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Solution-based approach to study binding to the eIF4E cap-binding site using CD spectroscopy

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ABSTRACT

The eukaryotic initiation factor 4E (eIF4E) is the key component of the translational initiation complex that recruits mRNA by binding to a unique "cap" structure located at the 5′ end of the mRNA. Overexpression of eIF4E has been implicated in the development of cancer, potentially as a result of increasing the cellular levels of proteins involved in processes that include proliferation and regulation of apoptosis. As a result, the cap-binding site of eIF4E has become a target for the development of anti-cancer therapeutics. The structure of eIF4E bound to the cap mimic 7-methyl-GDP revealed that two tryptophans from different loops in eIF4E sandwiched the 7-methylguanine group between them. This interaction gives rise to a strong exciton coupling signal between the two tryptophans that can be visualized by CD spectroscopy. eIF4E is a challenging protein to work with because of a propensity to aggregate under conditions used in biophysical techniques. CD spectroscopy provides a gentle, solution-based approach to study binding to the cap-binding site of eIF4E. Evidence is provided that the exciton coupling signal can be used to both qualitatively and quantitatively analyze the binding of cap analogs to eIF4E.

A key rate-limiting step in eukaryotic translation is attachment of the mRNA to the 43S translation initiation complex, a process that is facilitated by the heterotrimeric eukaryotic initiation factor 4E (eIF4F) complex [1]. eIF4E is the core component of the eIF4F complex that recognizes and recruits mRNA to eIF4F through a distinct moiety, termed the "cap," which is located at the 5′ end of the transcript [2]. eIF4E has been the subject of an intense amount of interest within the past decade as an oncology target [3–7]. The oncogenic potential of eIF4E is associated with an increase in its activity through elevated expression levels and/or inactivation of the 4E-binding proteins that bind to and inhibit eIF4E [6]. One potential effect of hyperactivity of eIF4E is an increase in the translation of a subset of mRNAs whose products are implicated in proliferation, regulation of apoptosis, angiogenesis, and metastasis [6]. The cap-binding pocket has therefore become a target for the development of anti-cancer drugs that inhibit the activity of eIF4E [8–10].

eIF4E is a 217-amino-acid protein that folds into an eight-stranded antiparallel β-sheet with three α-helices that pack against the concave surface of the β-sheet (Fig. 1A) [11]. The binding pocket for the cap is on the opposite side of the β-sheet with respect to the α-helices and is formed by three loops. Comparison of eIF4E in the absence and presence of 7-methyl-GDP, a cap mimic, reveals distinct structural rearrangements in the three loop regions that occur upon cap binding [11,12]. Two key residues drive the change in structure: Trp 56 and Trp 102 (Fig. 1B). In the absence of 7-methyl-GDP the two tryptophans are exposed to solvent in loops that adopt multiple conformations. In the presence of 7-methyl-GDP the two tryptophans sandwich the 7-methylguanine group between them. The positive charge on the 7-methylguanine is proposed to significantly increase the stability of the interaction owing to cation–π interactions that occur with the aromatic indole rings of the two tryptophans [13]. Trp 102 also promotes formation of a short α-helix through contacts made from the aromatic face opposite the 7-methylguanine binding face with residues in another loop.

The binding of 7-methyl-GDP to eIF4E can be directly visualized by the changes that occur in the CD spectrum of eIF4E. Between the wavelengths of 200 and 215 nm there is a deepening of the minimum, indicating an increase in secondary structure. There is also a distinctive increase in the small maximum that is centered around 230 nm. The small maximum probably corresponds to exciton coupling between Trp 56 and Trp 102. The strength of the signal from exciton coupling is dependent on the proximity and orientation of the tryptophans, with the strongest signal being obtained when the aromatic rings are in parallel planes [14]. The interaction between the 7-methylguanine group and Trp 56 and Trp 102 fixes their position and orientation to maximize the exciton coupling effect and therefore gives rise to an increased maximum at 230 nm in the CD spectrum.

There are technical difficulties with working with apo-eIF4E as it shows a strong tendency to aggregate over time even at

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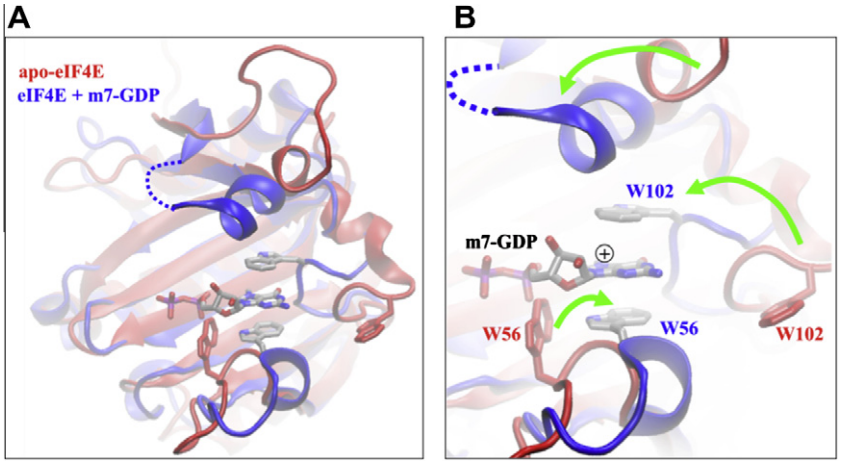

Fig.1. Structural rearrangement that occurs on 7-methyl-GDP (m7-GDP) binding to eIF4E. (A) Superposition of the apo-eIF4E and the 7-methyl-GDP-bound structure of eIF4E, represented in red and blue, respectively. 7-Methyl-GDP, together with the Trp 56 and Trp 102 side chains, are shown in a stick representation. The dashed line represents an unstructured loop region connecting two regions of eIF4E. (B) Close-up of the cap-binding site. The green arrows highlight the movement of loop regions and, in particular, Trp 56 and Trp 102 as the 7-methyl-GDP binds to eIF4E. The positive charge on the methylated N7 is indicated by a "+".

low-micromolar concentrations [15]. It is also sensitive to the mechanical process of mixing, which is an issue in common biophysical techniques such as isothermal titration calorimetry. CD spectroscopy provides a gentle solution-based approach to analyze binding to the cap-binding site. This paper provides evidence that the changes in the CD spectrum, in particular at 230 nm, can be used to both qualitatively and quantitatively study the binding of cap analogs to eIF4E.

Materials and methods

Protein expression and purification

The gene coding for eIF4E(28-217) was PCR-amplified and inserted into the vector pET28a using the restriction sites NcoI and XhoI. The vector was transformed into Rosetta (DE3) cells (Novagen). The cells were grown in TB medium at 37 °C until an OD (600 nm) of 0.6 was obtained. The temperature was reduced to 18 °C and the cells were induced with a final concentration of 1 mM isopropyl-β-D-1-thiogalactopyranoside. The cells were grown overnight and harvested by centrifugation. The cell pellets were resuspended in 50 mM Tris, pH 8, 500 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and lysed by passing through a microfluidizer (Microfluidics, Inc.). The suspension was centrifuged at 100,000*g* for 1 h and the supernatant discarded. The insoluble protein was resuspended in 8 M urea, 50 mM Tris, pH 8.0, 500 mM NaCl, and 1 mM DTT overnight at room temperature. The suspension was centrifuged at 100,000*g* for 2 h and the supernatant stored at 4 °C until use. The protein was refolded in batches to maintain a manageable final volume to load onto a cation-exchange column. For example, 30 ml of 10 mg/ml denatured protein was diluted to 0.25 mg/ml with 6 M urea, 20 mM Hepes, pH 7.0, 500 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.5 M arginine•HCl to give 1.2 L. This solution was dialyzed overnight at 4 °C into 15 liters of 20 mM Hepes, pH 7.0, 500 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.5 M arginine•HCl. The dialysis buffer was changed to 20 mM Hepes, pH 6.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT and the protein solution further dialyzed overnight at 4 °C. The final dialyzed solution was filtered and loaded onto two 5-ml HiTrap SP Sepharose FF columns. The running buffers for the column were as follows: buffer A, 20 mM Hepes, pH 6.5; buffer B, 20 mM Hepes, pH 6.5, 1 M NaCl. The column was preequilibrated with 10% buffer B. The protein was eluted using a linear gradient of 10–50% buffer B over 2 column volumes. The protein was dialyzed overnight at 4 °C into 20 mM Hepes, pH 7, 500 mM NaCl, 10% glycerol, 5 mM DTT, concentrated to between 2 and 3 mg/ml, and stored at −80 °C until use. The purity of the protein was analyzed by a Coomassie-stained SDS–PAGE gel and by passing over a Superdex 200 10/300 column (GE Healthcare) that was equilibrated in the dialysis buffer. The protein showed a tendency to precipitate after removal of the arginine, after elution from the column, during dialysis, and during concentration. Before being stored at −80 °C the protein was filtered. No precipitate was evident upon thawing the frozen samples. Before use, the protein was centrifuged for 5 min at 14,000 rpm. This was a preferred method as the protein showed a tendency to bind to spin filters. The final yields obtained after all steps and filtration were ~40 mg per liter of cells grown in TB medium and ~20 mg per liter of cells grown in LB medium.

CD spectroscopy

The protein was prepared for CD spectroscopy by dialyzing overnight into 10 mM phosphate, pH 7, 150 mM NaF, and 1 mM DTT. This solution is referred to as the "CD buffer." All data were collected on a Jasco J-815 CD spectrometer (Jasco, Inc.) equipped with a PTC-424S six-position Peltier-effect cell changer. The temperature was maintained at 20 °C for each experiment. To determine equilibrium dissociation constants from the titration experiments, five scans were collected using a 10-mm path-length cell. Otherwise three scans were collected using a 1-mm path-length cell. Each spectrum was collected with a scanning speed of 50 nm/min, data interval time of 1 s, band width of 1 nm, and data pitch of 0.1 nm. For each spectrum collected, the contribution from the solvent, and the solvent + cofactor where applicable, was subtracted from the final spectrum.

Comparison of cofactor binding to eIF4E(28-217)

To determine the effect of 7-methyl-GDP on the CD spectrum of eIF4E(28-217), four solutions containing 196 μl of 11 μM eIF4E(28-217) in CD buffer were prepared in 500-μl centrifuge tubes. To the first of these samples 4 μl of CD buffer was added to act as a control. To the remaining tubes, 1, 2, and 4 μl of 1 mM 7-methyl-GDP were added and made up to 200 μl by addition of CD buffer. The solutions were mixed by pipetting three or four times and incubating at room temperature for 20 min before transferring to four