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Kinetic Computational Alanine Scanning: Application to p53 Oligomerization

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We have developed a novel computational alanine scanning approach that involves analysis of ensemble unfolding kinetics at high temperature to identify residues that are critical for the stability of a given protein. This approach has been applied to dimerization of the oligomerization domain (residues 326–355) of tumor suppressor p53. As validated by experimental results, our approach has reasonable success in identifying deleterious mutations, including mutations that have been linked to cancer. We discuss a method for determining the effect of mutations on the location of the dimerization transition state.

Keywords: tumor suppressor p53; oligomerization; alanine scanning; ensemble unfolding kinetics; molecular dynamics simulation

Introduction

Tumor suppressor p53 is a "gatekeeper" of the genome, functioning at the center of a network of biological pathways that guard the cell from potential cancer. As a multidomain transcription factor, p53 is comprised of an N-terminal activation domain, a central DNA-binding domain, an oligomerization domain, and a C-terminal regulatory domain. The oligomerization domain enables p53 to adopt its biologically active tetrameric form. More than half of human cancers result from mutations in the p53 gene.[1] Although most of these mutations are in the DNA-binding domain, several studies have identified mutations within the oligomerization domain (p53tet) that are linked to increased incidence of cancer.[2–5] More cancer-associated mutations may yet be identified in p53tet, due to the fact that this region of the p53 gene was not sequenced in studies that searched for mutations in the p53 DNA-binding domain.[3]

Structures of p53tet have been determined by both NMR spectroscopy (Protein Data Bank 1PES[6] and 1SAK[7]) and X-ray crystallography (Protein Data Bank 1C26[8] and 1AIE[9]). This α/β domain, which is one of the smallest known protein oligomerization domains, assembles as a dimer of dimers.[10] Each dimer consists of an antiparallel β-sheet and a pair of antiparallel helices. The two dimers associate with one another to form the active tetramer *via* an extensive hydrophobic surface that is formed by the antiparallel helices of each dimer. Based on kinetics experiments and Φ-value analysis, tetramerization of p53tet is thought to involve induced-fit associations of monomers to form the dimer intermediates followed by "lock-and-key" associations of pre-organized dimers to form the tetramers.[10] A recent study of p53 biogenesis *in vitro* has shown that p53 dimerizes cotranslationally and then forms the tetramer post-translationally.[11] Given the kinetic advantage of dimerization before the p53 chains leave the polysome, mutations that affect the kinetics could play important biological roles. Consistent with the importance of kinetics in p53 function, molecular dynamics (MD) simulations of p53tet dimerization evolving from the rate-limiting transition state ensemble have revealed a nucleation-condensation mechanism in which L330, I332, and F338 from each monomer form a folding nucleus.[12]

Because of the structural symmetry of p53, point mutations in the oligomerization domain can reduce the stability of its tetramers significantly. For example, mutation of L330H in the folding nucleus is thought to disrupt the hydrophobic core of the dimers (Figure 1(a)) and has been linked to

Abbreviations used: p53tet, p53 oligomerization domain; MD, molecular dynamics; RMSD, root-mean-squared deviation.

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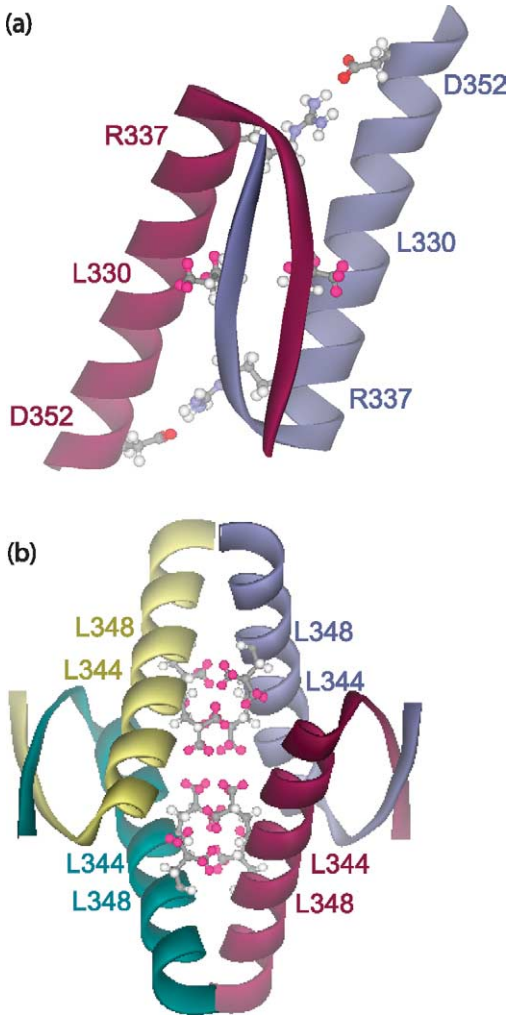

Figure 1. Amino acids that are mutated in the test set of mutant p53tet dimers: (a) L330 in the hydrophobic core of the dimer; R337, which forms a salt-bridge with D352 across the monomer–monomer interface; (b) L344 and L348, which are located centrally at the tetramer interface.

hepatocarcinoma and ovarian sarcoma.[1] As another example, an inherited mutation of R337C is associated with Li-Fraumeni-like syndrome (LFLS), in which the individual is predisposed to form a broad spectrum of tumors, including brain tumors, sarcomas, and adrenal cortical tumors.[3] Due to this mutation, the salt-bridges between R337 and D352 across the monomer–monomer interfaces no longer form; four such salt-bridges are lost in the tetramer (Figure 1(a)). The germline mutation of R337H also leads to loss of these salt-bridges and has been associated with pediatric adrenal cortical carcinoma (ACC).[5] Unlike many cancer-associated mutations in p53, this mutation is tumor-specific, leading only to pediatric ACC. Interestingly, malfunction of the p53 R337H mutant is pH-dependent, occurring only when the histidine residue, which has an elevated pK_a, is neutral as opposed to positively charged, when the mutant has near wild-type stability.[13]

Residues that are critical for the stability of the p53tet tetramer have been identified by systematically replacing nearly every residue in p53tet by alanine (alanine scanning) and experimentally measuring the effect on the stability of the tetramer.[14] The most critical of these residues are I332, L330, and F341, which lie in the hydrophobic core of the dimer; truncations of I332 in each of four monomers to alanine residues prevent folding while truncations of either L330 or F341 lead to folding at only high concentrations of protein or low temperature. Other critical residues are L344 and L348, which are located centrally at the tetramer interface; truncation of either residue leads to formation of stable dimers instead of tetramers (Figure 1(b)). To aid in the interpretation of folding kinetics data on p53tet, these mutants have been used as experimental models of the transient dimeric intermediates.[10] Residues that are strongly destabilizing when truncated ($\Delta\Delta G_u$ of 8.8–11.7 kcal/mol) lie in the periphery of the core (R337, F328, and F338) and at the tetramer interface (M340). Less critical, but still important ($\Delta\Delta G_u$ of 4.1–5.7 kcal/mol) are residues that are solvent-exposed (T329), involved in intermonomer hydrogen bonds (R333, N345, E349), or at the tetramer interface (A347).

To provide more efficient, alternative strategies for performing alanine scanning, computational approaches have been developed in recent years. The first computational alanine scanning study involved the MM-PBSA approach, in which molecular dynamics simulations with explicit water were used to generate relevant protein conformations and a continuum solvent model was applied to compute the free energies of the conformations.[15] Other computational mutagenesis studies involved the application of energy functions that were parameterized to reproduce experimentally measured changes in stability for a large database of mutations in proteins.[16,17]

While the computational mutagenesis approaches that have been developed thus far are based on thermodynamic analyses, the use of kinetic analyses can be effective as well. In particular, kinetic analyses can be used to identify thermodynamically destabilizing mutations if the mutations are also kinetically destabilizing, reducing the barriers to unfolding by destabilizing primarily the native, folded state of the protein. Indeed, it has been found for cancer-associated mutants of the p53 DNA-binding domain that, the more unstable the mutants, the faster they unfold.[18] With a few exceptions, destabilizing mutations to alanine residues in p53tet have little effect on unfolding rates of the tetramer, since the rate-limiting transition state to tetramer unfolding, which involves unfolding to the dimer intermediate, is suggested by Φ-value analysis to closely resemble the native tetramer.[10] However, these mutations may have a greater effect on unfolding rates of the dimer intermediate, since the transition state to dimer unfolding involves a transition state