Homeostasis in mRNA Initiation

WHEAT GERM POLY(A)-BINDING PROTEIN LOWERS THE ACTIVATION ENERGY BARRIER TO INITIATION COMPLEX FORMATION

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Previous kinetic binding studies of wheat germ protein synthesis eukaryotic initiation factor iso4F (eIFiso4F) and its subunit, eIF4E, with m7GTP and mRNA analogues indicated that binding occurred by a two-step process with the first step being too fast to measure by stopped-flow techniques (1). Further equilibrium studies showed that poly(A)-binding protein (PABP) enhanced the cap binding of eIFiso4F about 40-fold. The kinetic effects of PABP on cap binding and the temperature dependence of this reaction were measured and compared. Fluorescence stopped-flow studies of the PABP-eIFiso4F protein complex with cap show a concentration-independent conformational change. PABP did not significantly increase the rate of the conformational change, and because the initial secondary binding is essentially diffusion-controlled, the enhancement of cap affinity must reside in the dissociation rate. The dissociation rate was more than 5-fold slower in the presence of PABP. The temperature dependence of the cap binding reaction was markedly reduced in the presence of PABP. The reduced energy barrier for formation of a cap-eIFiso4F complex suggests a more stable platform for further initiation complex formation and a possible means of adapting to varying temperature conditions.

Most eukaryotic mRNAs contain both a 5′ cap (m7GpppX) and a 3′ poly(A) tail to increase synergistically translational efficiency and stability (2–4). These two elements have been investigated recently, and evidence suggests that they communicate during translation (3, 5). These interactions are mediated by interaction of poly(A)-binding protein (PABP) and the cap binding complex eIF4F or an isofrm of eIF4F found in plants, eIFiso4F. The small subunit of eIF4F (eIF4E) recognizes the cap structure, and the eIF4F or an isoform of eIF4F found in plants, eIFiso4F. The small subunit, eIF4G (eIF4E), recognizes the cap structure, and the large subunit (eIF4G) interacts with PABP (6, 7). The cap-associated proteins have a very high affinity for PABP in the absence of poly(A) in the wheat germ system (8) but require poly(A) in yeast (9). Interaction of PABP-eIF4F has been shown to enhance the binding affinity of both cap analogs and poly(A) by about 40-fold. Further studies have shown that both the cap and poly(A) tail can be bound by the protein complex simultaneously. It was concluded that at least part of the enhancement of translation could be accounted for by increased binding affinity for the 5′ cap by the protein complex.

The increase in binding affinity of the PABP-eIF4F complex for both cap analogs and poly(A) leads us to further investigate the kinetics and mechanism of these interactions. An increase in affinity for the cap structure can result from an increase in the association rate constant or a decrease in the dissociation rate constant or changes in both. Increases in the association rate constant may indicate an increase in the rate-limiting step, whereas decreases in the dissociation rate constant may indicate an increased stability for the intermediate complex in the protein synthesis pathway. We have previously investigated the mechanism of eIFiso4F or eIFiso4E interacting with cap (1). Kinetic studies indicated that the binding involved a two-step process with the first reaction being too fast to measure by stopped-flow techniques. The second reaction, a conformational change, was ~10-fold faster for eIF4E than for eIF4F.

Here, we report the effects of PABP on the binding kinetics of cap analog binding to eIFiso4F and the temperature dependence of these reactions. These data indicate that a large part of the enhancement in binding affinity resides in the dissociation rate and that one of the significant effects of PABP is to lower the Arrhenius activation energy of the eIF4F complex.

EXPERIMENTAL PROCEDURES

Materials—m7GTP was purchased from Sigma. Ant-m7GTP was synthesized and purified as described previously (10) with slight modification. After completion of the synthesis reaction, there are several products in the solution, Ant-m7GTP, m7GTP, isotopic anhydride, and anthraniloyl acid. Ethanol precipitation was used to separate Ant-m7GTP and m7GTP from the fluorescent dye. For the separation of Ant-m7GTP and m7GTP, a Sephadex G-15 column (1 × 120-cm, packed in deionized water) with a flow rate of 6 ml/hr was used. Ant-m7GTP was eluted as the second peak, which was characterized by UV absorbance and thin layer chromatography. Ant-m7GTP showed two absorbance maxima at 254 and 332 nm, a shoulder at ~280 nm, and a fluorescence spot on TLC plates. Ant-m7GTP was separated from Ant-m7GDP by TLC prior to use.

The preparation of PABP followed the procedure of Le et al. (8). Wheat germ eIFiso4F was obtained by mixing two subunits (P28 and P86) in a 1:1 molar ratio (11). The pure P28 and P86 subunits were prepared as described previously (12). All samples were dialyzed against Buffer A (20 mM HEPES-KOH, 100 mM potassium acetate, 1 mM CaCl2, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, pH 7.6) and passed through a 0.22 μm filter (Millipore) before the spectroscopy measurements were performed. Protein concentrations were estimated by the method of Bradford (13) using a Bio-Rad protein assay reagent (Bio-Rad Laboratories).

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exponential process. Ft is that fluorescence observed at any time, \( \text{F}_t \). Ant-m7GTP fluorescence was measured. Because of the high binding affinity of the complex, a large dilution, which could not be accomplished by stopped-flow, was necessary.

**RESULTS**

The stopped-flow data for a typical reaction curve for the binding of Ant-m7GTP to eIFiso4E and eIFiso4F are shown in Fig. 1. On the ordinate is the relative voltage, which is proportional to fluorescence intensity. Time course data were fitted by assuming a single exponential function. The data for the time course of the fluorescence intensity changes were recorded by computer data acquisition. Kinetic data were analyzed by nonlinear regression analysis using Global analysis software provided by Olis.

Steady-state fluorescence intensities were recorded on a Spex Fluorolog 2 spectrofluorometer. All measurements were performed at 20 °C. Stop-flow Fluorescence Measurements—As described elsewhere (10, 14, 15), \( P_{m7GTP} \) or eIFiso4F binding induced an increase in Ant-m7GTP fluorescence. The time course of the reaction was monitored using an Olis RSM 1000 stopped-flow system with a 1-ms dead time. Ant-m7GTP was diluted with eIFiso4E, eIFiso4F, or eIFiso4F-PABP. Formation of the eIFiso4F-PABP complex was determined as described previously (6). The excitation wavelength was 323 nm, and emission was monitored by use of a photomultiplier after passage through a 400-nm cut-on filter. A reference photomultiplier was used to monitor fluctuations in the lamp intensity. The temperature of flow-cell and solution reservoirs was maintained using a temperature-controlled circulating water bath. After rapid mixing of the protein with the complex of eIFiso4E and Ant-m7GTP, the data for the time course of the fluorescence intensity changes was recorded by computer data acquisition. Kinetic data were analyzed by nonlinear regression analysis using Global analysis software provided by Olis.

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Dissociation Rate Constants—To measure the dissociation rate constants, a complex of eIF4F-PABP-Ant-m7GTP was rapidly diluted 12-fold in a spectrofluorometer cuvette, and the resulting decrease in fluorescence was measured. Because of the high binding affinity of the cap analog to the complex, a large dilution, which could not be accomplished by stopped-flow, was necessary.

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protein was more able to accomplish the conformational change necessary for binding, although the equilibrium constants were similar for the two proteins.

The fact that the rate constant, $k_2$, for the two proteins is similar for the mononucleotide analog indicate that the second base must play a role in the binding. In the case of eIFiso4F, this slows down the binding, not only in comparison with eIFiso4E but also compared with the mononucleotide. The Ant- m$^7$GTP bound eIFiso4F about 3-fold faster than m$^7$GpppG.

Our previous studies using fluorescence energy transfer have shown that PABP has a large effect on the cap equilibrium with eIFiso4F. The dissociation equilibrium constant decreased from 8.9 to 0.21 µm in the presence of PABP. PABP has been shown to interact with eIFiso4F through the P$_{as}$ (eIFiso4G) subunit of eIFiso4F. Only a small effect on $k_2$ was observed for the PABP/eIFiso4F binding of cap analog. It is unlikely there is much effect on the initial second-order binding, because that rate is close to the diffusion-controlled rate and cannot account for the large difference in equilibrium constant. The most likely explanation is that the off rate, $k_{-2}$, is slower when PABP is present. To estimate the off rate, the dilution experiment described earlier was performed. This is not an ideal experiment, because concentrations of Ant-m$^7$GTP must be such that a significant change in the amount of cap analog that is bound occurs. There must also be sufficient eIFiso4F to assure that a significant eIFiso4F/PABP complex exists. Further, because these experiments are performed in a standard 1-cm fluorescence cuvette to allow mixing, large amounts of sample are required. Fig. 4 shows the difference measured. The curve in the presence of PABP represents dissociation from both eIFiso4F and eIFiso4F/PABP. We were unable to adequately resolve two rate constants from these curves, probably because about 10–15% of the reaction occurred during the mixing time. However, a significant difference is seen in the presence of PABP indicating the dissociation rate is indeed affected. The rates are at least 5-fold slower in the presence of PABP. This difference is an underestimate of the effect of PABP on the dissociation rate. More detailed kinetic studies are in progress.

The Arrenhius activation energies for the three protein systems, eIFiso4E, eIFiso4F, and eIFiso4F/PABP, were significantly different. The eIFiso4E and eIFiso4F had Arrenhius activation energies of 63.3 ± 1.4 and 81.5 ± 2.5 kJ/mol, respectively. The addition of PABP to eIFiso4F lowered the activation energy more than 2-fold. This reduced temperature dependence suggests that PABP succeeds in speeding the reaction by providing a path with a substantially lower energy barrier.

The kinetic effects of PABP enhancement on cap affinity must reside primarily in the dissociation rates. A likely possibility is that PABP, which interacts with the 4G subunit, induces a conformational change that is propagated to the cap binding site. The lower activation energy suggests an intermediate that more easily achieves a stable conformation. PABP reduces the dissociation of the complex and may enhance translation by forming a more stable platform for further assembly of the initiation complex. The reduced temperature dependence of the reaction may provide a means for adapting to variations in environmental temperatures encountered by plant systems. It would not be advantageous for rapid protein synthesis to occur during environmental stress.

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