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Biophysical and functional characterization of the zinc hook domain of the Rad50 protein and its homologues

Abstract

Proteins utilize a large array of cofactors to achieve a variety of the structural and functional properties. Of all inorganic cofactors, transition metal ions play a unique role in proteins. Among all of the transition metal ions present in all domains of life, zinc (formally Zn(II)) is one of the most widespread, reflecting the utilization of Zn(II) by proteins for a wide variety of biological functions. The vast majority of known zinc domains, such as the zinc fingers and catalytic domains in enzymes, have an intramolecular binding architecture, in which all protein-derived zinc ligands are located within a single polypeptide chain. Alternatively, Zn(II) can be intermolecularly bound, i.e., by two or more peptide chains, bridging these molecules to form a protein assembly, as in the case of the Rad50 protein dimer. In Rad50 dimer, two pairs of cysteines from conserved CXXCs motifs in both subunits form a unique intermolecular Zn(II) complex known as zinc hook. Rad50 is a rod-shaped molecule compromised zinc hook and globular domains linked at a distance of ~500 Å by long and extended antiparallel coiled-coil. The zinc hook domain is conserved in Rad50 homologs identified in all forms of life, from bacteria to mammals and even viruses. Formation of the zinc hook complex is required for functional association of the Mre11–Rad50–Nbs1 complex which plays a key role in DNA damage response (DDR) by sensing and repairing DNA double-strand breaks (DSB). The goals of this PhD thesis were to characterize the molecular assembly mediated by the hook domain and identify factors governing its stability, apply the optimized hook domain as the tool for reversible Zn(II) dependent protein dimerization and gain insight into functional role of the formation of the zinc hook complex.

The first part of the present study was focused on the characterization of the structural and thermodynamic effects governing the formation and stability of the zinc hook domain from *P. furiosus*. In order to dissect structural determinants of the stability of the zinc hook assembly a series a of peptides of various lengths ranging from 4 to 45 amino acid residues, from the CPVC motif required for intermolecular Zn(II) binding to the full-length domain. In addition, an alanine substitutions and the replacement of the amide bond with an ester bond were introduced to address the influence of certain residues and hydrogen bond formation in the peptide backbone on zinc hook complex stability. Next the spectroscopic potentiometric

and calorimetric studies were performed to measure the stability of the Zn(II)-peptide complexes, obtain structural information, determine the acid-base properties of the peptides, and study thermodynamics of the complex formation. Using these analyses it was established that the hook domain demonstrates extremely high stability of the metal mediated-dimer ($\log K_{12}$ = 20.74) and extensive structural changes upon Zn(II)-binding. It was established that the >650 000-fold increase of the formation constant of the dimeric complex of the full-length domain compared with the isolated metal-binding motif of the domain arises from favorable enthalpy. This favorable enthalpy reflects a reduction of the unfavorable enthalpy of cysteine thiol deprotonation ascribed to the β-hairpin forming fragment and the favorable enthalpy of interactions in the β-hairpin and coiled-coil structures of the domain, which are formed upon metal binding. The observed dependence of the enthalpy on the domain fragment length is partially compensated by the unfavorable change in entropy due to metal-coupled folding. Presented data indicate that hydrophobic interactions and the formation of the β-hairpin metal-coupled folding and the stable assembly of the hook domain.

High stability of the complexes formed by the zinc hook domain led to its application as a small recognition tag for stable and reversible protein homodimerization and the sequence-stability analysis of the hook domain from *P. furiosus* was used to design minimalized hook tag. Such tag of the length of 14-aminoacids compromising the central part of the zinc hook domain with stabilizing R449A mutation was incorporated at the C-terminus of cyan (CFP) and yellow (YFP) fluorescent proteins. Next the fusion proteins were purified and examined using FRET and DLS studies. The dimerization studies showed that the purified hook-tagged proteins form stable Zn(II)-mediated dimeric complexes ($\log K_{12}$ = 19.2) and thus the minimalized zinc hook can be used as a small, efficient tag for reversible Zn(II)-mediated protein dimerization.

The last objective of the performed research was to gain insight into functional role of the formation of the zinc hook complex by characterization of the effect of the mutations within the hook domain of Rad50 from *S. cerevisiae*. These mutants designated as *rad50-46*, *rad50-47* and *rad50-48* were identified and studied using *S. cerevisiae* by the collaborators and displayed a various extent of defects in DSB signaling and DSB repair functions *in vivo*. Using circular dichroism spectroscopy, thermal denaturation and spectroscopic methods, it was established that the these hook mutants impaired formation of the intermolecular Zn(II) zinc hook complex and showed less evident metal-coupled folding upon Zn(II) binding. These results along with *in vivo* observations, suggest the translation of the structural alterations in the hook to the coiled-coil and 500 Å distant globular domain of Rad50. To

further study this issue, an intragenic suppressor mutations (L703F, K700Q, I680V) of *rad50-46* allele found by the collaborators in the vicinity of the hook were studied. To asses the mechanism of suppression, a method for obtaining fluorescent labelled 130 – amino acid long fragments of Rad50 protein from *S. cerevisiae* encompassing zinc-hook domain with adjacent fragments of the coiled-coil was established. The studies performed using these protein models indicate that suppressor mutations of *rad50-46* does not compensate for the Zn(II) binding defect but rather influence the conformation within the coiled-coil region. Furthermore, the ability of suppressor mutations found for *rad50-46,* which is severely defective in both in DSB signaling and repair to suppress *rad50-48* which is defective only in DSB signaling, was tested *in vivo*. It was found that that L703F, K700Q and I680V suppressor mutations also suppressed *rad50-48* allele. These data underline the importance of the stability of the hook mediated dimerization and coiled-coil conformation on the functions of Rad50 in DSB sensing.