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Rational Design and Biophysical Characterization of Thioredoxin-Based Aptamers: Insights into Peptide Grafting

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Peptide aptamers are simple structures, often made up of a single-variable peptide loop constrained within a constant scaffold protein. Aptamers were rationally designed by inserting peptides into a solvent-exposed loop on thioredoxin (Trx). They were designed to interact with the proteins elongation initiation factor 4E (eIF4E) and mouse double minute 2 (MDM2) and were then validated by competitive fluorescence anisotropy experiments. The constructed aptamers interacted with eIF4E and MDM2 with apparent K_d values of 1.25±0.06 μM and 0.09±0.01 μM, respectively, as determined by isothermal titration calorimetry (ITC). The MDM2 aptamer (SuperTIP) interacted ~2-fold more tightly with MDM2 than the free linear peptide (12.1 peptide), while the eIF4E aptamer elongation initiation factor 4GI-SG interacted ~5-fold less strongly than the free linear peptide (elongation initiation factor 4GI). These differences in binding with respect to each aptamer's free peptide reveal that there are more factors involved than just constraining a peptide in a scaffold that lead to tighter binding. ITC studies of aptamer interactions reveal an enthalpic component more favorable than that for the free linear peptides, as well as a larger unfavorable entropic component. These results indicated that stapling of the free peptide in the scaffold increases the favorable enthalpy of the interaction with the target protein. Thermostability studies also revealed that peptide insertion significantly destabilized the Trx scaffold by ~27 °C. It is this destabilization that leads to an increase in the flexibility of the Trx scaffold, which presumably is lost upon the aptamer's interaction with the target protein and is the cause of the increase in unfavorable entropy in the ITC studies. The precise origin of the enthalpic effect was further studied using molecular dynamics for the MDM2–SuperTIP system, which revealed that there were also favorable electrostatic interactions between the Trx scaffold and the MDM2 protein itself, as well as with the inserted peptide. This work reveals that any increase in the binding affinity of an aptamer over a free peptide is dependent on the increase in the favorable enthalpy of binding, which is ideally caused by stapling of the peptide or by additional interactions between the aptamer protein and its target. These need to be sufficient to compensate for the destabilization of the scaffold by peptide insertion. These observations will be useful in future aptamer designs.

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Abbreviations used: Trx, thioredoxin; eIF4E, elongation initiation factor 4E; MDM2, mouse double minute 2; ITC, isothermal titration calorimetry; TrxA, *Escherichia coli* thioredoxin; CDK, cyclin-dependent kinase; eIF4G, elongation initiation factor G; eIF4GI, elongation initiation factor GI; FAM, carboxyfluorescein; FUV, far-UV; NUV, near-UV; TRX, oxidized Trx; TRXH, reduced Trx; FITC, fluorescein isothiocyanate; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; PDB, Protein Data Bank.

Introduction

The identification of protein scaffolds, which can be used to present peptides *in vivo* in order to retain their biological activity, have been the focus of much research.[1,2] *Escherichia coli* thioredoxin (TrxA), a small bacterial protein (12 kDa), is a commonly used scaffold that contains a tight disulfide-constrained solvent-accessible loop (–CGPC–), which is responsible for its function.[3] This solvent-accessible loop contains a naturally occurring RsrII site that allows peptides encoding oligonucleotide cassettes to be annealed in. Recent studies have shown that the insertion of peptides into the active loop of TrxA constrains the peptides and displays them in a manner that retains the peptides' activity.[3–5] This, however, also results in inactivation of the natural activity of TrxA. The new functionality given to the thioredoxin (Trx) scaffold after peptide grafting provides a powerful research tool for the dissection of protein function within complex molecular regulatory networks.[2]

Trx itself is a highly rigid protein with a reported melting temperature (T_m) in the mid 80s (at 189 μM Trx at pH 7.0, T_m was reported to be 85.13 °C) for the oxidized form[6] and in the mid 70s (at 161 μM Trx at pH 7.0, T_m was reported to be 75.48 °C) for the reduced form.[7] The double constraint that results due to the two points of insertion of the peptide within the protein chain distinguishes peptide aptamers from other man-made combinatorial protein molecules; the latter often consists of random peptidic sequences fused terminally to a carrier protein or to some other macromolecule.[8] The aptamer design renders peptide aptamers less vulnerable to proteases and enhances the average binding affinity of the peptides for their targets, presumably by reducing the conformational freedom of the peptides and thus reducing the entropic cost of binding of the peptides to their targets. There are several examples of proteins that have been used as scaffolds in the literature (e.g., Trx, green fluorescent protein,[9] staphylococcal nuclease,[10] and Stefin A;[11] for more comprehensive reviews, see Binz *et al.*[12] and Skerra[13]).

The strategy of inserting peptides into the active site of Trx has also been adapted for the display of large random libraries of peptides against a variety of targets.[14] TrxA was functionally presented on *E. coli* flagellum,[14] where it was inserted into the dispensable region of the flagellin (*fliC*) gene. Based on the flagellin fusion system, random dodecapeptide sequences were engineered into the active loop of TrxA to create a library with up to 1.77×10^8 unique clones. The isolated peptides were able to map the epitopes of several antibodies,[14] revealing a distinct consensus sequence. This technique was also used against proliferating cell nuclear antigen[15] and revealed peptides that interacted with the naturally occurring peptide interaction site. LaVallie *et al.*[3] and Fabbrizio *et al.*[16] have used a similar system where the TrxA active-site loop has been replaced by a 20-residue random sequence, which was then used in a yeast two-hybrid system to isolate peptides selected for binding to human cyclin-dependent kinase CDK2 and E2F, respectively. The highest affinity peptide isolated against E2F mimicked a portion of Dimerization Protein 1 (DP1) as well as preventing their heterodimerization with each other. In the case of CDK2, several aptamers displaying different cross-reactivities with the CDK family were isolated, implying that different surface features on CDK2 were being recognized.

To investigate peptide aptamer design in this current study, we chose two target systems: the translation elongation initiation factor 4E (eIF4E) and the ubiquitin ligase mouse double minute 2 (MDM2). eIF4E interacts directly with the cap-modified 5′ end of eukaryotic mRNAs. The cap plays a key role in facilitating the binding of the ribosomal 40S subunit to the 5′ end of mRNA[17] through interactions with eIF4E and the rest of the eukaryotic translation initiation factor 4F complex. Formation of the eukaryotic translation initiation factor 4F complex is negatively regulated by 4E-binding proteins that block the interaction of eIF4E with elongation initiation factor G (eIF4G). eIF4E overexpression has been observed in numerous human tumors, and it also appears to be related to disease progression. Although eIF4E regulates translation globally, it contributes to malignancy by selectively enabling the translation of a limited pool of mRNAs that encode key proteins involved in cell proliferation, angiogenesis, survival, and transformation (e.g., cyclin D1, vascular endothelial growth factor, c-*myc*, and matrix metalloprotease 9).[18,19] The other target system MDM2 (also termed HDM2 in humans) negatively regulates the powerful tumor suppressor p53[20] through a feedback mechanism.[21] There are two possible mechanisms by which MDM2 binding can regulate p53 activity—either by[22] inhibition of the transcriptional activity of p53 or by degradation of p53 via the ubiquitin–proteasome pathway, both of which are mediated via an interaction with the N-terminal domain of p53 (residues 15–29). Activation of p53 in tumor cells, by inhibition of its physical interaction with MDM2, is a desirable point for drug intervention, as validated with the nutlin series of compounds.[20]

Using a combination of different experimental and computational studies, we develop a mechanistic understanding of the structural and thermodynamic effects of the insertion of the peptides both on the scaffold protein Trx and on the interactions of the peptides with their targets. By elucidating the biochemical and biophysical details of Trx aptamer binding, we hope to develop insights into the general principles of aptamer design and the feasibility of using it as a tool for investigating eIF4E-related and MDM2-related oncogenesis.

Upon insertion of these two linear peptides, Trx, which is normally a highly thermostable protein, undergoes significant destabilization. It seems that this destabilization of Trx allows these two peptides, which form different elements of secondary structure, to be presented in a manner by which they can