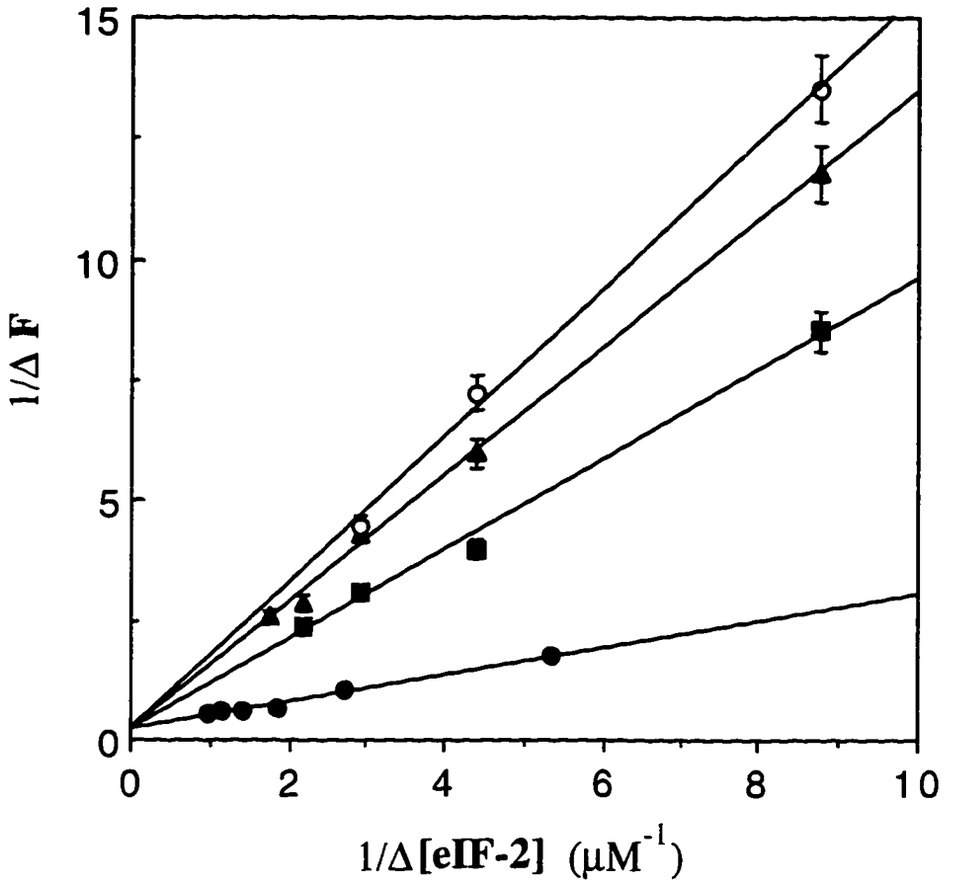

Fig. 9.7 Lineweaver-Burk plot of Ant-GDP and GTP in binding wheat germ eIF-2

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl at 10 °C with 1.0 μM Ant-GDP and GTP as indicated. The samples were excited at 332 nm. Filled circles: 0 μM GTP; squares: 10 μM GTP; triangles: 20 μM GTP; open circles: 30 μM GTP.

modifying on GTP and GDP ribose ring didn't change their eIF-2 binding property and this is consistent with previous observations for other proteins (6-10).

Protein binding environment and quenching study

To study the environment of Ant-GTP and Ant-GDP within eIF-2 complex, a KI quenching experiment was carried out to compare the ability of fluorescence of Ant-GTP and Ant-GDP free and bound to eIF-2 being quenched by KI. The Stern-Volmer plot was shown in Fig. 9.8. When bound to eIF-2, the iodide quenching of Ant-GTP or Ant-GDP fluorescence lines were lower than that of free analogs. Bound Ant-GTP or Ant-GDP were slightly more difficult to be quenched than free Ant-GTP and Ant-GDP. This showed that bound GTP or GDP was solvent accessible. This makes bound GTP or GDP exogenous accessible by GTP and GDP. Lax (1) has previously showed the exchange of bound GDP with exogenous GDP.

9.2 Guanosine nucleotide exchange

Guanosine nucleotide exchange in wheat germ eIF-2 complex: eIF-2 binding GDP was found to be only approximately 6 to 10 times stronger than binding GTP indicated that GDP bound to eIF-2 may be able to be displaced from eIF-2·GDP complex by GTP by increasing GTP concentration in solution. We tested this possibility by a substitution experiment. To a certain amount of Ant-GDP and eIF-2 reaction solution,

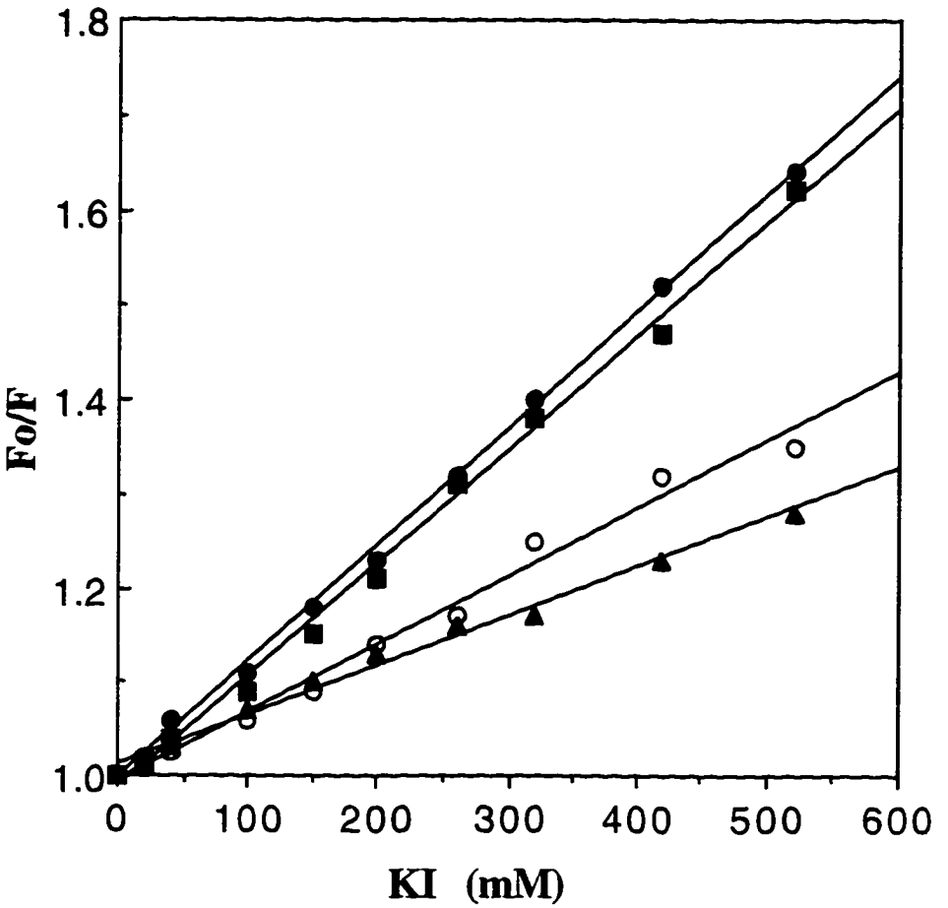

Fig. 9.8 Stern-Volmer plot of fluorescence quenching

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl at 10 °C with 0.4 μM Ant-GDP and 1 μM eIF-2. The samples were excited at 332 nm.

different amounts of unmodified GTP were added. It was found that Ant-GDP fluorescence, which was enhanced by reacting with eIF-2, gradually decreased. The results indicated that bound Ant-GDP was displaced by GTP. 10 μM GTP could displace over 50% of the bound Ant-GDP and 200 μM GTP could displace about 90% bound analog (Fig. 9.9). The presence of Mg^{2+} increased the binding affinity of GDP and caused the substitution slightly more difficult, but still able to substitute for bound Ant-GDP.

9.3 Discussion

In this chapter our results revealed the binding property of Ant-GTP and Ant-GDP with wheat germ eIF-2. The use of ribose modified nucleotides analogs in this study was very effective. The substitution experiment and Lineweaver-Burk plots showed that Ant-GTP and Ant-GDP bound eIF-2 and competed with unmodified GTP and GDP for the binding site. Compared with the filter binding assay, the fluorescence methods could fast trace the reaction process and without radioactive hazard. The dissociation constants and the ratio were consistent with observations from other experiments (1).

The quenching experiment showed that the bound Ant-GDP and Ant-GTP were solvent accessible. The small dissociation constant ratio of Ant-GDP and Ant-GTP bind wheat germ eIF-2 was different from that found for mammalian eIF-2. The substitution experiment showed that GTP could

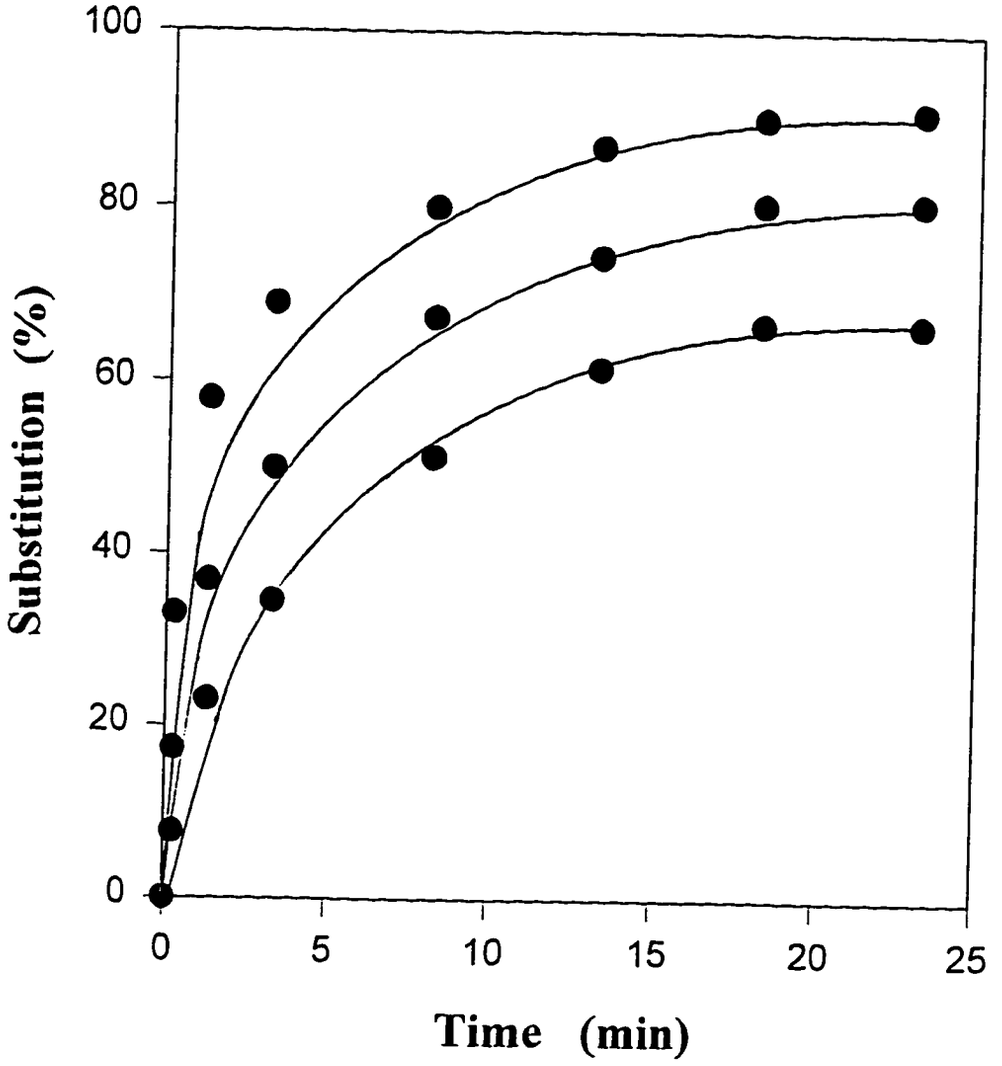

Fig. 9.9 Guanosine nucleotide exchange in wheat germ eIF-2

Spectra were measured in buffer containing 20 mM HEPES (pH 7.6), 2.4 mM DTT, 100 mM KCl at 10 °C with 1 μM Ant-GDP and 0.2 μM eIF-2. Different fraction of GTP was added. The samples were excited at 332 nm.

substitute GDP from wheat germ eIF-2 complex by controlling concentration ratio of GTP/GDP without the assistance of mammalian eIF-2B (GEF) like guanine nucleotide exchange factor. The fact is that a mammalian eIF-2B like protein has not been found in wheat germ up to now. The Co-eIF-2 (11-13) could stimulate the protein synthesis, but not act as guanine nucleotide exchanger. It was reported (14,15) that the phosphorylation of the eIF-2 α subunit blocked eIF-2 recycling in mammalian system. Because α subunit phosphorylated eIF-2 bound tightly to eIF-2B and blocked the guanine nucleotide exchange. In wheat germ system, Benne (16) found that α subunit phosphorylated wheat germ eIF-2 was not inactivated for protein synthesis. Therefore mammalian eIF-2B like protein might not be necessary to regulate the protein synthesis in wheat germ system, and it might not exist. A logical postulation is that GTP/GDP exchange in wheat germ eIF-2 complex may be processed by concentration difference of GTP/GDP without the assistance of mammalian eIF-2B like protein. Our substitution experiment results proved this possibility. This indicated a significant difference between mammalian and wheat germ eIF-2, except for their distinct subunits.

In mammalian system, eIF-2B was necessary for guanosine nucleotide exchange. Even concentration ratio of GTP/GDP high enough to compensate the dissociation constant ratio of eIF-2 •GDP/eIF-2•GTP could not process the guanosine nucleotide exchange without eIF-2B (16,18). The exceptional examples of guanosine nucleotide exchange without eIF-2B mediation were brine shrimp embryo Artemia eIF-2 (17,19) and Drosophila eIF-2 (20). By increasing GTP/GDP ratio, the Artemia binary complex readily exchanged bound GDP for free GTP

and eIF-2B was not necessary. The requirement for mammalian eIF-2B to promote GTP/GDP exchange could be bypassed. eIF-2B has not been isolated from shrimp embryo Artemia and Drosophila. Another distinct property of shrimp embryo Artemia and Drosophila was that the phosphorylation of Artemia eIF-2 and Drosophila eIF-2 did not depress the protein synthesis rate (19,20). This was very similar with wheat germ. Here we found the possibility that, in wheat germ system, mammalian eIF-2B like protein might be bypassed in guanosine nucleotide exchange and the necessary concentration ratio was smaller than in Artemia and Drosophila. Artemia, Drosophila and wheat germ might share some similarity in guanosine nucleotide exchange without eIF-2B.

In conclusion, we used GTP and GDP analogs to study the guanosine nucleotide binding and exchange of wheat germ eIF-2. The performance of wheat germ eIF-2 was found similar to mammalian eIF-2 at time course and binding preference for GDP than for GTP. The differences lied in the ratio of GDP/GTP binding affinity. The relative small difference of K_d ratio and substitution experiment showed the possibility of bypassing guanine nucleotide exchange factor in processing GTP/GDP exchange in wheat germ system.

Chapter 10 Helicase activity of Wheat Germ Protein Synthesis Initiation Factors eIF-4A and eIF-(iso)4F

10.1 Conditions of helicase reaction

Unwinding of duplex RNA by wheat germ eIF-4A and eIF-(iso)4F

By incubating with wheat germ eIF-(iso)4F and eIF-4A, the duplex RNA was found to be partially separated to single strand as shown in Fig. 10.1. This indicated that wheat germ eIF-(iso)4F indeed could replace eIF-4F in helicase reaction in wheat germ protein synthesis initiation. Following we tested the conditions for this helicase reaction.

Requirement of ATP and magnesium in the helicase reaction

The results presented in Fig. 10.2 show that eIF-(iso)4F and eIF-4A require ATP for unwinding, as do all helicases described to date (1-3). An ATP concentration of 2.0 mM is found to be saturating. We have previously reported (4) that ATPase activity was necessary for eIF-4A and eIF-(iso)4F complex formation and might also provide the energy needed for protein movement along the RNA substrate and/or for disruption of the hydrogen bonds between the two strands of the RNA duplex. The nonhydrolyzable ATP analog, AMP-PNP was used to replace ATP in the helicase assay. It was found that dsRNA could not be unwound (Table 10.1). The other nucleotide triphosphates, GTP, UTP, CTP, were also analyzed for their ability to support unwinding and found that they all failed to support any detectable unwinding (Table 10.1).

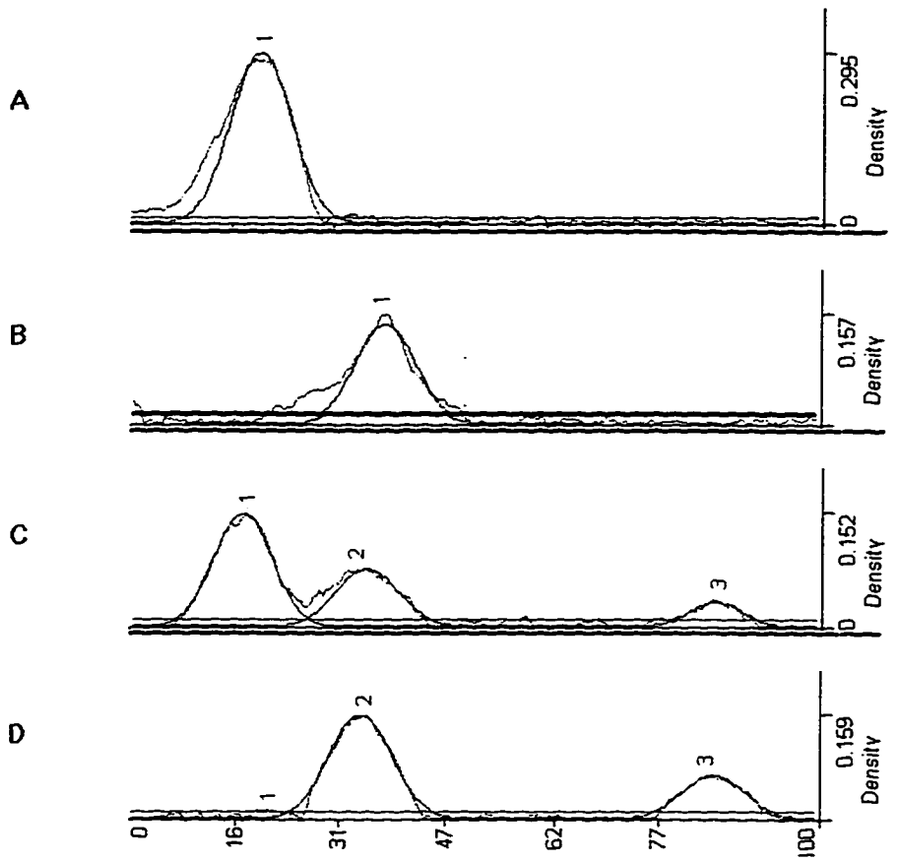

Fig. 10.1 Densitometer scans of gel demonstrating helicase activity of wheat germ eIF-(iso)4F and eIF-4A.

Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, incubated at 37 °C for 15 min. Panel A. is the double stranded RNA; panel B is the single stranded RNA B; panel C is the result of unwinding by eIF-(iso)4F and eIF-4A; panel D is the catalytic effect of eIF-4B on the helicase reaction

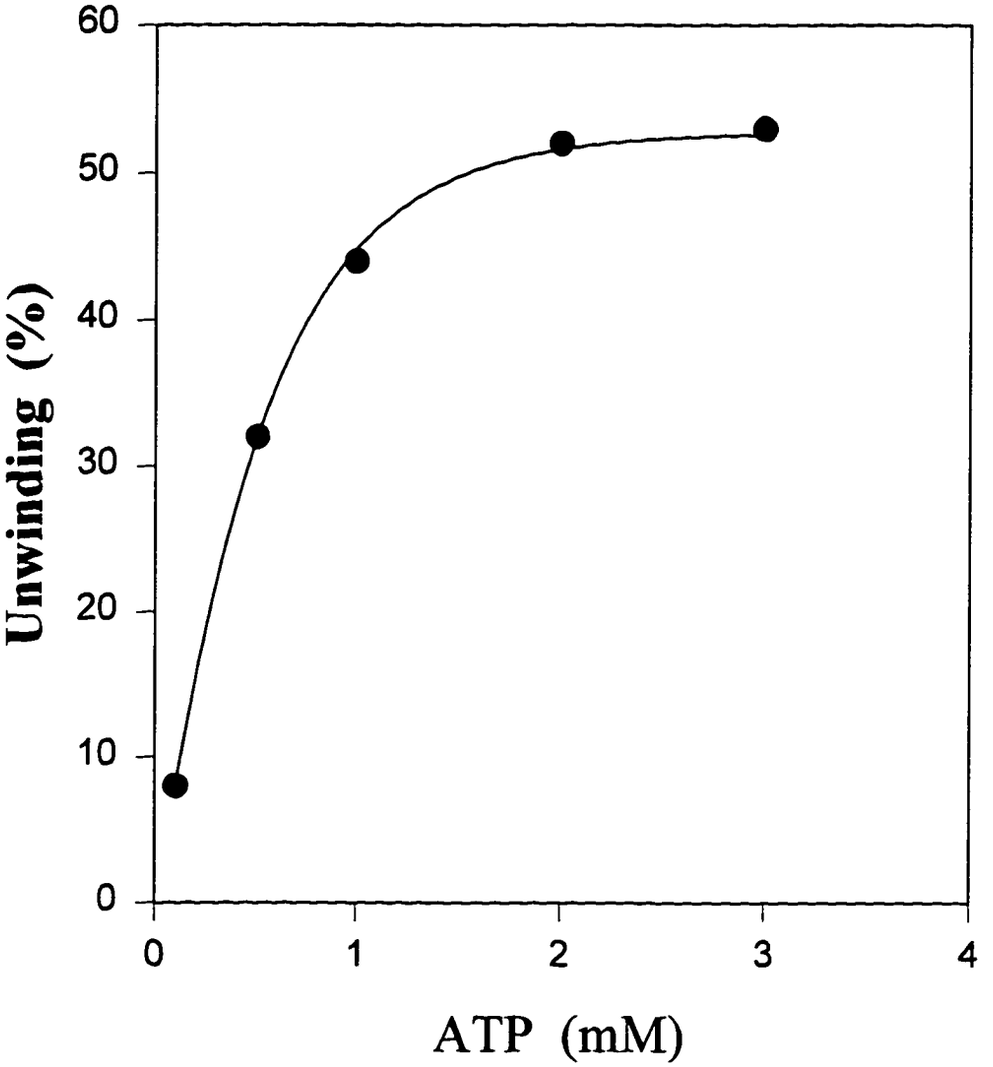

Fig. 10.2 ATP concentration dependence of helicase reaction
Reaction conditions: 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, incubated at 37 °C for 15 min.

Table 10.1. Requirement of nucleotide triphosphates in helicase reaction
Reaction conditions: 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, incubated at 37 °C for 15 min and 2 mM nucleotides listed in table.

	Density ratio (%)			unwinding (%)
	dsRNA	RNA A	RNA B	
ATP	50.5	36.2	13.3	50
AMP-PNP	100	0	0	0
CTP	100	0	0	0
GTP	100	0	0	0
UTP	100	0	0	0

There is an absolute requirement for magnesium in the helicase reaction. In the absence of Mg^{2+}, there is no detectable helicase activity. The effect of Mg^{2+} is shown in Fig. 10.3. This effect may be attributed to the requirement of Mg^{2+} for the hydrolysis of ATP by the formation of ATP-Mg^{2+} complex and therefore affect the energy needed for unwinding as described above.

Kinetics of helicase reaction

A kinetic investigation of the unwinding reaction showed that the helicase reaction displayed a small lag phase followed by a rapid increase and reached a plateau at approximately 50% unwinding (Fig. 10.4). At least 15 minutes incubation time was required for efficient unwinding. Unpublished studies indicate protein binding is rapid. This lag phase must be attributable to the unwinding reaction itself.

The effect of eIF-4A concentration upon the helicase reaction

eIF-4A has been reported (5-7) to be a factor responsible for helicase activity. Fig. 10.5 shows the effect of wheat germ eIF-4A concentration on unwinding activity in the presence of a constant amount of all other materials. With increasing amount of eIF-4A, there were an increase in the amount of RNA unwound and simultaneously decrease in the amount of duplex RNA. This activity leveled off when approximately 50% of the duplex RNA was unwound. It was found that a large excess of

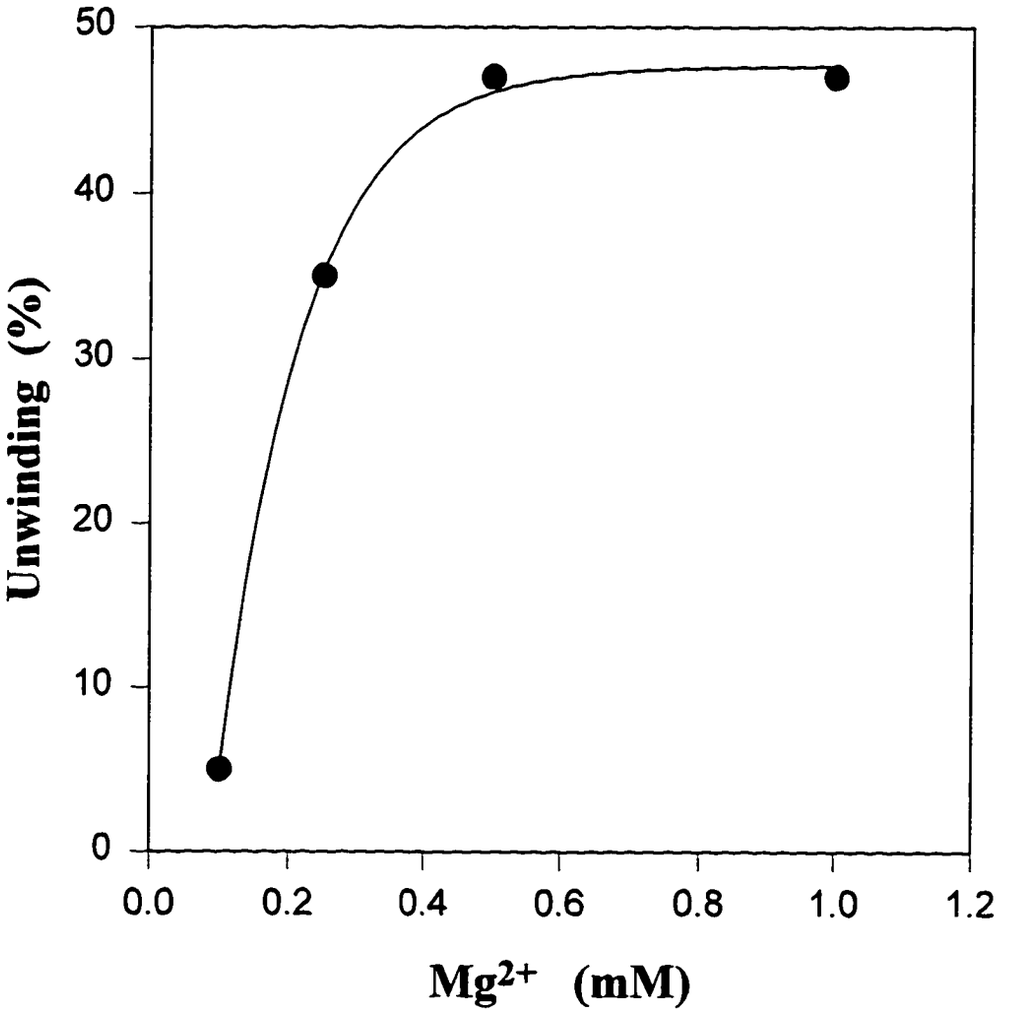

Fig. 10.3 Mg^{2+} concentration dependence of helicase reaction
Reaction conditions: 2 mM ATP, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, incubated at 37 °C for 15 min.

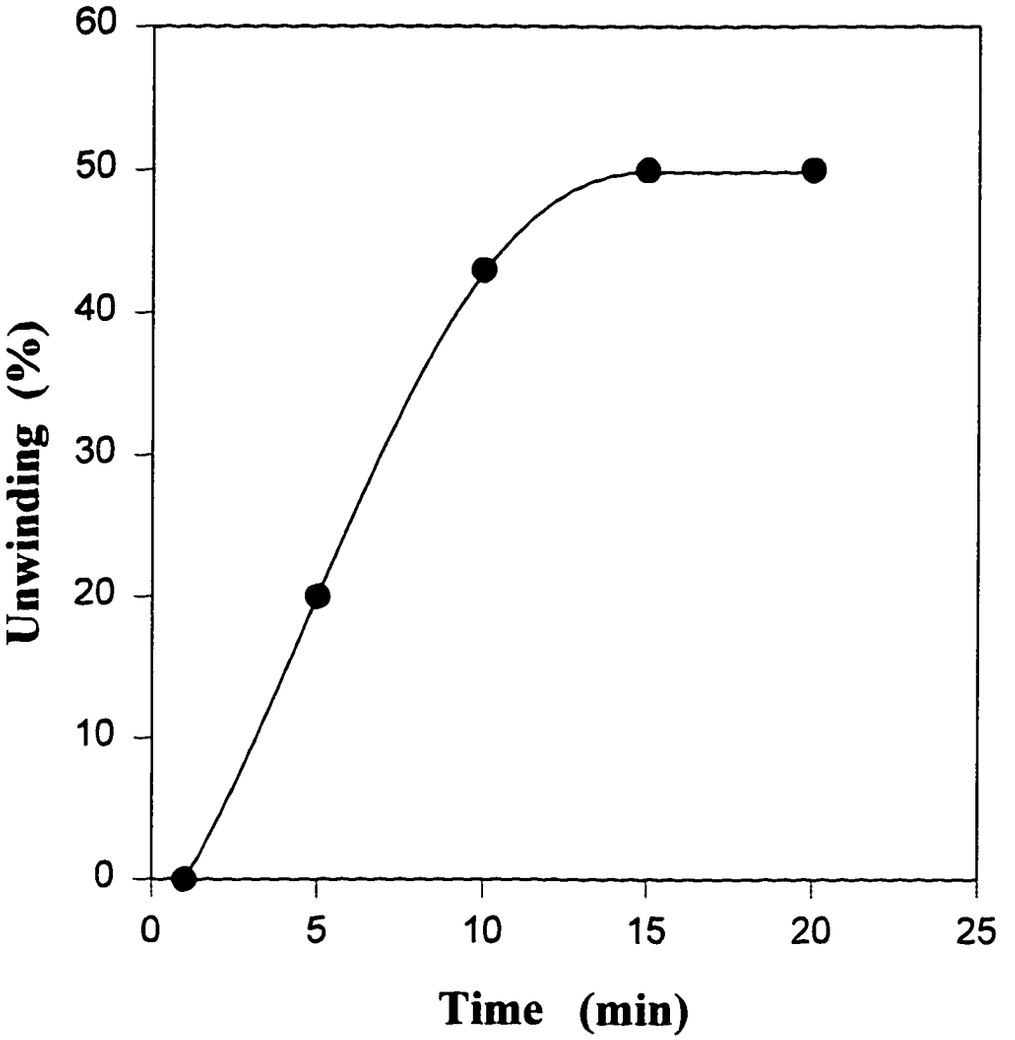

Fig. 10.4 Kinetics of helicase reaction

Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, incubated at 37 °C.

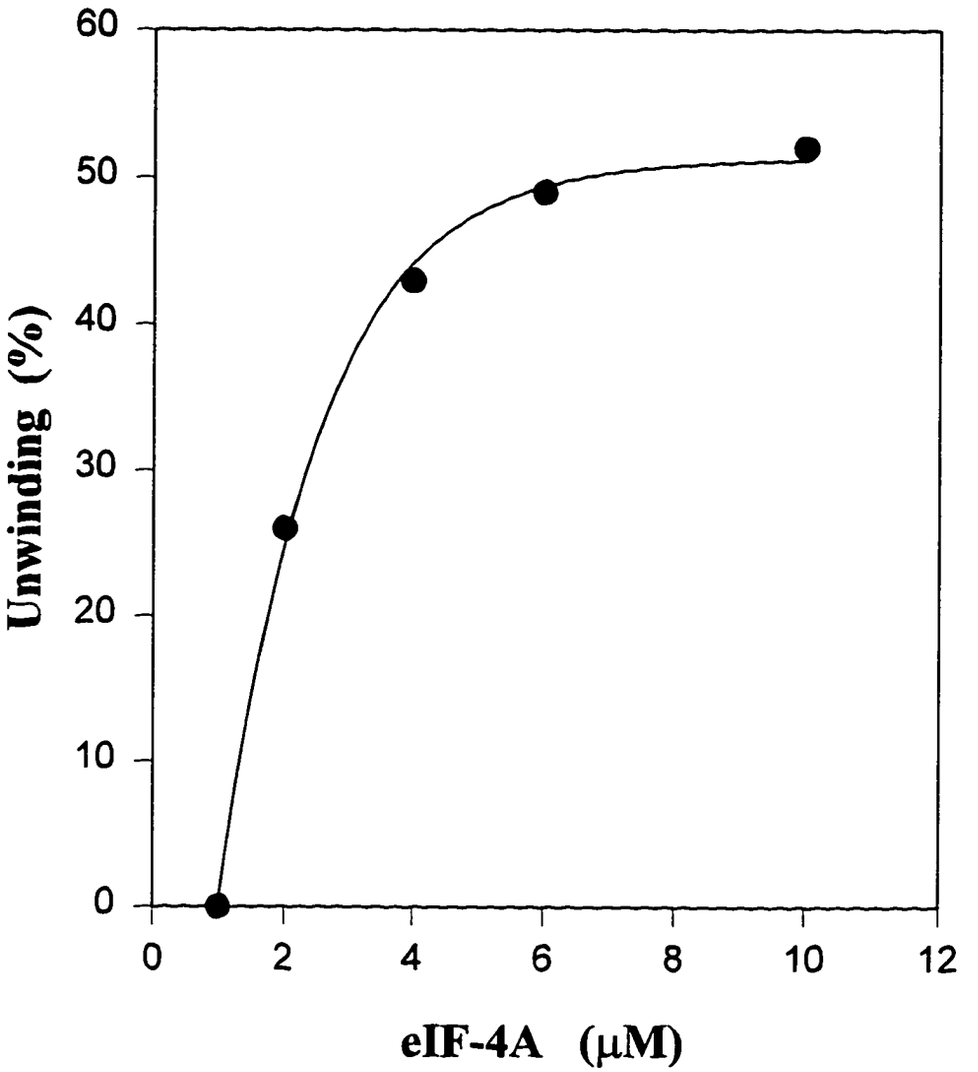

Fig. 10.5 eIF-4A concentration dependence of helicase reaction

Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 3.3 mM eIF-(iso)4F, incubated at 37 °C for 15 min.

eIF-4A was required to unwind dsRNA. The low efficiency of eIF-4A in the helicase assay may be due to the eIF-4A relatively low binding affinity (4).

The effect of eIF-(iso)4F concentration upon the helicase reaction

eIF-(iso)4F is also a required factor in the helicase reaction as shown in Fig. 10.6. The 28 kD subunit of eIF-(iso)4F is a cap binding peptide. The effect of eIF-(iso)4F may lie in forming a complex with eIF-4A and helping this complex bind to the 5' terminus of mRNA. Wheat germ eIF-4A itself is a poor mRNA binding protein (6).

10.2 RNA structures requirement for helicase reaction

The effect of cap structure upon the helicase reaction

To assess the importance of cap structure for unwinding activity, a similar dsRNA omitting cap but still with a single stranded region was used to investigate the requirement for a cap structure in the helicase reaction. The oligonucleotide structure is shown below.

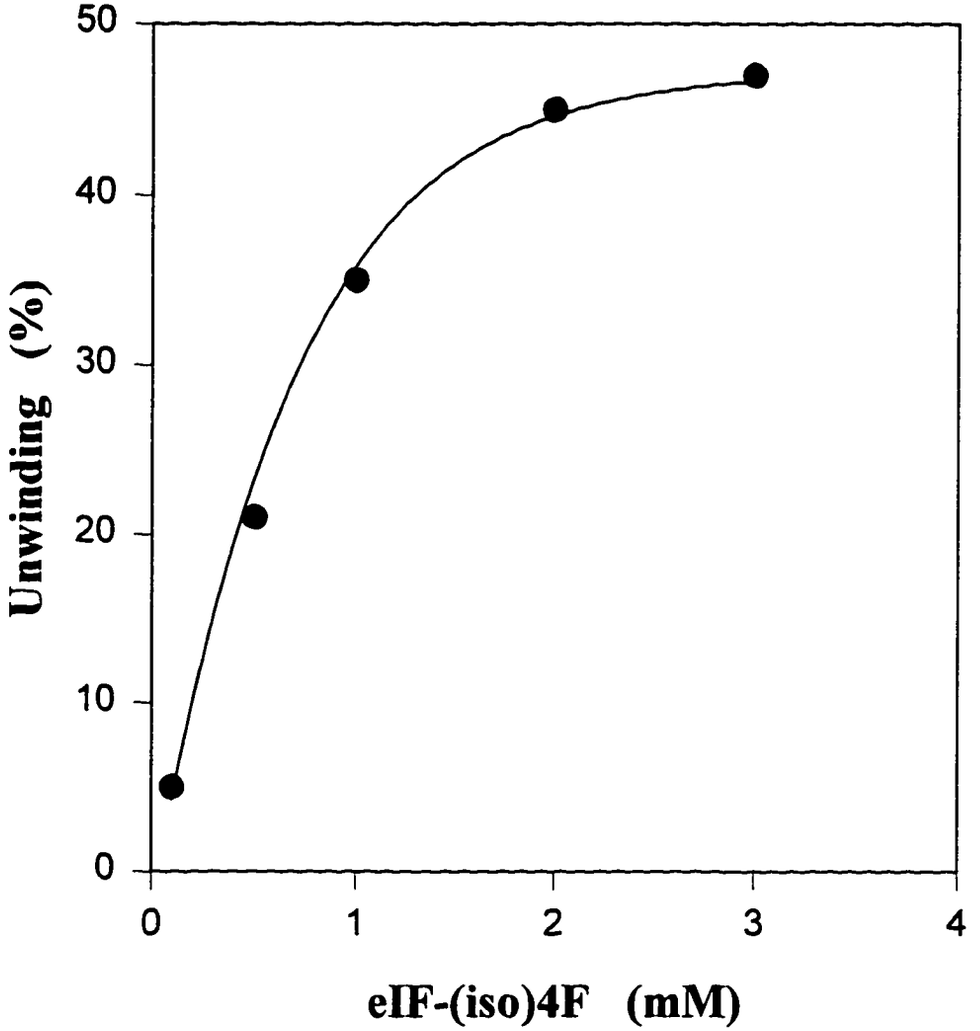

Fig. 10.6 eIF-(iso)4F concentration dependence of helicase reaction
Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 10 mM eIF-4A, incubated at 37 °C for 15 min.

3'-GCGACGCGACGGGG 5' A

5'-GGGACCAUGGAACAACAUUAUCGCUGCGCUGCCCC 3' B

Fig. 10.7 Description of uncapped RNA substrate used for cap-dependence assay

The results (Table 10.2) showed that the helicase reaction was prevented by omission of cap structure at the 5' end of RNA under the same conditions as capped RNA. No RNA monomer was detected. These data support the conclusion that one role of eIF-(iso)4F in helicase activity is to increase eIF-4A affinity and to locate the complex at the 5' end of mRNA.

The role of the single strand region

Previous studies showed that a single stranded RNA was necessary for initiation factors binding (8,9) and for ATPase activity (10). To investigate the importance of the requirement of the single stranded region, we utilized a RNA strand, designated C below, designed specifically for

this purpose. RNA strand C, completely complementary to RNA B, was annealed to RNA B and formed a blunt ended duplex RNA (Fig. 10.8).

3'-CCCUGGUACCUUGUUGUAAUAGCGACGCGACGGGG 5' C
5'-m7GpppGGGACCAUGGAACAACAUUAUCGCUGCGCUGCCCC 3' B

Fig. 10.8 Description of completely double stranded RNA substrate used for single strand dependence assay

This dsRNA totally abolished the ability of the duplex RNA to be unwound (Table 10.3) even in the presence of eIF-4B. The result showed that the helicase factors need single stranded region for binding and helicase initiation. This single stranded region is necessary for initiation factors landing and successively ATP hydrolysis and scan as for most helicase reactions (11).

Table 10.2 Requirement of m^7G cap in helicase reaction

Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, 1.7 μM eIF-4B, incubated at 37 °C for 15 min.

	ratio (%)		unwinding (%)
	dsRNA	RNA A	RNA B	
Capped RNA				
4A+(iso)4F	49.1	34.2	16.7	50
4A+4B+(iso)4F	0	73.7	26.3	100
Uncapped RNA				
4A+(iso)4F	100	0	0	0
4A+4B+(iso)4F	66.6	20.5	12.9	33

Table 10.3. Requirement of single stranded region in helicase reaction
Reaction conditions: 2 mM ATP, 1 mM Mg^{2+}, 10 mM eIF-4A, 3.3 mM eIF-(iso)4F, 1.7 μM eIF-4B, incubated at 37 °C for 15 min.

	ratio (%)		unwinding (%)	
	dsRNA	RNA A	RNA B	
With single strand				
4A+(iso)4F	49.1	34.2	16.7	50
4A+4B+(iso)4F	0	73.7	26.3	100
Without single strand				
4A+(iso)4F	100	0	0	0
4A+4B+(iso)4F	100	0	0	0

10.3 The catalytic effect of eIF-4B on the helicase reaction

A mammalian eIF-4B function like protein (12,13) was purified from wheat germ as described (12). The effect of eIF-4B upon eIF-(iso)4F and eIF-4A helicase reaction was tested. Results in Table 10.2 and Table 10.3 show that it was indeed able to catalyze the unwinding reaction of both 5' capped mRNA and uncapped mRNA. By adding eIF-4B, capped mRNA could be unwound up to 100%; uncapped mRNA could be unwound up to 33%. This also proved the importance of the cap structure and the cap binding subunit of eIF-(iso)4F in the helicase reaction.

10.4 Discussion

The results shown here provided a direct evidence of the helicase activity of wheat germ initiation factors eIF-4A and eIF-(iso)4F. In wheat germ eukaryotic protein synthesis system, eIF-(iso)4F was indeed able to replace eIF-4F in translation through double stranded RNA unwinding.

Like most helicases, ATP was necessary and ATPase provided the energy for unwinding the double stranded substrate; and Mg^{2+} mediated this hydrolysis reaction. This process was also single stranded RNA dependent. This is common for most helicases to land and bind to RNA or DNA strands (11, 17-19). A certain time of incubation was necessary for unwinding. Unwinding of the RNA duplex required a large excess of eIF-(iso)4F and eIF-4A over RNA. A similar requirement for a large excess of

enzymes over RNA or DNA was also observed with mammalian eIF-4F and eIF-4A etc DEAD box family and other RNA, DNA helicases (18,20,21).

By comparison with mammalian initiation factors eIF-4A and eIF-4F (14-16), the wheat germ helicase was more dependent on the cap structure for complete unwinding, but less dependent on auxiliary factor, eIF-4B. Without eIF-4B, double stranded RNA unwinding could start and go up to about 50% finishing. eIF-4B increased the unwinding efficiency up to 100%. Without eIF-4B, mammalian initiation factors could not efficiently unwind double stranded RNA (16).

Our results combining with the results from early fluorescence binding affinity studies (12) lead to a working model for the unwinding of mRNA by eukaryotic initiation factors eIF-(iso)4F, eIF-4A and eIF-4B in wheat germ. In the presence of ATP and Mg^{2+}, eIF-(iso)4F formed a complex with eIF-4A. The complex was bound to the m^7G cap by the 28 kD cap binding subunit then to RNA and this process was catalyzed by eIF-4B. Thereafter, at the assistance of the energy from ATP hydrolysis, the eIF-4A started to sweep along the single strand and broke the hydrogen bonds of the double stranded region it reached and causeed the strands to unwind. So in wheat germ system, there are two parallel ways for protein synthesis initiation, through eIF-4F or eIF-(iso)4F.

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