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Structure/Function Studies of Human p53 Protein

by

Nicole Magnasco Nichols

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
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Doctor of Philosophy

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Abstract

Structure/Function Studies of Human p53 Protein

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Cellular p53 response, including apoptosis and cell cycle arrest, is reportedly due to "activation" of otherwise "latent" protein, a process that has been mimicked in vitro by the use of agents that interact with the p53 C-terminal domain. We have therefore undertaken a biochemical investigation of the wild-type protein and an "activated" C-terminal mutant (S392E) to characterize the in vitro structure and function of the "latent" and "activated" forms of human p53 expressed in Escherichia coli.

Differential reactivity was observed for mutant and wild-type p53 to the DNA binding domain antibody, Ab1620. Reactivity with Ab1620 is not directly correlated with p53 functional activity as previously thought, since loss of this epitope occurs at temperatures at which specific DNA binding can still be measured. Fluorescence studies established that tryptophans in wild-type and S392E p53 are quenched but DNA binding and potential conformational changes due to Ab1620 binding result in increased fluorescence signal.
Structure prediction algorithms indicate that the great majority of β-sheet structure occurs in the p53 core DNA binding domain. Circular dichroism spectra demonstrate significant stability for the wild-type protein, with a transition midpoint of ~73 °C. The “activated” mutant, S392E, displays increased stability with no detectable loss of β-sheet structure even at 100 °C. Increased α-helical structure is also observed for the mutant protein. The persistent β-sheet CD signal of both proteins correlates with significant DNA binding ($K_d \sim$nM range) to temperatures as high as 50 °C. Surprisingly, the S392E mutant showed no increase in affinity for specific DNA compared to wild-type p53, although binding was maintained to a higher degree over the temperature range investigated. Interestingly, in comparison with wild-type p53, the mutant protein displays increased affinity for nonspecific DNA and for a DNA target containing both specific and nonspecific sequences. These data confirm the thermostability of both structure and specific DNA binding for both the “latent” and “activated” proteins and suggest that activation of p53 may derive from alterations in nonspecific binding by the C-terminal domain.
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I dedicate this thesis to my father.
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CHAPTER 1

INTRODUCTION
Cancer is not a single disease. Human cancers take on many forms, have a wide variety of causes, and derive from nearly all types of tissue. Tumors can result from significant tissue damage (e.g., smoking, prolonged exposure to ultraviolet light), from viral infection (e.g., human papilloma virus), or from genetic defects that result in the misregulation of key genes involved in cell growth or DNA repair pathways (1). In addition, cancer mortality and prevention rates vary dramatically depending on the cellular origin of the tumor (Table 1.1). For example, although a diagnosis of a lung carcinoma brings with it a low chance of survival (~16%), this is one of the most preventable forms of cancer, with smokers making up nearly 8 out of 10 patients (1).

The complexity surrounding the causes of malignant human tumor formation is equaled by the difficulties in effectively treating these diseases. To find cures for cancer, efforts are being made to determine similarities among different cancers that may be exploited for treatment. Regardless of the origin of a human cancer, certain properties are ubiquitous, including rapid cellular growth and division unchecked by the normal mechanisms of the cell. This growth is often the result of mutations in genes that are
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<sup>a</sup>Statistics published by the National Cancer Data Base ranging from 1985 – 1996.  
<sup>b</sup>Survival rate is expressed as a percentage of patients living 5 years post diagnosis and is adjusted for the normal life expectancy for patients of the same age, gender, and race/ethnicity.  
<sup>c</sup>Staging is according to the American Joint Committee on Cancer and describes the severity and degree of metastasis of a primary tumor. Stage IV describes the severest form of disease and is characterized by extensive spreading.
responsible for maintaining and regulating cellular and DNA integrity (1). The best known of these genes is p53. In the fight against cancer, a functional p53 protein is one of the body’s most significant warriors.

The discovery of p53 was much less ostentatious than its current moniker, ‘Guardian of the Genome’ (2), would imply. The discovery of a protein with an apparent molecular weight of 53 kDa was published by a number of laboratories nearly simultaneously, and results suggested two possible modes of action, both rather inauspicious. p53 protein was isolated bound to simian virus 40 (SV40) large T antigen and later to adenovirus E1B, both oncoproteins that aid in the neoplastic transformation of otherwise healthy cells (3-5). Shortly thereafter, isolates of this 53 kDa protein were combined with activated Ras protein and discovered to increase transformation of rodent cells (6-8). It was therefore not surprising that p53 was classified as an oncoprotein, a protein capable of causing tumor formation.

It was not until 1988, nearly 10 years after its discovery, that p53 was vindicated and its true function realized. Frequent inactivation of p53 in murine Friend virus-induced erythroleukemia cells led Ben-David and colleagues to hypothesize that p53 was in fact a tumor suppressor, a protein
whose normal function is the prevention of tumors but whose inactivation can lead to transformation (9). Soon after, transfection experiments demonstrated that the original p53 clones, capable of cell transformation, all contained mutations and that the wild-type protein suppressed transformation (10, 11). Both the gene and protein are well conserved throughout evolution and have been isolated from many species, including humans, monkeys, mice, *Xenopus laevis*, and most recently from *Drosophila melanogaster* (12-19). To date, no homologue of p53 has been found in either yeast or *E. coli*.

Human p53 is located on the short arm of chromosome 17 (17p13.1), spans approximately 20 kb of genomic DNA, and contains 11 exons and 10 introns (20) (Figure 1.1) that are largely conserved in both the murine and *Xenopus* genes (21, 22). The location of the p53 gene was found to correspond to the portion of chromosome 17 that is frequently deleted in many types of cancer cells. In order for cells to progress through meiosis and mitosis uncontrolled and unchecked, p53 must first be inactivated. Gross deletion of a large portion of chromosome 17 is one way to accomplish this inactivation. A variety of changes that silence p53 have been identified in cancer cells, including gene deletion, mutation, or
Figure 1.1 Human p53 gene, mRNA and protein. The human p53 gene is located on chromosome 17 and contains 11 exons and 10 introns with a large first intron. The mRNA transcription starts from within the second exon and yields a 2.8 Kb product. The 393 amino acid p53 protein is comprised of multiple segments and includes charged termini and a hydrophobic region of PXXP motif repeats (reviewed in 69).
improper protein localization (23-25). p53 mutations are frequent occurrences in human tumors and on average are found in ≥50% of all human tumors, although the exact percentage varies among tumor classes (26, 27). The percent of human tumors that are connected to p53 inactivation is estimated to be even more significant, since mutations in other genes in the p53 pathway (MDM2, p21^{waf1/cip1}) also occur (28, 29). A current understanding of the identity of the genes upstream and downstream of p53 is depicted in Figure 1.2.

The occurrence of p53 mutation in each type of cancer can be examined by accessing an extensive p53 database maintained by the World Health Organization's International Agency for Research on Cancer (30). p53 mutational information is catalogued in order to achieve a greater understanding of this tumor suppressor protein. For a disease that shares limited similarities from one person to the next, one hope for a cure rests upon exploiting any common factor of tumorigenesis. The interest in understanding the mechanisms of p53 and how its pervasive role in tumor formation can be exploited in cancer treatment can be seen in the immense volume of literature on the protein – 3744 publications in 1998 alone (31).
Figure 1.2 Diagram of p53 effectors. This schematic represents only a portion of the genes that have been shown to be involved in p53 pathways. p53 interacts directly and indirectly with products of both oncogenes (red circles) and tumor suppressor genes (green boxes). The pathways upstream of p53 have only been defined recently and rely on DNA damage sensing proteins like ATM to signal p53. Though only activation is diagrammed here, p53 is also able to repress transcription (adapted from 195).
Li-Fraumeni Syndrome

Mutations in p53 are most often somatic in nature, but families who carry germline mutations have been reported. Investigating the incidence of rhabdomyosarcomas in children, Frederick Li and Joseph Fraumeni discovered an abnormally high occurrence of cancers in other family members of the affected children (32, 33). In particular, the incidence of sarcomas, breast cancers, and leukemias were elevated and often occurred at an abnormally young age. Individuals with this inherited Li-Fraumeni syndrome (LFS) have a 50% incidence rate of cancer by the age of 30, compared to an approximately 1% chance in the general population. Li-Fraumeni families are identified through a strict classification procedure that describes relationship to the primary patient, the cell type of tumor origin, and the age at diagnosis (reviewed in 29).

In approximately 65% of the 100 or so families identified by the strict criteria of LFS, a germline mutation of one allele of the p53 gene was found to be the cause of the syndrome (34). Individuals within these families were discovered to carry one wild-type copy and one mutated copy of p53 (35, 36), although individuals with one mutated copy and one deleted allele have also been reported. As for the other 35% or so of LFS families, a number of
complexities surrounding genetic testing prevent a definitive statement about the presence or absence of a germline p53 mutation. There is now, however at least one example of a classic LFS family with two wild-type germline alleles of p53. The Lozano group sequenced the entire p53 gene as well as all known regulatory sequences to establish that no mutation in any region of p53 was responsible for the inherited disposition to cancer in this family (37). It is, however, entirely possible that this family carries a mutation in a gene either upstream or downstream of p53.

The p53 Knockout Mouse

The creation of a p53 null mouse by Donehower and Bradley in the early 1990’s resulted in a clarified and unpredicted view of p53 loss of function (38). The p53 gene was disrupted by gene targeting, and standard genetic crosses generated null mice with no intact p53 mRNA or protein. The ability of these mice to grow to adulthood revealed that p53 is not crucial to development, a fairly surprising result. Interestingly, effects of the p53 gene deletion soon became noticeable. By the age of 6 months, 74% of the homozygous null mice had developed tumors, and by 10 months every one of these mice had developed tumors or died. Heterozygous mice
containing only one copy of the wild-type gene developed tumors at a much slower rate than the null mice but still exhibited a significantly elevated incidence of tumor formation (38). These heterozygous mice have since served as a useful model for families with Li-Fraumeni Syndrome.

*p53 – Modes of Action*

p53 is involved in three major cellular pathways that interact to prevent tumor formation and maintain the integrity of the genome (Figure 1.3). In response to cellular stress, including DNA damage or nucleotide deprivation, p53 accumulates and can then initiate transcription of p21\(^{wafl/Cip1}\) which inhibits the Cdk complexes necessary for entry from G1 into the S phase of the cell cycle (39-43). Increased levels of p21\(^{wafl/Cip1}\) result in cell cycle arrest at the G1/S checkpoint and presumably stall the cell cycle long enough for repair to take place. Alternatively, p53 can upregulate the expression of the *bax* gene whose product dimerizes with Bcl-2 to elicit apoptosis (programmed cell death) to prevent the propagation of DNA mistakes (43-46). p53 also has been shown to regulate cellular replication. Its specific DNA binding activity and transcriptional activation increases, independent of any increase in protein levels, when cells reach the end of
**Figure 1.3 Modes of p53 action.** In response to cellular stress, p53 levels are increased and the protein is activated. Once activated, p53 signals the cell to undergo apoptosis or cell cycle arrest, potentially using the extent of DNA damage sensed through posttranslational modifications as a deciding factor on which of these two pathways is activated. Cell cycle arrest in the G1 gap is believed to stall the cell for time to repair DNA before continuing through the cycle. Mutation or inactivation of this protein can lead to tumor formation by uncontrolled cell growth and unchecked DNA replication. p53 has also been demonstrated to affect tumor cell senescence (reviewed in 47).
Normal cell

p53

DNA damage/stress

p53 mutation or deletion

Cell division with damaged DNA

Loss of cell viability due to DNA damage

Increased p53 levels (activation of p53)

p21(waf1/cip1)

Cell cycle stop at G1/S

S phase

Cyclin E Cdk2
Cyclin A Cdk2

Transcription

Bax/Bcl2

Programmed cell death (apoptosis)

Senescence
their replicative life span (48-50). More recently p53 has been shown to impose irreversible senescence upon cancer cells, although this mode of action is less well understood (51).

Normally low, p53 protein concentrations are increased in response to signals of DNA damage or stress, including hypoxia and nucleotide depletion (40, 42, 52-55). This increase is not due to upregulation of transcription but instead to a decrease in degradation rate. In undamaged cells, rapid degradation of p53 is largely accomplished by the MDM2 protein through the ubiquitin-dependent pathway (56-60). MDM2 appears to translocate p53 out of the nucleus into the cytosol where degradation takes place (61-63), although recent research demonstrates that nuclear export is not required for MDM2-mediated degradation (64).

MDM2 is a p53-specific E3 ubiquitin ligase (65), and its N-terminus interacts with a short region in the N-terminus of p53. This region of p53 has been demonstrated to fold into an α-helix upon MDM2 binding (66). MDM2 participates in an autofeedback regulation loop with p53 (67) (Figure 1.4). p53 protein binds to a p53-responsive element in the first intron of MDM2, upregulating transcription of the MDM2 protein. MDM2
Figure 1.4 MDM2-p53 autoregulatory loop diagram. MDM2 acts both directly and indirectly in the degradation of p53. p53 activates transcription of the MDM2 gene by binding to a regulatory site in intron 1. Once sufficient levels of MDM2 are present, it binds p53, and transports it to the cytosol where degradation occurs. Nuclear export and cytosolic degradation via the ubiquitination pathway (of which MDM2 is a member) decrease the pool of free p53, thereby downregulating transcription of MDM-2 until p53 levels are increased again. However, recent experimental results demonstrate that nuclear export is not required for MDM2-mediated p53 degradation (reviewed in 67).
then binds to p53, inactivating its ability to promote transcription and therefore downregulating MDM2 protein production.

*The p53 Protein*

p53 is a 47 kDa nuclear phosphoprotein of 393 amino acids, (Figure 1.5) with an acidic N-terminus and a basic C-terminus (68). A hydrophobic region containing PXXP motif repeats is located in the first half of the protein (69). p53 is able both to activate and repress transcription of genes involved in cell cycle progression, apoptosis, senescence, and DNA repair as well as genes whose products interact with p53 itself (Figure 1.6) (70, 71). The acidic N-terminus of p53 is responsible for its function as a transactivation factor, but the domain involved in repression has still not been identified. The N-terminus interacts with members of the basal transcription machinery including the TATA box binding protein (TBP), TBP – associated factors (TAFs), and transcription co-activators, CBP and p300 (reviewed in 72).

By proteolytic digestion, the core (residues 102-292) was established to be an independently-folded domain of p53 capable of specific DNA binding (73). The core domain has also been shown to exhibit exonuclease
MEEPQSDPSVEPPLSQETFSDDLWKLLPENNVLSPLPSQAMDDMLLSP
DDIEQWFTEDPGPDEAPRMPEAAPVAPAPAAAPTAPAAPAPAPAPSWPLS
SSVPSQKTYQGSYGFRLGFLHSGTAKSCTYSPALNKMFQCLAKT
CPVQLWVDSTPPPGTRVRAIAIKQSQHMTEVVRRCPHHERCSDS
GLAPPQHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIH
YNYMCNSSCMGGMNNRPILTITLEDSSGNLLGRNSFEVRVCACPGR
DRTTEENLRKKGEPHELPPGSKRALPNNTSSSPQPKKPKLDGEY
FTLQIRGRRERFEMFRELNEALELEKDAQAGKEPGGSRAHSSHLKSKKG
QSTSRHKKLMFKTEGPDS

**Figure 1.5 Amino acid sequence of human p53.** The 393 amino acid sequence of human p53 is depicted in single letter code. Basic residues are colored magenta (arginine, R; lysine, K; histidine, H) and acidic residues (aspartate, D; glutamate, E) green. The prolines (P) involved in the N-terminal repeats of the PXXP motifs are colored blue. Shown underlined in red is the CKII target serine, serine 392.
Figure 1.6 Domain organization of p53 shown on the linear amino acid sequence. The DNA binding domain (DBD) comprises approximately 50% of the protein. Roman numerals indicate the conserved regions of p53, most of which lie in this domain. Vertical lines represent the frequency of mutation found in human cancers at the indicated residue and highlight the importance of the DBD. Functions known to require specific portions of the protein are detailed in colored type under the appropriate domain. Little is known about the function of the SH2 signalling domain in p53.
activity, implying that p53 may not only be able to activate repair but also to participate in it directly (74). This domain is the largest within p53 and contains four of the five conserved regions of the protein and the majority of the mutation hotspots (73). Hotspots are amino acid positions whose DNA codons are most often found mutated in cells isolated from human tumors. Mutations in the p53 gene can vary in occurrence from one type of cancer to the next, and some have been linked to very specific environmental agents. For example, GC to TA transversion in codon 249 is fairly common in liver cancers in countries with high incidences of aflatoxin B_1 (associated with poor food storage) but is not common in liver cancers where the exposure to aflatoxin B_1 is low (75, 76). Similarly, ultraviolet light and the carcinogens in tobacco smoke including 3,4-benzopyrene leave signature mutations on the p53 gene, solidifying their role in tumor formation (77). Additionally, mutations in most Li-Fraumeni families map to the DNA binding domain of p53 between codons 245 and 258, with a hotspot at codon 248 (35, 36).

The C-terminus has been shown to mediate two distinct functions: oligomerization (78, 79) and the regulation of DNA binding (80-82). Residues 364-393 regulate DNA binding, whereas residues 322-355 are required for oligomerization (80, 83-85). The C-terminus is also capable of
binding single-stranded DNA, RNA, and non-specific double-stranded DNA, as well as participating in DNA/RNA strand exchange (86-89). Recently, this domain has been implicated in modulating transcriptional activation and apoptosis based on effects of C-terminal mutants (90).

**Protein Structure**

Crystallographic and NMR structures of p53 fragments/domains have aided efforts to understand its participation in multiple cellular processes. To date, structural information about four distinct regions of p53 have been published, though a complete picture of the structural arrangement of the intact protein remains elusive. From the N- to C-terminus these regions include a small portion of the transactivation domain, the majority of the DNA binding core domain, the tetramerization domain and most recently, the C-terminal regulatory domain.

The N-terminal transactivation domain of p53, with a net charge of negative 17, is a member of a family of acidic activation domains (AADs) (91, 92) that are inherently devoid of tertiary structure (93). In the entire N-terminus of p53 (~80 residues) the structure of only a small peptide, residues 17-27, co-crystallized with MDM2, has been solved (66) (Figure 1.7). This
Figure 1.7 The crystal structure of an N-terminal p53 peptide bound to MDM2. Residues 17-27 of p53 (yellow) were crystallized bound to an N-terminal fragment of the MDM2 protein (lavender) (66). The N-terminus of p53 shares similarity with a group of acidic transactivation domain proteins that are loosely structured. It has been suggested that this p53 peptide is only helical in the presence of MDM2 and that the entire N-terminus of p53 is fairly flexible (95). This and all structural figures (unless otherwise noted) were drawn in Ribbons v. 2.63 (94).
region folds into a small α-helix in the presence of an MDM2 peptide, shown in Figure 1.7 as a Ribbons diagram (94). Due to the low levels of p53 present in an undamaged cell, its rapid degradation, and the hydrophobicity of this domain, it is reasonable to conclude that p53 is continually bound by MDM2 in vivo. Recent NMR studies of the entire AAD reveal that this helix is moderately populated in the unbound form of the protein and is also accompanied by two nascent turns spanning residues 40-44 and 48-53 (95).

Residues 94-289 of the DNA binding core domain (DBD) of p53 were crystallized in the presence of DNA, and the structure – by far the most illuminating of the four – was solved to 2.2 Å resolution (96). This protein-DNA complex (Figure 1.8) reveals a mainly β-sheet sandwich containing 4 and 5 β-strands that form two β-sheets and a small loop-sheet-helix motif that binds in the major groove of DNA. The DBD tetrahedrally coordinates one zinc atom through three conserved cysteine residues, Cys 176, Cys 238, and Cys 242, and one histidine, residue 179 (96). Oxidation of p53 renders the protein inactive, suggesting the importance of Zn$^{2+}$ coordination for function (97, 98). Denaturation studies monitoring zinc presence in the
Figure 1.8 Crystal structure of monomeric p53 core DNA binding domain and a conDNA half-site. Residues 94-289 are shown complexed to a consensus DNA half-site at 2.2 Å resolution (96). Helix 2 and Loop 1 make direct contacts within the major groove of the DNA, and Loop 3 makes contacts with the minor groove. Shown in orange is a Zn$^{2+}$ atom required for structural integrity. The DNA is depicted in ball and stick form surrounded by a representation of each atom's van der Waals radius. The extensive β-sheet structure of the core is unusual for a DNA binding protein and illustrates how numerous mutations throughout this domain could propagate structural effects that would abrogate binding.
protein reveal a bound Zn\textsuperscript{2+} molecule even in the unfolded protein, confirming the importance of this metal to the structure of p53 (99).

The structure of the p53 DBD resembles that of a C-type immunoglobulin fold (100). The immunoglobulin-like fold is one of the most commonly found structures in animal proteins, yet p53 and NF-κB are the only two DNA binding proteins known to exhibit this fold. An overlay of p53 and NF-κB reveal that 43 carbon-alpha positions in the N-terminus of the p50 domain of NF-κB match to the corresponding positions of the fold in p53 with an RMSD of 1.6 Å (100, 101).

NF-κB, like p53, is a transcription factor. The extensive β-sheet domain shared by both proteins is unlike most traditional DNA binding domains that are composed of single small regions of high α-helical content. The β-sheets of the immunoglobulin-like fold provide extensive scaffolding on which a number of unstructured loops are positioned to make specific DNA contacts (96, 100, 102). This scaffolding suggests a reasonable structural explanation for the elimination of DNA binding activity by numerous mutations throughout the entire core of p53 (103-105). Conservative changes in residues not directly bound to DNA are able to disrupt the β-sheet structure, presumably misaligning the crucial protein-
DNA contacts (102). Two distinct types of common p53 DNA binding mutants are now classified by structural involvement: proteins with mutations in residues that make direct DNA contacts are Class I mutants, whereas Class II mutants involve residues in the core domain that do not directly contact DNA (96, 106). The Class II mutants are reported to have a dominant negative phenotype in vivo (107). This behavior has been linked to the ability of the mutants to form oligomers with the wild-type protein, creating tetramers unable to bind DNA or regulate cellular proliferation (103, 107-110).

Both X-ray crystallographic (111) and NMR structures (112-114) of residues 319-360 of the protein have demonstrated the presence of an \( \alpha \)-helical oligomerization domain (Figure 1.9, Panel A). This domain forms an anti-parallel 4 helical bundle in solution, providing structural evidence that confirms biochemical observations that p53 forms a tetramer (81, 115, 116). Tetrameric p53 may be organized as a dimer of dimers, with contacts from one set of monomers greater than contacts between the dimers (1543 Å\(^2\) vs. 1270 Å\(^2\)), implying that dimerization may be required before tetramerization (111, 113, 117). The tetramerization domain is very stable as a peptide, with a melting temperature (\( T_m \)) of 75-80 °C (118, 119).
Figure 1.9 X-ray/NMR structures of C-terminal p53 peptides. A. Both X-ray crystallographic and NMR structures reveal a tetrameric oligomerization domain for p53 (111, 113, 114). Each monomer (colored individually) is composed of a small β-sheet followed by an α-helix. Interactions between these antiparallel helices reveal that p53 is likely oriented as a dimer of dimers (red and yellow vs. blue and green). The N-terminal ends of each monomer are labeled for orientation. B. The fourth domain of the protein that has been accessible through structure determination methods is a small fragment of p53 that is C-terminal to the tetramerization domain (Figure 1.9, Panel B). Residues 376-387 are unstructured as a free peptide but form an α-helix upon binding to the S100B(ββ) protein dimer (120). Only one monomer is shown.
Though much has been learned from these domain structures, an integrated view of the intact protein is lacking. Because little is known about the orientation of one domain to another, speculation abounds concerning possible interactions between each of the domains within the monomer. Most notably, the C-terminus has been postulated to contact, or at least crowd, the DNA binding domain, and biochemical data supporting contacts between the N- and C-termini have also been presented (106, 121, 122). It is unclear whether the contact between monomers is limited to the tetramerization domain or if it occurs between other domains of p53.

Posttranslational Modifications

The role of various posttranslational modifications on the function of the p53 protein is a subject on which there is nearly constant debate and new information (reviewed in 123). Modifications on p53 appear to be concentrated at both ends of the protein with little to no modification within the DNA binding core domain (Figure 1.10). To date, there are reports of seven serines and one threonine in the N-terminus and five serines in the C-terminus that have been shown to be phosphorylated either in vitro or in vivo (124, 125). Additionally, acetylation has been reported at four C-terminal
Figure 1.10 Phosphorylation and acetylation sites of p53. Post-translational modifications of the p53 protein are concentrated to each of the termini, with no known modifications reported to occur in the DNA binding domain. Seven N-terminal and five C-terminal serine residues as well as one N-terminal threonine are reportedly phosphorylated either in vitro or in vivo. Acetylation occurs on three C-terminal lysine residues in vitro (reviewed in 47, 123). Shaded in blue is the CKII phosphorylation site, serine 392, discussed in Chapters 4 and 5.
lysines (123). There is also one report of O-glycosylation at the C-terminus of p53 in a cell line derived from a colon carcinoma (126), though a lack of similar findings in other cell lines make this an unlikely candidate for a universal form of regulation.

The debates surrounding p53 posttranslational modification have a number of origins, the most significant of which concerns the method of phosphorylation detection in vivo. Common methods of detecting protein phosphorylation include radiolabeling phosphates, but the energy of the radioactivity itself can induce DNA damage, which in turn can activate p53 and result in posttranslational modifications of the protein (47). The development of specific phosphoprotein antibodies (127-129) has made in vivo detection more reliable, though many of the modifications have still only been investigated in vitro.

There are four specific cases of p53 posttranslational modification that merit more investigation: MDM2 inhibitory phosphorylation (Ser 15 and Ser 37), damage-induced dephosphorylation (Ser 376), DNA binding activation (acetylation and Ser 392 phosphorylation), and UV vs. IR effects. In the case of MDM2 inhibitory phosphorylation, modification of p53 by DNA-PK, a DNA activated protein kinase that targets Ser 15 and Ser 37 in vitro
(130), is capable of inhibiting the interaction of MDM2 and p53 (127). Ser 15 phosphorylation has been demonstrated in vivo to be stimulated by DNA damaging agents, providing evidence for one possible mechanism (release of degradation) responsible for the increase in p53 protein levels upon DNA damage (127, 128).

The phosphorylation status of Ser 376 is intriguing, as it is the only residue that appears to be dephosphorylated in response to DNA damage (131). Both Ser 376 and Ser 378 have been reported to be constitutively phosphorylated in undamaged cells, but Ser 376 appears to undergo dephosphorylation in an ATM-dependent manner in response to DNA damage. ATM is a protein involved in ataxia-telangiectasia (AT), a degenerative disease that predisposes children to malignancies of the blood system. ATM appears to be a crucial protein messenger, relaying the presence of DNA damage to p53 by dephosphorylation at this serine as well as phosphorylation of MDM2 (132-134).

DNA binding activation has been reported as a result of both C-terminal acetylation and phosphorylation of Ser 392, the penultimate residue (80, 135). Ser 392 is conserved in all sequenced mammalian p53s and can be phosphorylated in vitro by CKII (125). Mutation of the murine protein at
the homologous CKII site (serine 389) to alanine generated a protein
defective in suppressing cell growth in a transfection assay, implying an *in vivo* role for CKII phosphorylation at this site (136). The increase in DNA
binding activity seen in response to the acetylation of either Lys 320 or Lys
382 as well as the phosphorylation of Ser 392 was described in the presence
of long competitor DNA molecules (137, 138). These results are but a few
examples of a growing number of investigations that are attempting to
clarify the role of the C-terminus in DNA binding and will be discussed in
more detail in both Chapters 3 and 4.

The last point to mention about p53 posttranslational modification
concerns the response patterns that arise from different modes of inducing
DNA damage. Reportedly, IR-induced DNA damage results in p53
phosphorylation in a different pattern than UV-induced DNA damage,
particularly at serine 20 (47, 139, 140). It has been shown extensively that
p53 can elicit distinct responses in the presence of DNA damage (cell cycle
arrest, apoptosis, and senescence), but this is the first evidence describing a
possible mechanism by which a pathway could be specified. These
examples of the effects of posttranslational modifications on the function of
the p53 protein emphasize why an *in vitro* investigation of p53 function
must be undertaken with a clear understanding of protein modification status. For this reason, studies described herein have utilized recombinant human p53 protein purified from bacterial cells. *E. coli* lacks the enzymes that have been reported to modify p53, thus providing a model for what has been termed the “latent” form of the protein.

*p53 Monoclonal Antibodies*

Monoclonal antibodies (Abs) are powerful tools commonly used for protein detection and identification during purification, but antibodies specific to p53 have had another interesting use. *In vitro*, protein denaturation is sufficient to abolish reactivity with a subset of these antibodies (141, 142). In addition, a temperature sensitive form of murine p53, later identified as an alanine to valine substitution at amino acid 135, was demonstrated to react with one subset of antibodies at 37 °C and a second subset at 30 °C (108, 143, 144). From these experiments, antibodies to p53 were hypothesized to exist in two major groups: those that recognized stable epitopes (*i.e.*, always reactive with p53, even after denaturation), including Ab421 (145) and DO-1 (146), and those that recognize unstable epitopes (*i.e.*, dependent on the conformation of p53), including Ab1620
(147, 148) and Ab240 (142, 149). Interestingly, epitopes in the N- and C-termini of p53 are frequently conformation-independent, and antibodies to these regions are useful for Western blotting. In contrast, the epitopes of the crucial DNA binding domain of p53 appear to be created by a flexible protein structure (Figure 1.11).

Functional conclusions have been drawn by the reactivity of p53 to antibodies that target regions of the DNA binding domain. p53 was hypothesized to exist in two distinct conformations – a wild-type conformation (Ab1620+/Ab240+) able to suppress cellular proliferation and oncogene mediated transformation and a mutant conformation (Ab1620-/Ab240+) that is believed to promote these activities (107, 108, 143, 144). Reactivity profiles to the conformational antibodies (Ab1620 and Ab240) have also been linked to DNA binding activity in vitro (150). Proteins with Ab1620+/Ab240+ reactivity have been shown to be capable of specific DNA binding, whereas proteins with Ab1620-/Ab240+ reactivity, either by mutation or thermal unfolding, have been linked to a loss in function (108, 144, 151-153). Experiments in Chapters 3 and 4 will investigate the reactivity of both "latent" p53 and an activated mutant as well as the validity of functional deductions based on reactivity with these structural probes.
Figure 1.11. Epitopes of commonly used p53 monoclonal antibodies.
Four commercially available p53 antibodies are illustrated against a linear protein sequence (conserved domains are shown in roman numerals for orientation). The exact epitope of each antibody (if known) is shown in parentheses. Antibodies to the N-and C-termini are conformationally independent, in contrast to the core domain antibodies. Ab240 recognizes an epitope available in unfolded or denatured p53 whereas the Ab1620 epitope is presumed available only in native p53 (reviewed in 150).
DNA Binding

The importance of DNA binding can be easily underscored by the significant percentage of mutant p53 proteins isolated from human tumors that no longer possess this function (26, 27). As a transcription factor, p53 is responsible for binding to sites within promoter or intron regions of numerous genes, including \( p21^{wafl/cip1} \), \( gadd 45 \), and \( MDM2 (41, 154, 155) \), termed p53 response elements (p53RE). p53 is capable of both activating and repressing transcription from these sites, and the human genome is estimated to contain as many as 200-300 p53REs (156).

An examination of over 100 different naturally occurring p53REs helped to define a p53 consensus sequence, termed p53CON or conDNA (157). ConDNA is two copies of \( 5'-PuPuPuC(A/T)\bullet(T/A)GPyPyPy-3' \) separated by up to 13 bases where \( Pu \) is either adenosine or guanine and \( Py \) is cytosine or thymidine (157). Funk and colleagues’ investigation of p53 sequence-specific binding generated a similar result using degenerate oligonucleotides (158). Additional studies have undertaken mutations at each site to verify specificity (82).

Not surprisingly, based on a history of misinterpretation, the C-terminus of p53, rather than the DNA binding core domain, was first
identified as the domain responsible for DNA binding. In an assay with
digested genomic DNA, deletion of the C-terminus of p53 resulted in a
dramatic decrease in detectable binding (104). These data are now
understood to have been a result of nonspecific binding events at the C-
terminus. After the identification of a number of p53REs, Bargonetti and
colleagues correctly identified the digestion-resistant core domain as
responsible for sequence-specific DNA binding using proteolytic digestion
experiments (159).

p53 appears to bind conDNA as a tetramer, and one tetramer is able to
engage the entire consensus sequence (96, 160). The conDNA sequence is
composed of four quarter sites or two half sites, oriented as seen in Figure
1.12, Panel A. Monomers of p53 have been shown to bind to quarter sites (5
nucleotides), and although a single half site can be recognized by tetrameric
p53, it is not sufficient to allow wild-type levels of transactivation to occur
in an in vivo luciferase-based assay (160). A significant separation between
the half-sites is tolerated, but quarter site separation diminishes DNA
binding significantly. Alterations in the orientation of half sites is also not
well tolerated, suggesting that p53 binds DNA as a dimer of flexible dimers
– with limited flexibility between the monomers of each dimer (157, 160).
Figure 1.12  p53 specific DNA binding. A. Consensus DNA (conDNA) is composed of four inverted repeats of a pentameric quarter site. Separation of the half-sites can accommodate up to 13 base pairs before a decrease in specificity is seen. B. One model depicts the core and C-terminal domains of p53 oriented on consensus DNA as a dimer of dimers with flexibility between the dimer pairs (160). Not shown are the N-terminal and C-terminal regulatory domains.
A model of one possible orientation consistent with these data is shown in Figure 1.12, Panel B.

Efforts to crystallize tetrameric p53 in complex with conDNA have been largely unsuccessful. Contacts describing only monomeric DNA binding domains to a consensus half site have been published. Figure 1.8 depicts only one of the monomeric DBDs that was found attached to this half site – the monomer whose contacts are consistent with all other previously published data (73, 96). Unexpectedly, Cho and others found two other monomeric DBDs bound to DNA in their crystals (Figure 1.13). One was bound to a pseudo half-site created at the interface of two DNA fragments, and the other appeared to participate in protein-protein interactions that stabilize crystal packing and suggest a possible orientation of monomeric binding domains on consensus DNA.

Activation

Clearly, p53 function requires tight regulation, lest we experience rampant cell death, and, to that end, p53 protein levels are kept in check. The origins of the p53 activation hypothesis lie in a number of observations, including gel shift DNA binding experiments that illustrated that p53 was
Figure 1.13 Crystal structure of three monomeric p53 DNA binding domains. Two of the three monomers crystallized with a conDNA half-site demonstrate specific DNA contacts (blue and green) in similar orientations (96). The monomer shown in blue contacts the DNA at the junction of two strands formed by the crystal lattice. The green monomer is rotated $180^\circ$ horizontally from its depiction in Figure 1.8. The third monomer (yellow) shows nonspecific contacts to the DNA backbone as well as protein-protein interactions with the green monomer.
not functionally active (i.e., no *in vitro* DNA binding) without additional modification or interactions (54, 144, 161-163). Combined with the knowledge that in normal cells p53 is not active, whereas in response to DNA damage it is capable of initiating apoptosis and cell cycle arrest, the beginnings of an activation model were formed (163, 164). This model (Figure 1.14) implies the inability of p53 to bind specific DNA in its ‘latent’ form. Only after activation, achieved *in vitro* by phosphorylation of penultimate residue serine 392, acetylation of the C-terminus, deletion of the C-terminus, or binding of C-terminal antibody Ab421, can p53 demonstrate measurable specific DNA binding (54, 135, 144, 163, 164).

A growing number of studies, including this one, are calling into question the accuracy of this widely accepted model. Recently, release of MDM2 degradation has been shown clearly by a number of laboratories to be sufficient for *in vivo* p53 function (apoptosis, etc.) (134, 165-169). p53 activation through the release of degradation is also consistent with the observation that MDM2 null mice are embryonic lethal in a wild-type p53 background but are rescued by deletion of both p53 alleles (170). Additionally, *in vitro* experiments have demonstrated the inhibitory effect
**Figure 1.14 p53 activation model.** "Latent p53" is kept at low concentrations in a normal cell and is not capable of specific DNA binding and transactivation at p53 response elements (p53RE). "Activated" p53 is found in high concentration in a compromised cell and is capable of transactivation leading to apoptosis and cell cycle arrest. Because there are numerous differences *in vivo* between "latent" and "activated" p53, including degradation status and multiple posttranslational events, the requirements for activation have not been clearly determined. *In vitro* efforts to characterize activation have been complicated by the use of p53 isolated from baculovirus (and therefore modified) as well as by the presence of various DNA competitors in traditional assay mixtures (see text).
Latent p53
no ability to bind specific DNA

Active p53

Specific DNA binding

Cell Cycle Arrest

p21

Apoptosis

bax

MDM2
Degradation

Association with MDM2

DNA Damage

p53RE
of commonly-used additives in DNA binding studies that have led to extensive misinterpretation of binding results (171, 172).

Protein Structure - Theories and Algorithms

Though a protein sequence can be readily determined using its coding DNA sequence, protein structure is not so easily understood. Two basic schools of thought predominate the landscape of protein folders - the first describes protein folding as starting with a hydrophobic collapse of the peptide strand into a more compact globule from which portions of structure can then emerge. The second describes the initial formation of a local structural element, based on nearest neighbors, folding first to present a type of scaffold onto which the remaining portions of the protein could fold (173). Realistically, in vivo protein folding is most likely a combination of these two scenarios, with predominantly β-sheet proteins following the former and predominantly α-helical proteins preferring the latter (reviewed in 173).

Numerous algorithms have been written since the 1970s to attempt to predict secondary structure from primary protein sequence to solve the protein folding problem (e.g., 174-180). Despite many advances in this
field, algorithms still only average 70% accuracy – often predicting the location of the structures but not their orientation. Another shortcoming of structure prediction algorithms is an inherent bias against β-sheet structure. β-Sheet structure is not as well understood or easily defined as α-helical structure. α-Helices form in globular proteins when certain criteria are met – strong n/n+4 interactions, an absence of prolines, and sufficient helix-capping residues – all of which can be incorporated into a predictive algorithm. The inherent complexity of β-sheet structure arises from its lack of strict dependence on sequential amino acids. Helices can only be formed by local, linear, one-dimensional, structure elements, but β-sheets are often formed by local three-dimensional structure elements. Without knowledge of the protein fold, it is difficult to determine proximity in three-dimensional protein space, thus creating a loop where the algorithm requires as input what is sought as output.

Despite these limitations, in the absence of X-ray crystallographic or NMR structures, protein structure prediction algorithms remain powerful tools in the analysis of protein folding. When possible, these algorithms should be used in conjunction with both biochemical and biophysical information to generate a more complete picture of protein structure. Such
corresponding information is often gained by the analysis of *in vitro* protein folding, often investigated in the reverse, as protein *unfolding*.

*Protein Structure – Stability*

Numerous parameters and conditions can be used to denature proteins, including pH, temperature and chaotropic agents (urea and guanidine hydrochloride - GuHCl) (181, 182). The extent to which protein structure is disturbed generally follows this relationship: GuHCl > urea > high temperature > extremes of pH (181). The amount of denaturant required to unfold a protein can then be related to the inherent stability of the starting structure in the absence of denaturant if two conditions are met: equilibrium conditions and reversibility. Extremes of pH and temperature often result in irreversible unfolding whereas chemical denaturants frequently provide reversible unfolding conditions. Inherent in the concept of reversibility is the ability of the protein not only to fold into its native tertiary structure but also to resume any quaternary interactions.

Protein stability can be affected by conformation, hydrophobic effects, salt bridges, hydrogen bonding, disulfide bond formation, oligomerization, and ligand binding to name a few. The concept of protein stability can be
approached from at least two different schools of thought, and it should be noted that they do not describe precisely the same phenomenon.

Thermodynamic stability will be discussed in this thesis and quantitatively describes the stability of the native structure relative to the unfolded or denatured structures. Alternatively, it is not uncommon for protein stability to be defined based on cellular stability: the half-life of a protein \textit{in vivo}.

Protein half-life may indeed be related to inherent thermodynamic stability, but numerous other factors must also be considered. For instance, in a normal cell the half-life of p53 is on the order of minutes, but to assume that this short time frame corresponds solely to a low thermodynamic stability would be to ignore effects of MDM2-mediated degradation (40, 183). p53 protein stability has become increasingly important since the discovery that many naturally occurring p53 mutations result in destabilized DNA binding core domains (99, 184). Exploitation of this stability differential could prove useful in cancer therapies.

\textit{Objectives of This Study}

The potential use of p53 in cancer therapies drives the massive volume of research on this protein. The majority of p53 research has been
undertaken by cell and molecular biologists, creating a wealth of *in vivo* information on this crucial nuclear phosphoprotein. Less well understood are the biophysical properties of this protein, including DNA binding function, thermodynamic structural stability, and the relationship of one to the other.

Until the publication of a complete tetrameric structure of p53 bound to DNA, our understanding of protein structure and function will be shaped by biochemical and biophysical investigations. To expand that knowledge, we have investigated native p53 protein structure using predictive algorithms, circular dichroism, fluorescence spectroscopy, and conformational antibody analysis. An examination of protein assembly and stability was accomplished with the use of chemical and thermal denaturation, chemical crosslinking, and analytical ultracentrifugation. The picture of p53 structure that emerges from these studies is that of a surprisingly stable protein.

To address whether this stable structure could be related to the persistence of function, DNA binding was investigated. Previously published *in vitro* experiments outlining the inhibitory effect of commonly used additives in most p53 DNA binding studies (171, 172) encouraged us
to thoroughly investigate the nature of "latent" p53 function and subsequent activation effects. Therefore, these experiments were conducted in the absence of inhibitors and demonstrated a correlation between structural stability and functional stability of the wild-type, "latent" protein as well as for an activated mutant protein, S392E. These experiments are the first to quantitate both specific and non-specific binding for the full-length, unmodified wild-type and S392E proteins. This study describes a model for p53 activation in vitro, consequent in vivo implications, and provides a potentially useful characterization of p53 for protein stability-based cancer therapies.
CHAPTER 2

MATERIALS AND METHODS
Plasmids and Cell Strains

The human p53 gene cloned into the pET15b expression plasmid and hybridoma cells expressing the monoclonal p53 antibody Ab421 were generous gifts from the laboratory of Dr. G. Lozano (M. D. Anderson Cancer Center, Houston, TX). The p53 gene was excised from pET15b and cloned into a pRSET plasmid using XbaI and BamHI restriction enzyme sites. The pRSET vector contains an F1 origin, necessary for single stranded mutagenesis. This insert contained the p53 gene as well as 491 base pairs of genomic sequence at the 3' end of the gene, and the resulting construct was named SET53 (Figure 2.1). Bacterial strain DH5α was used to prepare all double stranded DNAs. BL21(DE3) cells, with or without the pLysS episome (Novagen), were used for p53 expression.

Site-specific Mutagenesis

Single-stranded mutagenesis (185) was performed on SET53 to generate a glutamic acid substitution for the wild-type serine at position 392 (S392E), the penultimate amino acid. A mutagenic primer, 5'-GAGAATGTCAGTCTTCGTCAGGCCCTTCTG, was designed and analyzed using the computer program Amplify (University of Wisconsin)
Figure 2.1 Map of SET53 plasmid. The human p53 gene was cloned into the pRSETb vector from the pET15b vector using restriction enzymes XbaI and BamHI. Use of XbaI at the 5'-end of the gene ensured proper transcriptional alignment of p53. Approximately 400 base pairs of genomic sequence can be found at the 3'-end of the p53 gene, between the p53 stop codon and the BamHI site. The restriction enzyme AlwNI was used to verify proper insertion of the gene as well as assess the integrity of the plasmid through propagation.
and synthesized on a Biosearch 8600 DNA synthesizer. DNA sequencing was used to identify colonies containing S392E mutations, and full gene sequencing on the final clone at the time of isolation verified the integrity of the remainder of the p53 gene. Full gene sequencing was repeated before protein production began. The following four primers were used to generate sufficient redundancy to read most portions of the gene sequence twice.

\[
\begin{align*}
T7 & \quad 5'-TAATACGACTCACTATA \\
564s & \quad 5'-GCGCTGCTCAGATAGCGATGGTCTG \\
a640m & \quad 5'-GTCGAAAAGTGTGTTCTGTCATCC \\
s1070m & \quad 5'-AGGAGCCAGGGGAGCAAGGGCTCA
\end{align*}
\]

**Protein Purification**

Cells transformed SET53 plasmid containing wild-type or mutant human p53 were grown overnight on LB agar plates supplemented with 50 μg/mL ampicillin. Individual colonies were transferred into 12 L of LB media containing ampicillin, and the bacteria were allowed to grow slowly overnight with moderate shaking (~32 °C at ~150 rpm) to maximize soluble protein production. When the cells had reached an absorbance at 600 nm of 0.6-0.8, IPTG was added to 1 mM to induce protein production, and the
cultures were allowed to shake gently at room temperature (~100 rpm). Cells were harvested 4 hours post-induction and resuspended in 20-30 mL breaking buffer containing 0.01 M Mg(CH$_3$COO)$_2$, 1 mM Zn(CH$_3$COO)$_2$, 0.1 M Tris-HCl, pH 7.6, 0.3 M KCl, 5 mM DTT, 1 mM PMSF, and 10% (v/v) glycerol and frozen overnight in the presence of ~0.4 mg/mL lysozyme at ~20 °C.

After thawing and complete cell lysis, a trace amount of DNaseI was added and the mixture allowed to incubate for 30-60 min. The cell debris was removed by centrifugation. Ammonium sulfate was added slowly to 20% saturation and the suspension allowed to sit for 1 h at 4 °C. The solution was centrifuged, and the pellet was discarded. Ammonium sulfate was slowly added to the supernatant to 35% saturation, and this mixture was allowed to precipitate overnight at 4 °C. Following centrifugation, the pellet was resuspended in <5 mL 0.2 M KPB (potassium phosphate buffer, pH 7.5, 5 mM DTT, 10% (v/v) glycerol and 5% (w/v) glucose) and dialyzed against 0.2 M KPB for 2 hours with 3 buffer changes. Overnight dialysis was avoided since it resulted in significant protein loss. The dialysate was then loaded onto a small, ~2 mL phosphocellulose column, and both wild-type and S392E p53 protein were eluted approximately midway through a 0.2 M-
0.5 M KPB gradient. Fractions were confirmed to contain p53 by Western analysis with either monoclonal antibody Ab421 (145) or Ab240 (149). Protein purity was determined by SDS-PAGE analysis and densitometry of the stained protein bands and ranged from ~ 80-90% per preparation. Protein concentration was determined by a Bio-Rad assay (186) and by absorbance at 280 nm using the Beer-Lambert Equation where the extinction coefficient was calculated from amino acid content. Because of dramatic protein loss upon concentration or dialysis, the purified protein samples were used in all assays as eluted from the column in ~0.35 M KPB or diluted into a lower molarity potassium phosphate buffer as specified.

**Determination of Assembly**

Glutaraldehyde crosslinking experiments were conducted at both room temperature and 50 °C to determine that p53 remains tetrameric over this temperature range. Protein at 5.0 x 10^{-7} M tetramer (MW_{tet} is 188,000 Da) in 0.3 M KPB was incubated with 0.01% glutaraldehyde for 5 or 20 minutes before samples were prepared and loaded on a 1.5 mm step 5% and 7.5% SDS-PAGE. Gels were either silver stained or transferred onto nitrocellulose. Western blotting was conducted at room temperature with
the conformationally-independent p53 monoclonal antibody Ab240 to confirm p53-containing bands.

A Beckman XL-A analytical ultracentrifuge was used in sedimentation velocity and equilibrium experiments. Two-channel sector cells were used for velocity experiments, and six-channel sector cells were used for equilibrium experiments. Experiments were conducted at 22 °C, and absorbance was monitored at 220 nm. Equilibrium experiments were conducted at three concentrations (1.1 x 10^{-6}, 9.0 x 10^{-7}, and 7.5 x 10^{-7} M_{tot}) and three speeds (7000, 9000, and 11000 rpm) to eliminate possible effects of either concentration or speed to the final analysis. Data for both wild-type and S392E were similar under all conditions monitored. The presence of higher-order species prevented quantitative analysis of the data.

*Secondary Structure Prediction*

Structure prediction algorithms were accessed through the Biology Workbench, Protein Tools (University of Illinois; [http://biology.ncsa.uiuc.edu/](http://biology.ncsa.uiuc.edu/)) using the sequence for the wild-type protein. Output files were examined for the exact number of amino acids calculated to be involved in either α-helical or β-sheet structure. The determined
regions of secondary structure from published NMR and X-ray
crystallographic structures were used to calculate the percentage of α-helical
and β-sheet structure. The percent contribution to the entire protein was
calculated by summing the amino acids for each type of structure. These
values were determined using the beginning and ending points of helices and
sheets reported in the published structures and dividing by 393, the number
of amino acids in a human p53 monomer.

_Circular Dichroism – Thermal Denaturation_

Circular dichroism spectra were collected on an AVIV Model 62A DS
circular dichroism spectrometer equipped with Star 3.0 Stationary. 10-
Camphorsulfonic acid was used as an internal standard. For thermal
denaturation experiments shown in Chapters 3 and 4 (Figures 3.3, 4.2), wild-
type p53 was at a protein concentration of 0.15 mg/mL (8.0 x 10⁻⁷ Mₜₚ) in
the buffer in which the protein was purified, ~0.35 M KP. Experiments
were also completed with protein concentrations from 0.1-1.0 mg/mL as
well as for protein diluted into 0.05 M KP to result in a final buffer condition
of ~0.2 M potassium phosphate buffer, pH 7.5, 2.5 mM DTT, 5% (v/v)
glycerol and 2.5% (w/v) glucose – referred to as Buffer D (dilution) with
similar results. S392E protein was either at $7.2 \times 10^{-7}$ or $1.4 \times 10^{-6}$ M\textsubscript{tet}, in either $\sim 0.35$ M KPB or Buffer D. After corrections for concentration were made, all conditions yielded similar spectra for each protein.

A quartz cuvette with a 0.2 cm pathlength was used to minimize loss of light due to scatter and absorption of DTT present in the protein sample. A scanning macro was written using an AVIV macro writer on the DOS system to allow full wavelength scans to be taken as a function of temperature. Scans were collected in 0.5 nm increments from 255 nm to 200 nm with an 8 second/point averaging time. Baseline buffer spectra were subtracted from sample spectra prior to averaging the points along each temperature curve. Data points were converted to $\Delta \varepsilon$ using the Beer-Lambert Equation,

$$\Delta \varepsilon = \varepsilon(L) - \varepsilon(R) = \frac{\Delta A}{c \ell} \tag{2.1}$$

where $c$ is the concentration of p53 expressed in M tetramer, $\ell$ is the cuvette pathlength in cm, and $\Delta A$ is the measured CD signal. The spectra were then smoothed (bounce, n=7) and plotted in Igor Pro (Wavemetrics, Lake Oswego, OR). Temperature was monitored at the sample compartment with a computer-controlled Peltier device. For each temperature, the sample was
equilibrated in the cell holder for 3 min before full wavelength scans were
collected to assess the contributions of both α-helix and β-sheet to the
signal. Spectra were collected from 0 to 100 °C in 10 degree steps. The
results from multiple independent runs were averaged. Similar data were
obtained on at least three different protein preparations.

Data points at 218 nm and 210 nm were expressed as a fraction of the
initial signal (at 0 °C) as a monitor of β-sheet and α-helical content,
respectively. The data were fit to a modified Michaelis-Menten equation to
estimate the midpoint of irreversible unfolding. Dynode voltage was
monitored to assess the signal to noise ratio as a function of wavelength
during scanning and made it clear that one of the more traditional
wavelengths to monitor α-helical content, 208 nm, could not be used due to
the increased interference of DTT in this range. The other common α-
helical wavelength to monitor, 222 nm, was also discarded due to difficulties
discerning contributions from the strong and persistent β-sheet signal,
traditionally monitored at 218 nm. Data at 210 nm were selected as a
compromise. CD spectra of p53 were also collected as a function of time
(up to 120 minutes) to verify that equilibrium was reached at each
temperature in the range investigated (data not shown).
Spectra of unfolded p53 shown in Figure 3.3 were generated by incubation of the protein in ~7 M guanidine hydrochloride, and data were collected in 1 nm steps with a 3 second averaging time. Unfolded p53 spectra were corrected as described above, averaged, and plotted from 210 nm to 255 nm in Igor Pro. Data collected at wavelengths lower than 210 nm were complicated by interference from both DTT and guanidine hydrochloride.

**Chemical Denaturation**

Stock solutions of guanidine hydrochloride (GuHCl) and urea were made from ultra-pure chemicals (Fluka) according to refractive index measurements using the following equations,

\[ M_{GuHCl} = 57.147(\Delta N) + 38.68(\Delta N^2) - 91.60(\Delta N^3) \]  \hspace{1cm} (2.2)

\[ M_{urea} = 117.66(\Delta N) + 29.753(\Delta N^2) + 185.56(\Delta N^3) \]  \hspace{1cm} (2.3)

where \( \Delta N \) is the difference in the index of refraction between the stock GuHCl sample and the corresponding buffer at the sodium D line (I81). To allow for proper dilution of the hygroscopic denaturants, 2X-5X buffer stocks were made into which the denaturant was then added before filtration.
and concentration measurements were conducted. Stock solutions of urea were made fresh daily to prevent the formation of cyanate ions (181).

*Circular Dichroism – Chemical Denaturation*

CD data of wild-type and S392E p53 monitored at 218 nm were collected at 22 °C during titration with the chemical denaturant guanidine hydrochloride (GuHCl). A GuHCl stock solution in 0.4 M KP, pH 7.5, 10% (v/v) glycerol, 5% (w/v) glucose, without DTT was used for experiments with both wild-type and S392E proteins to prevent additional dilution requirements into lower salt concentrations. Additional DTT was not introduced to limit the interference from its absorbance.

Data were collected on an AVIV Model 62A DS circular dichroism spectrometer fitted with a stir motor and a double Hamilton syringe pump titrator accessory. Unfolded protein stock solutions were generated by incubation of the protein for 30 min in the presence of denaturant. Incubations from 10 min to 4 hours indicated that unfolding of both wild-type and S392E occurs within 10 min of denaturant addition. Thirty minutes was chosen for the incubation because it corresponded to the time necessary to ready the instrument.
For most experiments, a sample of native protein [0.1 mg/mL (8.0 x 10^-7 M) in 0.4 M KPB, 0 M GuHCl] was monitored at 218 nm in the sample compartment in a 1 x 1 cm quartz cuvette while a stock of unfolded protein [0.1 mg/mL (8.0 x 10^-7 M) in 0.4 M KPB, 5 M GuHCl] was controlled by the syringe pumps. A volume of sample was then aspirated from the cuvette and replaced with an equal volume of unfolded protein sample from the syringe. This method assumes reversibility: the protein in the high concentration of GuHCl that is being added to the folded or native sample is assumed to refold upon dilution into lower GuHCl concentrations. Because reversibility is crucial to determine thermodynamics of the system, this assumption must be tested and proven. To that end, reverse experiments were conducted with the unfolded protein in the cuvette titrated with a folded protein solution in the syringe to monitor the refolding of p53. Data points from both folding and unfolding experiments overlapped.

Additional folding experiments were conducted with the wild-type protein from 4.6-3.1 M GuHCl to provide data points in the range of 4.6-3.6 M GuHCl that could not be provided by the unfolding experiments. Since wild-type p53 was purified at a lower concentration than S392E, collection of data points at GuHCl concentrations higher than 3.6 M was only possible
during refolding. The additional points from 3.6-3.1 M GuHCl were collected to serve as an overlapping region of comparison between the two types of experiments.

For both folding and refolding experiments, the sample was allowed to stir for 120-360 seconds before CD measurements were taken, during which time the slit-width was kept closed to prevent photobleaching of the sample. Measurements at each GuHCl concentration were averaged over a 10 second collection window. Identical results were achieved by mixing the samples for various times (120-360 seconds) after each denaturant addition. Representative points along the unfolding curve were monitored for longer time intervals (hours) and demonstrated no additional spectroscopic changes.

For each denaturation experiment, ellipticity values were corrected to reflect the starting ellipticity of the sample, reflecting the fraction of folded protein remaining. Assuming reversibility and that only unfolding of the monomer is detected by the CD transition, the equilibrium constant ($K$) of this presumed two-state process can be described by

\[
K = \frac{y_{obs} - (y_f + m_f D)}{(y_u + m_u D) - y_{obs}}
\]  
(2.4)
where $y_{obs}$ is the measured observable (CD signal expressed as a fraction of initial signal), $y_f$ and $y_u$ and the y-axis intercepts of the folded and unfolded baselines respectively, $m_f$ and $m_u$ represent the slopes of those baselines, and $D$ is the molar concentration of denaturant. Setting equation 2.4 equal to Gibbs Free energy, $\Delta G = -RT\ln K$, and solving for $y_{obs}$, results in equation 2.5;

$$y_{obs} = (y_f + m_f D) + (y_u + m_u D) \cdot \exp\left(\frac{mD - \Delta G}{RT}\right)$$ (2.5)

where $R$ is the gas constant (1.987 cal/mol•deg) and $T$ is the temperature in degrees Kelvin (295 for these experiments). Data were fit to equation 2.5, using Igor Pro.

Circular dichroism spectra were also collected in the presence of urea, although the weaker nature of this denaturant required significant protein dilution to generate samples with sufficiently high concentrations of urea to observe unfolding. Protein concentrations precluded the use of the titrator for these experiments; therefore separate protein-urea samples were generated at a number of concentrations of urea. Spectra were then collected in a 0.2 x 1 cm quartz cuvette at 22 °C. Urea concentrations up to 3.5 M were not sufficient to unfold either protein.
**Fluorescence**

Scans were collected on either a modular SLM Amino MC200 fluorimeter or an SLM Amino-Bowman Series 2 Luminescence Spectrometer. Samples were made at $5 \times 10^{-7} \text{M}_{\text{tet}}$ in 0.05 M potassium phosphate buffer, pH 7.5, and scanned at room temperature from 310-370 nm at 2 nm/sec with an excitation of 285 nm. A Corning filter 7-54 was used in the excitation pathway to allow only light of wavelengths 240 through 420 nm to pass to the sample. Unless otherwise indicated, fluorescence emission values were taken at 336 nm ($\lambda_{\text{max}}$ for native wild-type p53). For comparative studies with NATA (N-acetyl-L-tryptophanamide), p53 protein concentration was converted to monomeric molarity and multiplied by the number of tryptophans in each monomer (4) to yield molar tryptophan concentrations. Spectra of p53 protein and NATA samples at the same molar tryptophan concentration were then collected as previously described.

**Fluorescence - Additives**

Fluorescence spectra from 320-360 nm were also collected at 1 nm/sec of protein in the presence of DNA and conformational antibody,
Ab1620. Samples contained protein at $8.8 \times 10^{-8} \text{M}_{\text{tet}}$. Fluorescence intensity was monitored as indicated previously upon the addition of equimolar DNA (conDNA or 40DNA) and $3 \times 10^{-9} \text{M}$ Ab1620 as indicated. Fluorescence spectra were also collected for protein samples upon addition of equal volumes of buffer to correct for dilution and photobleaching effects. Effects of additives on protein fluorescence were immediately detected and did not change over time (up to 20 min). Data reported are after baseline correction as a fraction of fluorescence of the protein sample in the absence of additives.

Potassium iodide quenching experiments were performed on the modular MC200 fluorimeter fitted with a stirring motor. Fluorescence spectra were collected during the titration of a protein sample with a potassium iodide solution. A 5 M KI, 1 mM sodium thiosulfate stock was made to allow addition of insignificantly small volume additions (5 µl into 1.0 mL protein sample) via Hamilton syringe, after which the solution was mixed for 25 seconds before a scan was collected. The presence of thiosulfate was required to prevent the formation of I$_2$. The experiment was repeated with buffer additions to a protein solution to provide correction values for non-iodide sources of quenching. These corrections, including
both protein dilution and photobleaching, were made using the following equation:

\[ \frac{F_0}{F} = \frac{F_0}{F_n - (B_n - B_0)} \] (2.6)

where \( F_0 \) is the fluorescence in the absence of quencher, \( F_n \) is the fluorescence at point \( n \) in the presence of quencher, \( B_n \) is the fluorescence of the protein plus buffer solution at point \( n \) and \( B_0 \) is the fluorescence of the protein in the absence of buffer additions. The term \((B_n - B_0)\) is then the factor to correct the fluorescence for dilution and photobleaching. The Stern-Volmer constant, \( K_{sv} \), was determined by the slope of the linear fit of the data in Igor Pro according to equation 2.7 (187).

\[ \frac{F_0}{F} = 1 + K_{sv}[Q] \] (2.7)

**Fluorescence – Chemical Denaturation**

Guanidine hydrochloride and urea stock solutions were prepared from ultra-pure grade chemicals (Fluka) in 0.05 M KP buffer, pH 7.5 by refractive index measurements. Samples of \( 3 \times 10^{-7} \) M\textsubscript{tot} p53 protein, either wild-type
or S392E, were allowed to incubate in denaturant from 2-24 hrs before spectra were collected from 310-370 nm at room temperature as indicated previously. Data at 340 nm or 355 nm were then expressed in either fluorescence units or as a fraction of the fluorescence in the absence of denaturant.

*Conformational Antibody Experiments*

Purified wild-type and S392E p53 proteins were diluted to a final concentration of $3.0 \times 10^{-7} M_{tet}$ in 0.3 M KPB with 0.1 mg/mL BSA. Samples were incubated at either 0, 5, 16, 22, 37, 42, 50, or 65 °C for 30 min before rapid filtration at room temperature onto a nitrocellulose filter presoaked in potassium phosphate buffer. Temperature measurements for samples containing buffer in the absence of protein were used to establish that no significant temperature change occurred during filtration. The nitrocellulose filter was blocked with 10% dry milk in PBST (phosphate buffered saline, pH 7.4, 0.05% (v/v) Tween 20) for 30 min and exposed to either Ab1620 (conformation dependent) or Ab421 (conformation independent) overnight at 4 °C. After thorough washing with PBST, the filter was incubated with a secondary antibody complexed to horseradish
peroxidase for 1 h at room temperature followed by PBST washes. The use of an ECL kit (Amersham) and subsequent film exposure allowed visualization of the reaction. Reactivity was quantitated by a Molecular Dynamics Computing Densitometer equipped with ImageQuant software and normalized to the reactivity of the 0 °C sample for each experiment. Normalized data from independent runs were averaged and reported.

DNA binding

A variation of the 96-well nitrocellulose filter assay first described by Wong and Lohman (188) was used to determine equilibrium dissociation constants for p53 binding to its consensus sequence, conDNA, as well as to a nonspecific sequence, NS2, and a 40mer DNA containing the consensus sequence, 40DNA, under non-stoichiometric conditions. The conDNA sequence, 5'-AGACATGCCTAGACATGCCT, and its complement were commercially synthesized (Great American Gene Co.) and are based on the p53 consensus DNA operator, consisting of two repeats of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy (157, 158). DNA binding assays to determine nonspecific DNA binding by p53 employed the 20 mer sequence 5'-TCCAGATGTACCCAAACGTG and its complement. NS2 was
designed to contain the identical percent composition as conDNA while at the same time using as many of the least used residues as possible at each position (Figure 2.2). 40DNA was designed as a double stranded DNA target to contain the conDNA sequence as its internal 20 base pairs with 10 base pairs of random sequence on both the 5'-and 3'-ends. Complementary DNA's were annealed to form double-stranded targets and labeled at the 5'-ends using γ-[32P]-ATP and polynucleotide kinase incubated at 37 °C for 1 h. Protein from 1.0 x 10^{-7} to 1.0 x 10^{-11} M_{tet} was incubated with either 2 x 10^{-11} M conDNA or 40DNA or 2 x 10^{-10} NS2 DNA, all well below the $K_d$s of the respective interactions.

Buffer used to dilute the protein samples contained 0.1 mg/mL BSA to minimize aggregation and adherence of protein. The protein-DNA samples were incubated for 30 min at the temperature indicated (from 0 to 50 °C) in 0.05 M potassium phosphate buffer, pH 7.5, before rapid filtration through nitrocellulose paper (Schleicher & Schuell). The nitrocellulose was dried at 65°C for 5 min and then exposed to a phosphorimaging plate at room temperature. A Fuji Phosphorimager was used to quantitate the pixels, and a value for a background point with no protein was subtracted from each corresponding condition with protein (Fuji MacBas Software).
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**ConDNA:** A G A C A T G C C T -

A G A C A T G C C T

**NS2:** T C C A G A T G T A -

C C C A A A A C G T G

**Figure 2.2 Comparison of conDNA and NS2 to the p53 consensus site.**
A p53 consensus site was determined from numerous naturally occurring p53 response element sequences and determined to be comprised of 2 repeats of the half site 5'-RRRCWGWGY, where R is any purine, Y any pyrimidine, and W is A or T ([157]). ConDNA is two repeats of a sequence whose pattern matches the consensus site while NS2 matches many of the least used base pairs per position as possible while maintaining an identical composition to conDNA: 10 G-C and 10 A-T pairs. Only one strand of each double-stranded target sequence is depicted.
Values from multiple sets of experiments were fit (Igor Pro) according to the following equation,

\[ R = Y_{\text{max}} \frac{[p53]^n}{[p53]^n + K_d^n} \]  \hspace{1cm} (2.8)

where \( R \) is the amount of bound complex at a specific protein concentration divided by the amount of bound complex at saturating protein, \( Y_{\text{max}} \) is a correction factor that allows for the \( R \) value at saturation to float, \( K_d \) is the apparent equilibrium dissociation constant, \( [p53] \) is the concentration of total p53 protein expressed in tetramer, and \( n \) is the Hill coefficient. The use of DNA concentrations well below the \( K_d \) for the interaction makes the concentration of free protein approximately the same as the concentration of total protein. This condition allows the concentration of total protein (easily determined) to be used for the concentration of free protein (not easily determined) in the interpretation of the data.

Binding isotherm data are the compilation of multiple assays performed in duplicate and were similar over at least two different protein preparations. The value of \( n \) for wild-type-conDNA and S392E-conDNA did not vary significantly as a function of temperature, though Hill coefficients for nonspecific binding were significantly decreased for both
proteins. DNA binding at temperatures exceeding 50 °C was not possible due to the melting temperature of the 20 bp dsDNA targets ($T_m \sim 55$ °C).

Protein activity assays were conducted both at room temperature and at 50 °C for the wild-type protein and conDNA as described above, but with conDNA concentration above the $K_d$ at $2 \times 10^{-8}$ M and protein concentrations from $5.0 \times 10^{-9}$ to $3.0 \times 10^{-7}$ M tetramer to reach saturation levels. Activity assays were also conducted for both proteins with 40DNA. Protein concentrations for each experiment to determine equilibrium dissociation constants were corrected to reflect the relative activity of the sample, which was ~30-50% for each preparation.

**DNA Binding - Competition**

Effects of nonspecific DNA sequences and Ab421 on consensus DNA binding were also examined using nitrocellulose filtration. Where indicated, Ab421 was added at $2.4 \times 10^{-8}$ M (420 ng), just over a stoichiometric concentration to p53, which was held constant at either 1 or $2 \times 10^{-8}$ M. Radiolabeled conDNA was then added at $2 \times 10^{-11}$ M. When present, nonspecific DNA was added last at $2 \times 10^{-6}$ M, a concentration above the $K_d$ determined for nonspecific binding. The experiments were conducted as
indicated for DNA binding analyses with an additional 10 min incubation on ice to allow for Ab421-protein binding prior to the addition of either DNA. Data were corrected to fractional activity of p53-conDNA binding alone at each temperature.

Thermodynamic Analysis of DNA Binding

DNA binding data collected as a function of temperature were analyzed using a van't Hoff plot fit to the following equation modified from Ha et al. (189).

\[
\log K_{obs} = \frac{\Delta C_{p, obs}}{2.303 R} \left( \frac{T_H}{T} - \ln \frac{T_S}{T} - 1 \right)
\]  

(2.9)

where \( \Delta C_p \) is the change in the heat capacity for the p53-DNA complex formation, \( K_{obs} \) is the apparent equilibrium association constant for DNA binding, and \( T_H \) and \( T_S \) are the temperatures at which \( \Delta H \) (enthalpy) and \( \Delta S \) (entropy) are zero, respectively. The subscript 'obs' indicates that the thermodynamic values are a function of solution variables, including salt and pH. Thermodynamic parameters for enthalpy, entropy and Gibbs free energy (\( \Delta G \)) were calculated from \( \Delta C_p, T_H \) and \( T_S \) according to equations 2.10 – 2.12 (189).
\[
\Delta H_{obs}^\circ = \Delta C_{p, obs}^\circ (T - T_H)
\]  
(2.10)

\[
\Delta S_{obs}^\circ = \Delta C_{p, obs}^\circ \ln \frac{T}{T_S}
\]  
(2.11)

\[
\Delta G_{obs}^\circ = \Delta H_{obs}^\circ - T\Delta S_{obs}^\circ
\]  
(2.12)

Values for \(\Delta G\) calculated from Equation 2.12 were compared with values calculated from the experimental data as determined by Equation 2.13.

\[
\Delta G_{obs}^\circ = -RT \ln K_{obs}
\]  
(2.13)
CHAPTER 3

DNA BINDING AND THERMOSTABILITY STUDIES OF

WILD-TYPE P53
INTRODUCTION

The ability of p53 to bind to specific double-stranded DNA (dsDNA) segments and to act as a transcription factor in response to DNA damage is essential for its protective function (41, 52, 157, 158, 190, 191). The majority of p53 mutants isolated from human tumors produce proteins with abrogated dsDNA binding function, reflecting the importance of DNA binding for in vivo functions (30, 104, 105, 159, 192, 193).

Under normal cellular conditions, p53 is maintained at low concentrations through ubiquitin-mediated degradation regulated by interaction with MDM2, an E3 ubiquitin ligase (56-58, 60, 65, 194). Upon DNA damage, p53 degradation decreases, and cellular levels of p53 increase (40, 58, 60, 139, 194, 195). In addition to elevated p53 levels, DNA damage also results in post-translational modifications that alter DNA binding and transcriptional regulation (40, 121, 123, 196). In fact, in vitro experiments have demonstrated the requirement for an activating agent (e.g., C-terminal antibody binding, phosphorylation, acetylation) to achieve high affinity dsDNA binding by p53 (54, 144, 161-163). Consequently, the protein was deduced to exist naturally in a “latent” form with diminished DNA binding ability until activated by a variety of mechanisms (163, 164). However, in
vivo studies have suggested that blocking MDM2-induced degradation of p53 is sufficient to result in an "activated" p53 phenotype (134, 165-169).

An alternative explanation for the apparent activation requirement has been presented based on the effects of nonspecific competitor DNA present in the most commonly used assay conditions (171, 172). Added nonspecific DNA, including both plasmid DNA (pBluescript) and poly d(I-C), inhibits specific dsDNA binding, apparently by affecting the C-terminus of the p53 protein (171, 172). Due to the presence of these nonspecific competitors, p53 requires artificial "activation" by C-terminal antibody binding, phosphorylation, or other agents in order to relieve the negative effects of nonspecific DNA. These "activating agents" can therefore be considered "anti-inhibitors", blocking the inhibition of specific DNA binding by nonspecific DNA. Collectively, these data call into question the validity of the commonly accepted "latent-activated" switch hypothesis. Herein, a quantitative determination of the dsDNA binding properties of full-length "latent" unmodified p53 casts further doubt on the necessity for DNA binding activators.

Based on both in vivo and in vitro results, full-length, "latent" p53 protein has been deduced to be relatively unstable, in terms of both
degradation and folded structure. In response to DNA damage, the half-life of p53 is increased from <30 min to hours (40, 183, 194). The in vivo half-life of p53 may depend not only on the rate of MDM2-mediated degradation, but also on p53 conformational stability (197). Examination of dsDNA binding in the presence of competing nonspecific DNA showed significantly diminished protein activity at 37 °C and higher (141, 153). Loss of reactivity between 37 and 42 °C with Ab1620, an antibody widely used as a determinant for folded and active p53 protein (142, 148), has also been reported (151). Further, unfolding studies of the isolated DNA binding domain yielded a $T_m$ of ~42 °C (99). These observations have provided support for the hypothesis that intact p53 is not a thermodynamically stable protein and that its DNA binding activity therefore exhibits thermal sensitivity (108, 141, 151-153).

We have used circular dichroism (CD) in conjunction with sequence-based structure prediction algorithms to examine the secondary structure of full-length p53 produced in E. coli without the post-translational modifications that occur in eukaryotic organisms. In contrast to expectations from previous domain studies, the intact, full-length, wild-type protein is surprisingly thermostable, with an estimated transition midpoint of ~73 °C.
Circular dichroism signal corresponding to significant amounts of β-sheet structure persists to temperatures as high as 100 °C. Further, at temperatures up to 50 °C, the bacterially derived, “latent” p53 demonstrates high affinity dsDNA binding to its consensus sequence that requires no “activation”. However, we did observe loss of Ab1620 antibody reactivity between 37 and 42 °C, similar to behavior observed previously (151). These data suggest that the stable β-sheet structure observed by CD reflects a folded and active DNA binding domain that persists even after loss of reactivity with Ab1620. In the context of the full-length, unmodified protein, the DNA binding domain maintains a much more stable structure than previously presumed, and Ab1620 reactivity does not correlate directly with specific dsDNA binding capacity.

RESULTS

Protein Purification

The full-length, wild-type, human p53 protein was expressed in bacterial cells and purified using the purification protocol outlined in Chapter 2. Elution from a phosphocellulose column midway through a 0.2-0.5 M KPB gradient resulted in a highly purified protein band recognizable
by monoclonal p53 antibodies. Protein purity (~80-90%) was assessed by SDS-PAGE analysis (Figure 3.1) and confirmed by densitometry.

*Structure Prediction Algorithms and Solved Structures Describe p53*

Sequence-based structure prediction algorithms from the Biology Workbench were used to predict the secondary structure elements of the human p53 protein. These predictions were then compared to the previously determined NMR and X-ray crystallographic structures for p53 fragments. A small N-terminal α-helix (residues 17-27, Figure 1.7; Box 1, Figure 3.2) of p53 was crystallized with the N-terminus of MDM2, defining the basis for this interaction (66). The DNA binding core domain (DBD, residues 94-312, Figure 1.8; Box 2, Figure 3.2) was crystallized as a monomer bound to DNA (96). The structure reveals that the DBD is primarily composed of β-sheet structure with small α-helical segments. Both NMR and X-ray crystallography were used to determine that the structure of the C-terminal oligomerization domain (residues 319-356, Figure 1.9; Box 3, Figure 3.2) is organized as a dimer of dimers (111, 113, 114). Each dimeric unit contains an antiparallel, two-stranded β-sheet and two antiparallel α-helices with
Figure 3.1  SDS-PAGE of wild-type p53 purification. Samples were loaded onto a 7.5% SDS-PAGE after elution from a phosphocellulose column by a postassium phosphate buffer (KPB) gradient. Lane 1 is a protein standard, and approximate sizes are indicated in kDa; lanes 2-5 represent fractions that eluted midway through a 0.2-0.5 M KPB gradient. p53 protein is indicated by an arrow and was confirmed by Western analysis using p53 specific monoclonal antibodies (not shown).
Figure 3.2 Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) structure prediction algorithms. The 393 amino acid p53 protein is depicted in linear sequence format. Peaks in each line represent predicted structure of turns, α–helices, or β-sheets, by either CF or GOR algorithms (174, 175). Dashed boxes 1-4 outline the p53 fragments for which a structure has been published: (1) the MDM-2 recognition helix (66); (2) the β-sheet rich DNA binding domain (96); (3) the tetramerization domain (111-113); and (4) the C-terminal S100B recognition peptide (120). These four solved structures identify ~19% β-sheet and ~13% α-helical structure in p53, whereas the CF and GOR algorithms predict 19.8% and 17.8% β-sheet and 16.5% and 18.0% α-helix respectively.
interactions between the dimers strictly limited to the helices. A fourth structure published recently demonstrates that a fragment of p53 C-terminal to the tetramerization domain that is unstructured as a free peptide (residues 376-387, Figure 1.9; Box 4, Figure 3.2) forms an α-helix upon binding to the S100B(ββ) protein dimer (120).

The percentage of β-sheet structure identified in p53 from the four structures shown in Figures 1.7-1.9 is ~19%. Results from two sequence-based structure predication algorithms, Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) are shown in Figure 3.2 (174, 175). Both algorithms predict all four regions of known structure reasonably well. The predicted percentages of β-sheet structure are 19.8% and 17.8% by CF and GOR methods, respectively, indicating that the β-sheet rich DBD and the C-terminal β-sheet in the oligomerization domain account for nearly all β-sheet structure in the full-length protein.

The percentage of α-helix within the known structures is ~13%, whereas the predicted values for the full-length protein are 18.0% and 16.5% from the GOR and CF algorithms, respectively, suggesting that additional, as yet unsolved, portions of p53 may contain α-helical regions. Five other
algorithms from the Biology Workbench yielded similar results (Table 3.1) and also predicted structure consistent with the solved domains (176-180).

Circular Dichroism Shows p53 Thermostability

The secondary structure of p53 was examined using circular dichroism spectroscopy to determine the effects of increasing temperature on p53 stability. The shape and intensity of the CD spectrum of intact purified p53 protein at temperatures less than 60 °C were consistent with a folded protein. Major contributions of both β-sheet and α-helical structure were indicated by the spectral minima observed at ~218 and 208 nm, respectively (Figure 3.3). This finding correlates well with the emerging picture of p53 from both structures of individual domains and the prediction algorithms used in this study. The mixed α/β structure can be observed at temperatures from 0 to 60 °C. With increasing temperature, the signal at ~210 nm that reflects α-helical content gradually decreases until ~70°C, at which point the shape of the spectrum is consistent with an all β-sheet protein and minimal α-helical content. Strikingly, significant loss of the β-sheet structure signal at 218 nm is not observed until temperatures exceed 60 °C. The presence of
<table>
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<td>(\beta)-sheet</td>
</tr>
<tr>
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<td>17.8%</td>
<td></td>
</tr>
<tr>
<td>CF(^b)</td>
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<td>19.8%</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Total(^d)</td>
<td>18%</td>
<td>16%</td>
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\(^a\)Results from Garnier-Osguthorpe-Robson structure prediction algorithm (175). \(^b\)Results from Chou-Fasman structure prediction algorithm (174). \(^c\)Results calculated from the four X-ray and NMR structures of p53 domains (66, 96, 114, 120). \(^d\)Total refers to results averaged from seven different structure prediction algorithms (see text).
Figure 3.3 Circular dichroism spectra of wild-type p53 as a function of temperature. CD spectra were collected from 0 to 100 °C in 10 degree intervals as described in Chapter 2. Samples contained purified full-length p53 protein at a concentration of 8.0 × 10⁻⁷ M tetramer in 0.35 M KPB. Spectra were baseline corrected and smoothed before being plotted. Data points are expressed as a change in extinction coefficient, Δε, as determined from equation 2.1. Spectra are shown in 20 °C intervals. (●) 0 °C, (△), 20 °C (■) 40 °C, (O) 60 °C, (▲) 80 °C, (□) 100 °C. An average spectrum of p53 unfolded by ~7 M guanidine hydrochloride is shown for comparison (◆). For this spectrum, wavelengths less than 210 nm are excluded due to the small signal to noise ratio.
~10% glycerol in the sample allowed measurements to be taken at 100 °C where considerable β-sheet structure persists. Attempts to reach temperatures high enough to generate spectra characteristic of random coil were not possible due to substantial sample evaporation. It was therefore necessary to incubate p53 in the presence of ~7 M guanidine hydrochloride to generate a spectrum for the unfolded protein for comparative purposes (Figure 3.3).

Thermal unfolding was irreversible (data not shown), consistent with findings reported previously for the core domain alone (99). For this reason, thermodynamic calculations of unfolding energies were not possible. However, analysis of the data for β-sheet structure at 218 nm indicates a midpoint of ~73 °C for the transition (Figure 3.4), a value which is reproducible over multiple experiments, several preparations of protein, and two different buffer conditions (Chapter 2). In an attempt to determine the $T_m$ for the α-helical unfolding transition, data points at 210 nm were examined as a function of temperature (Figure 3.4). The increased interference of DTT at wavelengths lower than 210 nm precluded examination at 208 nm, a standard wavelength monitored for α-helical structure. The midpoint of the 210 nm data is at ~68°C, but this value
Figure 3.4 Assessment of temperature dependence of α-helical and β-sheet structures in wild-type p53. CD data at 218 nm (●) and 210 nm (O) as a function of temperature were averaged (n=3) to assess the fraction of residual structure (R) at wavelengths that monitor β-sheet (218 nm) and α-helical (210 nm) structure. Values are expressed as a fraction of the initial signal at 0 °C. The solid lines are a fit of the data points to a modified Michaelis-Menten equation to determine the midpoint of the unfolding curve. Because thermal unfolding of p53 is irreversible, the resulting transition midpoints of 73 °C at 218 nm and 68 °C at 210 nm are useful primarily for comparative purposes. Error bars indicate one standard deviation.
appears to be a high estimate, as spectra above 60 °C appear to have minimal \( \alpha \)-helical content and the baseline is complicated by the presence of the persistent \( \beta \)-sheet signal (see Figure 3.3). Based on both known and predicted structures, the \( \beta \)-sheet structure signal monitored by CD can be correlated with the core DNA binding domain of the protein. We therefore conclude that the DNA binding domain in the full-length protein is much more stable than the isolated domain, which has an apparent \( T_m \) of 42 °C (99).

Specific DNA Affinity of “Latent” p53 as a Function of Temperature

Whereas CD can monitor substantial structural changes in a protein, subtle conformational changes that may influence protein function are not necessarily detectable. We therefore examined the DNA binding activity of p53 as a function of temperature to establish that the significant \( \beta \)-structure observed in CD spectra at elevated temperatures corresponded to a functionally intact DNA binding domain. Using a p53 consensus sequence, conDNA, DNA binding was examined at temperatures at which significant \( \beta \)-sheet stability was indicated by CD experiments. Consistent with an intact and active core domain, high affinity dsDNA binding by p53 was observed
at all temperatures assayed (Figure 3.5, Table 3.2). Measurement of DNA
binding at temperatures greater than 50 °C was precluded by the $T_m$ of the
20bp target sequence. At room temperature, specific p53 DNA binding
shows an apparent $K_d$ of $1.6 \times 10^{-9}$ M tetramer (Equation 2.8), confirming an
intact DNA binding domain. At 42 °C, a temperature at which p53 has been
reported to be inactive in the presence of nonspecific DNA competitors
(151), high affinity binding in the absence of these competitors is still
observed. Specific dsDNA binding was observed even at 50 °C, with an
apparent $K_d$ of $7.2 \times 10^{-9}$, only 6-fold lower than that measured at room
temperature (Table 3.2). Additionally, measurements of activity under
stoichiometric conditions indicated no difference in the fraction of active
protein at elevated temperatures (data not shown). Thus, the β-sheet DNA
binding core not only retains significant secondary structure at elevated
temperatures, as indicated by the CD data, but this region also remains
functionally active.

*Thermodynamic Analysis of Specific DNA Binding*

For protein-ligand interactions, the heat capacity change of the
reaction can be a useful determinant of the amount of conformational
Figure 3.5 Wild-type p53 specific DNA binding as a function of temperature. Isotherms for full-length, wild-type p53 binding to the specific dsDNA target, conDNA, are shown. Protein at the indicated concentration (expressed in moles/liter of tetrameric protein) was incubated for 30 min at temperature indicated on the graph. Labeled DNA concentration was held constant at 2 x 10^{-11} M. Data at each temperature were derived from at least 3 experiments conducted in duplicate. The protein concentration was corrected based on measured percent protein activity. Fractional saturation (R) was determined by the amount of bound complex at a specific protein concentration divided by the amount of bound complex at saturating protein. Solid lines are fits to all data points at each temperature to Equation 2.8 to determine the dissociation constant ($K_d$) for dsDNA binding shown in Table 3.2.
<table>
<thead>
<tr>
<th>Temp</th>
<th>$K_d$</th>
<th>$n$</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>37</td>
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</tr>
<tr>
<td>42</td>
<td>$3.6 \pm 0.2 \times 10^{-9}$</td>
<td>1.7</td>
</tr>
<tr>
<td>50</td>
<td>$7.2 \pm 1.1 \times 10^{-9}$</td>
<td>1.6</td>
</tr>
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</table>

Table 3.2 Observed dissociation constants ($K_d$)$^a$ and Hill coefficients ($n$)$^b$ for wild-type sequence specific DNA binding.$^c$

$^a$Dissociation constants are averaged from 3-6 experiments, performed in duplicate, and are corrected for protein activity. $^b$Hill coefficients were derived from Equation 2.8. $^c$Binding was examined using a double-stranded 20mer base pair target sequence, conDNA.
rearrangement linked to the binding process (189). $\Delta C_p$ can be derived from fitting a plot of $\log K_a$ vs $1/T$ (Figure 3.6, Equation 2.9). The more concave this curve, the greater the negative change in the heat capacity and the larger the presumed surface area buried in the p53-DNA interaction. $\Delta C_p$ for consensus DNA binding to wild-type p53 is relatively small, at approximately -0.5 kcal/mol•K, indicating no substantial conformational change upon DNA binding.

The contributions of enthalpy and entropy to the protein-DNA interaction can also be determined from the van't Hoff plot. The temperature at which $\Delta H = 0$, $T_H$, for wild-type-conDNA binding occurs at 15.2 °C, corresponding to the temperature at which the two species have the highest binding affinity over the range of temperatures studied (Figure 3.7). However, the temperature at which $\Delta S = 0$, $T_S$, is 40 °C, indicating that the most thermodynamically favorable temperature for complex formation (i.e., most negative $\Delta G$) occurs near physiological temperature. Below $T_H$, the interaction is driven by entropy since the enthalpy term is positive in this region. Above $T_S$, 40 °C, the $\Delta S$ term becomes negative (and thus, $-T\Delta S$ positive), and the interaction is driven by enthalpy (Equations 2.10-2.12). At all temperatures between $T_H$ and $T_S$, both enthalpic and entropic forces drive
Figure 3.6 Van't Hoff plot of wild-type specific DNA binding data. The fit (solid line, Equation 2.9) to the data points generated from binding isotherms yields the thermodynamic parameters, $\Delta C_p$, $T_H$, $T_S$, of wild-type p53 binding to conDNA (see text). The slight curvature of the fit highlights the small change in binding affinity over the temperatures studied and corresponds to a $\Delta C_p$ of approximately -0.5 kcal/mol•K. Error bars indicate one standard deviation.
the wild-type p53-DNA interaction. Evident in Figure 3.7 is the absence of significant changes in the free energy of binding across the temperatures investigated, with a total differential of only \(\sim 1.2\) kcal/mol.

**Antibody Reactivity to Monitor DNA Binding Function**

The accessibility of an epitope in the DNA binding domain of p53 was assayed at the temperatures at which significant DNA binding was demonstrated. Monoclonal antibody Ab1620 has been used previously to monitor a region in the core domain of p53 that is presumed to be exposed only in the wild-type conformation but is not available in mutant or unfolded p53 (108, 142). The loss of the Ab1620 epitope at temperatures >37 °C in full-length p53 protein has been used to diagnose the presence of inactive protein, while positive reactivity has been used to indicate folded and active protein sample (151). In contrast, monoclonal antibody Ab421 is specific to an epitope in the C-terminus of p53 that is conformation-independent (145, 151).

Ab1620 was able to recognize the purified protein after incubation at 0, 5, 16, 22, and 37 °C but not after incubation at 42, 50 or 65 °C (Figure 3.8). This result contrasts with the high affinity consensus dsDNA binding
Figure 3.7 Determination of the contributions of $\Delta H$ and $\Delta S$ to the Gibbs free energy change for WT p53-conDNA binding. Values of $\Delta C_p$ determined from the fit of the data presented in Figure 3.6 were used according to Equations 2.10-2.12 to determine the contributions of $\Delta H$ (dotted line) and $T\Delta S$ (dashed line) to $\Delta G$. $T_H$ (15 °C) and $T_S$ (40 °C) are marked to illustrate the tightest binding and most favorable $\Delta G$, respectively. The solid line corresponds to $\Delta G$ determined by $\Delta G = \Delta H - T\Delta S$, and the data points were determined by $\Delta G_{\text{obs}} = -RT\ln K_{\text{obs}}$. Note the narrow range (~1.2 kcal/mol) for $\Delta G_{\text{obs}}$ over these temperatures.
Figure 3.8 Wild-type conformational analysis via antibody reactivity as a function of temperature. A. Wild-type protein was incubated at the indicated temperature for 30 minutes, filtered onto nitrocellulose, and exposed to either Ab1620 or Ab421 monoclonal p53 antibody. Radiography was used to detect antibody reactivity following ECL use (Amersham) to generate signal. B. Densitometry was used to quantitate reactivity, and the results of at least three separate experiments at each temperature were averaged. Fractional reactivity (R) was determined at each temperature by comparison to reactivity at 0 °C. Error bars indicate one standard deviation.
activity observed at both 42 and 50 °C (see Figure 3.5, Table 3.2). Ab421 was positive for samples at all temperatures assayed. The antibody binding results are consistent with those reported previously (151), but the CD and DNA binding studies indicate that Ab1620 reaction does not correlate directly with the folding or activity of the full-length protein.

_Nonspecific Binding Affinity of Wild-type p53_

For comparison to conDNA, a nonspecific DNA (NS2) was designed. This sequence is comprised of many of the least used bases at each position of the consensus sequence (157, 158), while at the same time maintaining the number of G/C and A/T base pairs (Figure 2.2). The affinity of p53 to NS2 was sufficiently low that it was not possible to generate complete binding curves (Figure 3.9). However, an estimate of the $K_d$ indicated a value >$5 \times 10^{-8}$ M at both room temperature and 50 °C, significantly lower than that for wild-type-conDNA binding at any measured temperature (Equation 2.8).
Figure 3.9 Wild-type p53 nonspecific DNA binding at room temperature and 50 °C. Isotherms for wild-type p53-nonspecific DNA (NS2) binding are shown. Experiments were conducted similarly to those for conDNA binding, but fractional saturation data could not be generated in the absence of a measurable saturation level, and bound radioactivity is reported. The estimated $K_d$ values are $>5 \times 10^{-8}$ M, significantly higher than values for wild-type specific DNA binding, and do not appear to vary significantly with temperature.
Effect of Nonspecific DNA and C-terminal Antibody on ConDNA

Binding

Previous studies have demonstrated that nonspecific DNA inhibits p53 binding to consensus DNA sequences and that this effect is mitigated by inclusion of antibody specific for the C-terminal region of p53 in the assay mixture (171, 172). To confirm that the bacterially produced protein examined in this study demonstrated similar behavior, binding studies were performed at 0 °C, 22 °C, and 50 °C. As evident in Figure 3.10, the addition of 2 x 10^{-6} M nonspecific DNA, for which binding was measured directly (see Figure 3.9), inhibited binding to consensus DNA substantially. Stoichiometric addition of Ab421 restored binding to levels approaching those in the absence of nonspecific DNA, whereas the addition of Ab421 alone had no significant effect on wild-type p53-conDNA binding. The behavior observed was similar at all temperatures examined.

Wild-type p53 Assembly

Glutaraldehyde crosslinking experiments, previously reported to monitor p53 assembly state (116), were used to determine whether the 6-fold loss of DNA binding affinity from room temperature to 50 °C was related to
Figure 3.10 Effects of nonspecific DNA and Ab421 on wild-type binding to consensus DNA at 0, 22 and 50 °C. p53 at 1 x 10^{-8} M (tetramer) was mixed with radiolabeled consensus DNA at 2 x 10^{-11} M. Nonspecific DNA (unlabeled) was added to 2 x 10^{-6} M to determine its effect on p53-conDNA binding. Significant inhibition of binding is evident at all three temperatures assayed (Condition 2). Addition of 2.4 x 10^{-8} M Ab421 antibody alone did not significantly alter the binding of purified full-length p53 to conDNA (Condition 3). However, addition of Ab421 to the mixture of labeled conDNA and unlabeled nonspecific DNA restored conDNA binding to a significant degree (Condition 4). By design, these experiments are very sensitive to small variations in reaction components; as a consequence, the error range is large, and no distinctions between 0 °C (solid bar), 22 °C (plain bar), and 50 °C (diagonally striped bar) were detectable. Data are reported as a fraction relative to conDNA binding to p53 alone (Condition 1) at each temperature and error bars indicate one standard deviation.
the inability of the protein to form tetramers. Incubation of p53 with glutaraldehyde at either room temperature or 50 °C followed by SDS-PAGE resulted in a cross-linked protein band of ~200,000 Da, an expected value for tetrameric p53 (Figure 3.11). These bands were reactive with the p53 monoclonal antibody Ab240. Although glutaraldehyde crosslinking occurs on a faster time scale at 50 °C than at room temperature, the final amounts of tetrameric p53 observed are similar.

DISCUSSION

The critical response of p53 accumulation to the stimulus of DNA damage is generated by inhibiting MDM2-mediated degradation of p53 and/or stabilizing p53 structure (58, 60, 65, 67, 194). Modifications of the accumulated protein, including phosphorylation and acetylation, have appeared essential to “activate” high affinity DNA site recognition (54, 135, 144, 161-163, 166, 195). However, studies on the full-length p53 protein without post-translational modification have been incomplete. The ability of this unmodified protein to recognize the p53 dsDNA consensus sequence has not been assessed in the absence of nonspecific DNA competitors that have been shown to interfere with specific binding (171, 172). Previous
Figure 3.11 Glutaraldehyde crosslinking of wild-type p53 at room temperature and 50 °C. Purified p53 was incubated at either room temperature (lanes 3 and 5) or 50 °C (lanes 4 and 6) in the presence of 0.01% glutaraldehyde to verify the presence of tetrameric p53 in solution. At both temperatures, samples were allowed to incubate for 5 min (lanes 3 and 4), or 20 min (lanes 5 and 6) before being prepared for electrophoresis. Molecular weight markers in lane 1 are expressed in kDa, and lane 2 contains purified p53 in the absence of glutaraldehyde. Arrows mark monomeric (m), and tetrameric (t), forms of p53. Both species were reactive to p53 monoclonal antibody Ab240 (data not shown).
studies have indicated that p53 is relatively unstable at physiological temperatures, suggesting that thermodynamic instability of the full-length protein may play a role in regulating p53 cellular response (108, 141, 151-153). We therefore sought to examine thermal stability and DNA binding capacity, both central to understanding structure and function of p53, using full-length protein produced in Escherichia coli and therefore lacking post-translational modifications characteristic of eukaryotic organisms.

*Predicted and Solved Structures Confirm Stable β-Sheet CD Signal Represents Core Domain of p53*

To address directly the question of thermodynamic stability of full-length p53 protein, we measured circular dichroism spectra as a function of temperature. The spectra were compared to results from predicted secondary structure algorithms and to solved structures of four p53 fragments. Using this approach, the vast majority of β-sheet structure in the full-length protein can be assigned to the DNA binding domain (DBD). This β-sheet CD signal corresponding to the DBD is remarkably stable to very high temperatures. The estimated midpoint of the irreversible unfolding for
the full-length protein is 73 °C, significantly higher than that observed for the DBD as an isolated fragment (42 °C) (99).

This increased thermostability of the p53 DNA binding domain in the context of the full-length protein may result from a variety of factors, in particular oligomer formation. Previously published unfolding results investigating the stability of the tetramerization domain as a fragment determined a melting temperature of ~75-80 °C for this α-helical motif, suggesting that p53 may remain tetrameric up to this temperature (118, 119). Because the β-sheet structure signal of the much larger DNA binding domain overwhelms the expected CD signal for the small α-helical tetramerization domain, we are unable to confirm the presence of this α-helical region at elevated temperatures. However, glutaraldehyde crosslinking results indicate that the protein remains tetrameric to 50 °C. The persistence of tetramer and the high $T_m$ of the tetramerization domain lead us to propose that the observed loss of α-helical CD signal at elevated temperatures represents unfolding of the p53 N-terminus and the unstable C-terminal α-helix that binds S100B (120) rather than the tetramerization domain. The N-terminal region is predicted to have both α-helical and large
random coil regions (see Figure 3.2), and biochemical experiments suggest that this region is not highly structured (73).

The fold of the p53 β-sheet DNA binding domain is not common among DNA binding proteins and may provide potential for interactions that increase stability of the full-length protein relative to the isolated DNA binding domain. The β-sheet structure within the monomer may be extended to form additional inter-monomeric β-sheet associations that stabilize the tetramer. Indeed, DNA bending that enhances p53 affinity for its DNA target site (198-200) may also increase monomer-monomer interactions. Interactions between various domains of p53 may also contribute to the enhanced stability of the core DNA binding domain in the full-length protein. Although widely separated in the primary sequence, thermal studies of deletion mutants indicate that the N- and C-terminal domains influence activity of the folded protein (122). Additionally, alterations in the N-terminal domain can influence the properties of the DNA binding domain, further illustrating that a variety of interactions may contribute to the increased stability of the full-length protein (121).
"Latent" p53 Demonstrates High Affinity for ConDNA and is Inhibited by the Presence of Nonspecific DNA

The ability of the DNA binding domain to recognize specific sites on dsDNA is a critical property for p53 function as a regulator of transcription. The apparent p53 requirement for "activation" of high affinity DNA binding by a variety of agents gave rise to the "latent-activated" conformational hypothesis for p53 function (54, 82, 144, 163). However, more recent experiments demonstrate that the observed increase in p53 binding to consensus dsDNA sequences in response to activating agents derives, at least in part, from releasing the inhibitory effect of non-specific DNA in the assay mixtures employed (171, 172). To explore the inherent ability of p53 without "activation" to recognize its consensus dsDNA target, we have examined the DNA binding properties of full-length p53 isolated from E. coli. High affinity binding (\(K_d \sim 1.6 \times 10^{-9} \text{ M}_{\text{tot}}\)) of the bacterially-grown, "latent" form of the protein to a 20 bp dsDNA consensus sequence for p53 dispels the view that this protein requires activation for effective DNA binding. The binding affinity for consensus DNA determined in this work is comparable to the highest affinities reported previously for an "activated" form of the protein that was generated by C-terminal truncation (160). Thus,
the requirement for "activation" does not derive from compensation for an inherent deficit in the DNA binding capacity of the unmodified protein, but rather from inhibition by competing non-specific dsDNA. This competition was clearly demonstrated upon addition of nonspecific DNA (NS2) to p53-conDNA. Further, the addition of the "activator" Ab421 had no significant effect on specific DNA binding in the absence of nonspecific DNA but restored p53-conDNA binding in the presence of NS2. These studies confirm that the activation requirement for "latent" p53 to bind specific dsDNA is dependent on the presence of competing nonspecific DNA and does not reflect a deficit in the ability of purified unmodified protein to bind conDNA (171, 172).

*DNA Binding Function Persists at Elevated Temperatures*

The demonstrated thermal stability of secondary structure corresponding to the DNA binding domain suggested the possibility that DNA binding might also persist to high temperature in the full-length protein. In the absence of non-specific competitor DNA, "latent" p53 is not only active at physiological temperature but retains high affinity binding to temperatures as high as 50 °C with only a 5-6-fold decrease in affinity for
conDNA. This loss in affinity does not derive from a decrease in the fraction of active protein, as stoichiometric assays at room temperature and 50 °C yield similar levels of active protein. The decrease in affinity at 50 °C is also not due to a switch to nonspecific binding, since p53 binding to a nonspecific sequence (NS2) results in an apparent affinity substantially less that of conDNA at 50 °C. Further, the diminished activity at elevated temperature cannot be ascribed to alteration in oligomeric structure, as glutaraldehyde crosslinking assays demonstrate similar levels of tetrameric p53. The persistence of high affinity DNA binding in the full-length protein to at least 50 °C provides functional evidence that confirms the structural integrity of the β-sheet structure corresponding to the DNA binding domain.

*Ab1620 Reactivity Does Not Accurately Predict DNA Binding Function*

A monoclonal antibody (Ab1620) has been deduced to react with only the “active” form of p53 and has been applied in numerous studies to assess thermal stability and predict the ability of p53 proteins to bind DNA with specificity (82, 108, 141, 148, 151). In our hands, reactivity with Ab1620 at elevated temperatures is similar to previously reported results (151): the epitope for this antibody is reactive at temperatures up to 37 °C, but no
reactivity is observed above this temperature. However, this pattern
does not track with direct measurements of DNA binding. One possibility is
that Ab1620 detects a small conformational change that correlates with the
6-fold decrease in DNA binding observed over the temperature range
examined; however, this alteration that abolishes the Ab1620 epitope does
not completely abrogate DNA binding activity. Of importance for future
studies is that reactivity with Ab1620 alone cannot be utilized to determine
DNA binding capacity or the folded state of p53 with confidence.

**Wild-type p53 Does Not Undergo Dramatic Structural Rearrangements
Upon ConDNA Binding**

Temperature dependence of protein-DNA binding has been used to
assess the apolar surface area buried on formation of the complex (189,
201). For DNA binding reactions in which protein folding is linked with
complex formation, large negative changes in heat capacity of the reaction
have been observed and attributed to a combination of both hydrophobic and
hydrogen bonding forces (189, 201, 202). Interestingly, the temperature
dependence of p53-conDNA binding suggests a relatively small change in
the exposed apolar surface area upon complex formation (189, 201). The
$\Delta C_p$ for p53-conDNA interaction was determined to be approximately 0.5 kcal/mol K. For comparison, lac repressor, another tetrameric DNA binding protein, exhibits a $\Delta C_p$ of -1.2 kcal/mol K. This large $\Delta C_p$ for lac repressor is ascribed to the folding of the hinge domains of the protein concomitant with operator DNA binding (201). The relatively low value for p53-conDNA suggests that large alterations in protein fold or extensive changes in exposure of apolar residues to solvent are not associated with this binding interaction. A rough estimate of the number of amino acid side chains buried in this interaction, calculated from equations in (201) is ~4 per monomer, a value that suggests minimal folding/rearrangement occurs in complex formation. The low $\Delta C_p$ value combined with the observed thermostability of the DNA binding domain in the intact protein suggests a relatively rigid fold for this domain that is able to "fit" the DNA binding surface without significant conformational alteration.

Motivation for this study was derived from the central importance of this tumor suppressor protein in vertebrate systems and the potential to employ p53 in cancer therapies based on structural and functional information. The results presented demonstrate unanticipated structural and functional stability for the DNA binding domain within the full-length
unmodified p53 protein. These results contrast with earlier work that
deduced lower stability and the requirement for activation of the protein for
effective binding to its target DNA sites (54, 82, 108, 141, 151-153, 163).
The stability and function of p53 are influenced by interactions with multiple
ligands — damaged DNA, dsDNA, ssDNA, and a multitude of other
proteins — and modifications that include phosphorylation, acetylation, and
alternative splicing (121, 123, 196). Demonstration that the sequence
scaffold for p53 assumes a stable and highly functional structure in the
absence of other modifications provides a baseline against which other
studies can be compared.
CHAPTER 4

CHARACTERIZATION OF AN "ACTIVATED" MUTANT
INTRODUCTION

Posttranslational modifications of the p53 protein include phosphorylation, acetylation, and potentially even glycosylation in response to various cellular signals (reviewed in 123, 124). p53 phosphorylation occurs extensively at both the N- and C-termini of the protein. Phosphorylation of serine 392 (serine 389 in murine p53) is a well-established p53 modification and has been reported to increase sequence-specific DNA binding (162, 164, 203, 204). Casein kinase II (CKII) has been implicated in the modification of this residue and has been reported to co-purify with p53 in SV40-transformed cells and to be immunoprecipitated with p53 from insect cells (205, 206). The presence of the C-terminal monoclonal p53 antibody, Ab421, in the immunoprecipitation assay resulted in a drastically reduced amount of CKII, verifying that the antibody epitope and site of phosphorylation overlap (206).

CKII is present in both the cytoplasm and nucleus of eukaryotic cells and has a range of substrates, including Myc, Fos, the adenovirus E1A protein, the SV40 large T antigen, and p53 (205, 207, 208). CKII has also been shown to be tightly associated with the nucleus of actively growing cells, and its activity can be increased in response to various mitogenic
signals (208-211). These results suggested a potential form of p53 regulation in response to cellular signaling pathways and led to increased interest in the CKII serine including mutational studies to alter this residue.

Mixed results from substitutions of the CKII serine have yet to clarify the functional importance of phosphorylation at this site. Substitution of the murine residue at position 386 with either alanine or aspartic acid demonstrated a clear difference in the ability of the two mutant proteins to suppress cell growth in a transformation assay (136). The alanine mutant (S386A) did not suppress cell growth at all, but the aspartic acid mutant (S386D) exhibited only a slightly diminished function from the wild-type protein. Milne and colleagues reported similar results in both transformed and normal cell lines (136) and concluded that abolishing the CKII site by alanine substitution prevented normal p53 function. However, substitution of a negatively charged amino acid (aspartate) was sufficient to mimic phosphorylation and partially rescue the effect.

More recent mutation experiments have demonstrated that p53 CKII phosphorylation selectively regulates p53 function (212). In 1996, Hall and colleagues reportedly found no difference between the murine S386A, S386D and wild-type proteins using a transformed cell assay to monitor
transcriptional activation of a reporter plasmid containing either multiple repeats of the p53 consensus sequence or the naturally occurring p21\textsuperscript{waf1/cip1} promoter (212). However, these serine substitutions dramatically affected the ability of the proteins to repress transcription in a similar assay using the SV40 promoter sequence. Similar to the report from Milne et al. (1992), the S386D mutant was able to repress transcription at nearly wild-type levels, but the S386A mutant was severely incapacitated (212). Further, substitution of the murine CKII serine to glutamic acid (S386E) also activated transcription in contact-inhibited, but not actively growing, NIH3T3 cells, whereas substitutions at six other phosphorylation sites had no effect (203). Conclusions from these studies underscored the complexity of the system, since p53 appears to be regulated in both a promoter and cell-type specific manner. Additionally, wild-type protein appears to be activated in transformed cells; therefore, the ability of the murine S386D and S386E proteins to mimic wild-type function suggests that these mutant proteins are also activated.

\textit{In vitro} studies involving CKII phosphorylation have also proved intriguing, providing evidence that the wild-type protein's ability to bind sequence-specific DNA is enhanced by this modification (80). In fact,
numerous modifications of the C-terminus of p53, including Ab421 binding and C-terminal deletion yield increased activity, implicating the C-terminus as a negative regulator of specific DNA binding (80, 161-163). However, as outlined in Chapter 3, these data belong to a subset of experiments conducted in the presence of excess non-specific DNA whose presence is now known to alter specific DNA binding results.

Analysis of the DNA binding activity of synthesized C-terminal phosphopeptides indicated that phosphorylation at serine 392 (but not at other phosphorylation sites in the C-terminus of human p53) resulted in decreased non-specific DNA binding but not in increased specific DNA binding (213). A thorough in vitro investigation of a protein with a negative side chain substitution at the CKII site is essential to determine the effects of an “activating” mutation on the structure and function of p53. To provide an unmodified background, against which a glutamate substitution could be assayed, the human S392E protein was purified from E. coli. We have found that this mutation does not increase in vitro sequence-specific binding, yielding dissociation constants similar to the wild-type protein at room temperature. Strikingly, the mutant displays an increased affinity for nonspecific DNA and an increased thermal stability of both β-sheet and α-
helical structure as monitored by circular dichroism. Increased thermal
stability is also evident in the largely unaltered affinity for specific DNA as a
function of temperature as well as increased reactivity to the conformational
antibody Ab1620 at 42 °C compared to the wild-type protein.

RESULTS

Mutagenesis and Protein Purification

Mutational substitution of serine 392 with glutamic acid (S392E) was
achieved by single-stranded mutagenesis following the method of Kunkel
(185). The entire gene was sequenced to confirm the absence of additional
mutations. S392E was expressed in bacterial cells and purified using the
wild-type purification protocol outlined in Chapter 2. Elution from a
phosphocellulose column midway through a 0.2-0.5 M KPB gradient
resulted in a highly purified protein band recognizable by monoclonal p53
antibodies. Protein purity (~80-90%) was assessed by SDS-PAGE analysis
(Figure 4.1) and confirmed by densitometry.
Figure 4.1 SDS-PAGE of S392E purification. Samples were loaded onto a 7.5% SDS-PAGE after elution from a phosphocellulose column in potassium phosphate buffer (KPB). Lane 1 contains protein standards with approximate sizes indicated in kDa; lanes 2-4 represent fractions that eluted immediately after the start of a 0.2-0.5 M KPB gradient and should not contain p53; lanes 5-10 are fractions that were eluted mid-way through the KPB gradient. p53 protein (confirmed by p53 monoclonal antibody reactivity) is indicated by an arrow.
**Thermal Denaturation of Mutant p53**

The secondary structure of S392E was examined using circular dichroism spectroscopy to determine the effects of this "activating" mutation on the thermal stability of the protein. Similar to wild-type p53, contributions from both β-sheet (~218 nm) and α-helical (~208 nm) structure are evident at the lowest temperatures (Figure 4.2). It is important to note that sources of error arise from both the instrument and the determination of protein concentration, and significant differences are generally considered those greater than 5-10% (Drs. Stephen Edmondson and Wayne Bolen, personal communication). Given these limits, the general shape of the S392E CD spectrum does not differ significantly from that of the wild-type protein. However, the intensity of the 208 nm minimum is significantly increased compared to wild-type p53, suggesting a greater contribution of α-helical structure in the mutant protein than seen in the wild-type structure. Notably, alterations in the α-helical structure due to increasing temperature follow a similar pattern in both proteins with the signal at ~208 nm gradually decreasing until ~70°C when the shape of both protein spectra switches from that consistent with mixed α/β structure to all β-sheet structure with minimal α-helical content. Interestingly, significant
Figure 4.2 Circular dichroism spectra of wild-type and S392E proteins as a function of temperature. The loss of structure in the wild-type protein, is more pronounced than in the mutant as can be seen in the decrease in negative minima at both 208 and 218 nm. S392E protein displays little loss of β-sheet structure even at 100 °C. Spectra were collected from 0-100 °C and are shown in 20 °C intervals. Before plotting, spectra were baseline corrected and smoothed (n=7). Symbols are as follows; (●) 0 °C, (▲) 20 °C, (■) 40 °C, (○) 60 °C, (▲) 80 °C, (□) 100 °C.
spectral differences can be observed between the two proteins at temperatures greater than 60 °C. Whereas the wild-type protein loses signal characteristic of β-sheet structure (~218 nm) from 60-100 °C, the mutant displays no observable loss in this signal at any of the temperatures investigated. Hour-long incubations of S392E were conducted at 90 °C to verify this result (normal incubations are on the order of 3-5 minutes), and no protein unfolding could be detected under these conditions.

Although analysis of the data for wild-type β-sheet structure at 218 nm indicates a midpoint of ~73 °C for the unfolding transition (Figure 3.4), a similar estimate is not possible with S392E since no decrease in β-sheet signal appears to occur. To determine the $T_m$ for the α-helical unfolding transition, data points at 210 nm were examined as a function of temperature (Figure 4.3). As described in Chapter 3, the increased interference of DTT at wavelengths lower than 210 nm precluded examination at 208 nm, a standard wavelength monitored for α-helical structure. The midpoint of the 210 nm wild-type data occurs at ~68 °C. The midpoint of the S392E data is estimated to occur at ~80 °C; however, the absence of a plateau at elevated temperatures complicates the determination of this value. Because both known and predicted structures (Figures 1.7-1.9, 3.2) allow the β-sheet
Figure 4.3 Thermal stability comparison of wild-type and S392E proteins. Plot of the fraction of residual structure of S392E (black triangles) and wild-type (red circles) as a function of temperature. The amount of structure present at 0 °C is defined as 1 for each experiment. Data are taken from two wavelengths to monitor α-helical (210 nm, open symbols) and β-sheet (218 nm, filled symbols) structure. Because thermal denaturation is not reversible, unfolding midpoints are estimated based on a modified Michaelis-Menton equation and can be used only for comparative purposes. Values for wild-type protein, as reported in Chapter 3 are ~73 °C at 218 nm and ~68 °C at 210 nm. An unfolding midpoint for the mutant could only be estimated from the data at 210 nm (~83 °C) since no unfolding was observed for the β-sheet structure at 218 nm.
structure to be correlated with the core DNA binding domain of the protein, we conclude that the C-terminal glutamate mutation provides thermal stabilization to the core domain. Increased thermal stability can also be demonstrated in the \( \alpha \)-helical structure of S392E and could possibly correspond to an increased extent of helix in this mutant. Whether these effects on \( \alpha \)-helical structure are within the C-terminal region itself or other regions of the protein cannot be discerned.

*S392E ConDNA Binding*

Based on previously published experiments (80, 161-163), phosphorylation or mutation at the CKII site would be anticipated to result in an increased affinity for specific DNA binding. In light of results detailed in Chapter 3 describing the inhibitory nature of nonspecific DNA in previously published experiments, we sought to re-examine the nature of S392E specific binding in the absence of these inhibitors. The double-stranded 20mer sequence, conDNA, was used to assay S392E specific binding in a nitrocellulose filter binding experiment described in Chapter 2 (Figure 4.4). The apparent dissociation constant for S392E-DNA at room temperature \( (K_d = 2.5 \pm 0.2 \times 10^{-9} \text{ M}_\text{tet}) \) was only slightly higher than that of
Figure 4.4 Comparison of wild-type and S392E specific conDNA binding at room temperature. Binding isotherms for both proteins are presented as a function of protein concentration expressed in moles/L of tetrameric protein. R describes the fraction of protein bound to DNA. Circles represent data points from multiple experiments (n≥4). Solid lines are fits to the data generated from Equation 2.8 and reveal little difference in the apparent affinity of the two proteins to conDNA (Table 4.1).
the wild-type protein ($K_d = 1.6 \pm 0.1 \times 10^9 \text{ M}_\text{tet}$), indicating that no significant increase in specific DNA binding could be detected in vitro.

*Thermal Insensitivity of S392E Specific DNA Binding*

The DNA binding activity of S392E as a function of temperature was examined to determine whether the increased structural stability would correlate with a decreased thermal sensitivity for in vitro DNA binding function. Specific DNA binding was examined at temperatures from 0-50 °C and compared to wild-type values. High affinity dsDNA binding by the mutant protein was detected at all temperatures assayed (Figure 4.5, Table 4.1), and observed dissociation constants did not vary dramatically. Whereas wild-type specific DNA binding values decrease 5-6 fold over this temperature range, S392E decreases only ~2 fold, with an apparent $K_d$ of $5.8 \times 10^{-9} \text{ M}_\text{tet}$ at 50 °C. Values of the Hill coefficient ($n$), which describe the slope of the isotherms, were somewhat higher for the mutant protein than for wild-type and ranged from 1.6-2.4, suggesting that an increase in cooperativity may contribute to the decreased variation in apparent $K_d$ values.
Figure 4.5 S392E specific conDNA binding as a function of temperature. The concentration of conDNA was held constant at $2 \times 10^{-11}$ M while the concentration of tetrameric S392E protein was varied as indicated on the X-axis. $R$ describes the fraction of protein-DNA complex. Numerous experiments ($n=3-5$) were conducted in duplicate to generate data points (circles). The solid line in each graph represents the fit of the data to Equation 2.8. Temperature has only a minor effect on binding with a 2-fold decrease observed in apparent dissociation constants ($K_d$) over the range investigated (Table 4.1).
<table>
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<td>2.4</td>
<td>$7.2 \pm 1.1 \times 10^{-9}$</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Table 4.1  Observed dissociation constants ($K_d$)\(^a\) and Hill coefficients ($n$)\(^b\) for S392E and wild-type sequence specific DNA binding.\(^c\)*

\(^a\)Dissociation constants are averaged from 3-6 experiments, performed in duplicate, and are corrected for protein activity. \(^b\)Hill coefficients were derived from Equation 2.8. \(^c\)Binding was examined using a double-stranded 20mer base pair target sequence, conDNA.
**Thermodynamic Analysis of S392E-ConDNA Binding**

The extent of structural change upon protein-DNA binding can be inferred from measurements of DNA binding affinity constants over a variety of temperatures by determining the heat capacity change for the interaction. As discussed in Chapter 3, the difference in heat capacity ($\Delta C_p$) from the unliganded to bound protein states is related to changes in the exposed apolar surface of both the protein and the ligand (i.e., DNA) and can be used to estimate the surface area that is buried in the binding process (189, 201). The fit of a van't Hoff plot ($\log K_a$ vs. $1/T$) yields a concave line whose curvature describes the negative heat capacity change of the interaction; the more concave the shape, the greater the heat capacity change and the more nonpolar surface area buried in the interaction. Not surprisingly then, nonspecific binding is often characterized by a straight line (201).

Shown in Figure 4.6 is the van’t Hoff plot of S392E-conDNA specific binding from 0-50 °C. Wild-type results discussed in Chapter 3 are shown for comparison. The S392E data yields a curve that lacks significant concavity, a result not unexpected given the insensitivity to temperature seen in the dissociation constants (Table 4.1). Data points from the two proteins
Figure 4.6 Van't Hoff analysis of S392E DNA binding. The log of the association constants ($K_a = 1/K_d$) from specific DNA binding experiments are plotted as a function of inverse temperature in degrees Kelvin to determine the heat capacity change upon DNA binding. The more concave the curvature of the fit (solid line) to wild-type (●) and S392E (▲) data points, the greater the negative change in the heat capacity upon binding. Error bars indicate one standard deviation. Temperature in degrees Celsius is shown on the top axis for orientation.
follow similar trends, differing notably only at the higher temperatures, where wild-type affinity drops more dramatically than S392E affinity. This difference creates a more concave profile for wild-type, whereas the fit of the S392E data approaches a straight line. Quantitatively, this translates into a change in heat capacity for S392E specific DNA binding of approximately half the already diminutive wild-type value (S392E, ~ -0.28 kcal/mol•K; WT, ~ -0.50 kcal/mol•K). The lower negative heat capacity change in the mutant indicates an interaction devoid of significant conformational changes in either DNA or protein and suggests that S392E buries even less nonpolar surface area upon DNA binding than wild-type p53. This result indicates that the character of the specific DNA binding process for the mutant protein is similar to that for nonspecific interactions. An examination of S392E binding to nonspecific DNA would therefore be predicted to yield a higher affinity than the wild-type-nonspecific interaction.

Conformational Antibody Reactivity of S392E

For wild-type p53, the loss of the Ab1620 epitope at temperatures >37 °C has been used to diagnose the presence of inactive protein, and positive reactivity has been used to indicate folded and active protein sample (151).
We have shown that even though reactivity is lost at high temperatures (42 and 50 °C), significant levels of specific DNA binding are still observed (Figure 3.5, Table 4.1). Similar experiments were conducted with the S392E protein to determine the ability of this antibody to detect the increased thermal stability of S392E apparent in CD experiments.

After incubation at 0, 5, 16, 22, 37, 42, and 50 °C, the protein was filtered onto nitrocellulose and probed with p53 monoclonal antibody Ab1620 (Figure 4.7). Reactivity at 0 °C is defined as 1 for each experiment. Protein incubated at either 0, 5, 16, 22, or 37 °C displayed reactivity levels undiminished from those observed at 0 °C, whereas incubation at 50 or 65 °C resulted in a dramatic loss of reactivity, similar to results for the wild-type protein. At 42 °C, however, reactivity of S392E, though diminished compared to data at 0 °C, was significantly greater than reactivity of the wild-type sample at the same temperature. Although reactivity with Ab1620 did not correlate with DNA binding function at elevated temperatures, this antibody does appear to monitor a small conformational change that occurs in the protein just above physiological temperatures.
**Figure 4.7 Ab1620 reactivity to wild-type and S392E proteins.** A. Film exposure of one conformational antibody experiment. Samples are filtered onto nitrocellulose after a 30 min incubation at the temperature indicated above and below the image. The membrane is then probed with Ab1620 in a Western-like experiment and quantitated by densitometry. B. Quantitative representation of reactivity data (n=3-5) for wild-type (green bars) and S392E (blue bars). Reactivity at each temperature was corrected to reflect the relative reactivity of the specific protein at 0 °C for each experiment (defined as 1). Significant differences between the mutant (blue) and wild-type (green) proteins could only be detected at 42 °C (see arrows in Panel A). Error bars indicate one standard deviation.
S392E Nonspecific DNA Binding

Binding of S392E to a nonspecific DNA (NS2) was examined at 0, 22, and 50 °C to compare to both wild-type nonspecific binding and S392E specific binding. Phosphorylation or mutation at the CKII site leads to an activated p53 phenotype in vivo (136, 203, 212). Therefore, some variation between the wild-type and a protein modified at this site must exist to account for the observed phenotype. One mechanism to achieve activation is an increase in DNA binding affinity; however, S392E DNA binding differs significantly from the wild-type only in its decreased sensitivity to thermal variation. An alternate way to generate apparent activation is to increase the difference between specific and nonspecific DNA binding, particularly important if nonspecific binding is an inhibitor of specific DNA binding.

To determine whether S392E binding to nonspecific DNA was altered, affinity for the NS2 sequence used in wild-type experiments was measured. The apparent dissociation constant measured for S392E-NS2 binding was 1 x 10^{-8} M_{\text{diss}} and did not vary significantly over the temperature range investigated (Figure 4.8, Table 4.2). Wild-type affinity on the other hand was low enough that it was not possible to generate complete binding
Figure 4.8  S392E nonspecific DNA binding as a function of temperature. Isotherms from DNA binding experiments that were conducted at 0, 22 and 50 °C with the nonspecific double-stranded 20 base pair sequence, NS2, and purified S392E protein are shown. R is the fraction of bound complex and is presented as a function of tetrameric protein concentration. The affinity of the protein for this target sequence (apparent $K_d \equiv 1 \times 10^{-8} \text{M}_{\text{tot}}$) does not vary significantly over this temperature range. Data points are the results of three experiments conducted in duplicate and were fit (solid line) to Equation 2.8. Hill coefficients ($n$) for S392E nonspecific binding are significantly lower than those observed for S392E specific binding (Table 4.2).
Table 4.2  Observed dissociation constants ($K_d^a$) and Hill coefficients ($n^b$) for S392E and wild-type sequence specific and nonspecific DNA binding.\(^c\)

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<tr>
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<tr>
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<td>7.2 ± 1.1</td>
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\(^a\)Dissociation constants are averaged from 3-6 experiments, performed in duplicate. \(^b\)Hill coefficients were derived from Equation 2.8. \(^c\)Binding was examined using double-stranded 20mer base pair target sequences, conDNA and NS2. \(^d\)The absence of a saturation plateau for wild-type-NS2 experiments precludes a more rigorous analysis of this interaction. \(^e\)ND, not determined.
curves (Figure 3.9). An estimate of the $K_d$ for wild-type-NS2 indicated a value $>5 \times 10^{-8} \text{M}_{\text{tot}}$, significantly lower than that for the mutant. What is more striking is that the S392E-NS2 affinity is only 2-fold different from that of S392E-conDNA at 50 °C, revealing that the difference between specific and nonspecific binding has decreased for this mutant (Table 4.2).

**Effect of Nonspecific DNA and Ab421 on S392E Specific DNA Binding**

Previous *in vitro* studies have demonstrated that nonspecific DNA inhibits p53 binding to consensus DNA sequences and that this effect is mitigated by inclusion of antibody specific for the C-terminal region of p53 in the assay mixture (171, 172). Experiments with the bacterially expressed wild-type protein confirm these results (214). We have demonstrated that the S392E mutant binds to nonspecific DNA with a greater affinity than the wild-type protein, though the affinities for conDNA do not differ substantially at room temperature. The effects of nonspecific DNA and C-terminal antibody (Ab421) on specific DNA binding affinity are of significant consequence given the C-terminal location of the S392E mutation. Binding studies were performed at 0 °C, 22 °C, and 50 °C in the presence of constant protein and specific DNA concentrations as described
previously. As evident in Figure 4.9, the addition of excess nonspecific DNA substantially inhibited binding of the mutant protein to consensus DNA. Interestingly, the levels of inhibition are somewhat diminished compared to those seen for the wild-type protein at both 0 °C and room temperature, i.e., more specific DNA is retained by S392E than by the wild-type protein in the presence of non-specific DNA (Figure 4.9, Condition 2). Stoichiometric addition of Ab421 restored binding to levels approaching those in the absence of nonspecific DNA, and the addition of Ab421 alone had no significant effect on S392E-conDNA binding, similar to results described for the wild-type protein. The data from the addition of C-terminal antibody to S392E-conDNA experiments allow us to attribute the specific DNA binding to the core domain alone, confirming that this mutation does not directly affect specific DNA binding (Figure 4.9, Condition 3). Similarly, the addition of Ab421 to S392E-conDNA in the presence of NS2 restores binding levels to those measured in the absence of either modifier, indicating that the C-terminus of S392E is still responsible for the observed inhibition by nonspecific DNA (Figure 4.9, Condition 4).
Figure 4.9 Effects of nonspecific DNA (NS2) and C-terminal antibody (Ab421) on S392E specific DNA binding. Binding of $2 \times 10^{-8} \text{ M}_{\text{tet}}$ protein to $2 \times 10^{-11} \text{ M}$ conDNA was defined as 1 for each experiment at 0, 22, and 50 °C (Condition 1). Addition of excess NS2 ($2 \times 10^{-6} \text{ M}$) to the protein-conDNA mixture resulted in a dramatic decrease of specific DNA retained by both wild-type (filled bars) and S392E (empty bars; Condition 2). Addition of stoichiometric amounts of C-terminal antibody ($2.4 \times 10^{-8}$) in the presence of both specific and nonspecific DNA (Condition 4) reversed the effect of the nonspecific DNA, returning binding to levels approaching those observed in the absence of NS2 (Condition 1). The presence of Ab421 in the protein-conDNA mixture had no effect on specific binding (Condition 3). Asterisks indicate significant differences between the mutant and wild-type proteins.
Though the S392E mutant displays an increased apparent affinity for nonspecific DNA (Figure 4.8), the presence of NS2 in this specific DNA binding experiment does not compete as well for the mutant as for the wild-type protein. A kinetic investigation of specific and nonspecific DNA binding for both the mutant and the wild-type proteins demonstrated similar off rates (on the order of 1-2 minutes), eliminating a kinetic difference as a possible explanation for these competition results (data not shown).

**DNA Binding of S392E and Wild-type p53 to a 40mer Sequence**

A double-stranded 40 base pair target DNA sequence (40DNA) was constructed to contain conDNA as its central 20 bases with 10 base pairs of flanking nonspecific sequence at both the 3' and 5' ends. This DNA was designed to investigate whether nonspecific sequences influenced specific DNA binding by the S392E protein. DNA binding assays with 40DNA were conducted similarly to all other DNA binding assays (Figure 4.10) and revealed a slight increase in S392E apparent affinity for 40DNA over the 20mer conDNA in the absence of flanking nonspecific sequence (40DNA, $K_d = 1.6 \pm 0.1 \times 10^{-9} \text{ M}_{\text{tet}}$; conDNA, $K_d = 2.5 \pm 0.2 \times 10^{-9} \text{ M}_{\text{tet}}$). Wild-type 40DNA binding levels however, were slightly decreased compared to those
Figure 4.10 Specific DNA binding of wild-type and S392E proteins to a 40 base pair target DNA. Ten base pairs of nonspecific DNA were added both 3' and 5' to the 20 base pair cDNA sequence to create a 40 bp double-stranded target sequence. Duplicate DNA binding experiments were conducted in triplicate at room temperature and resulting data (circles) were fit (solid line) to Equation 2.8.
measured for conDNA (40DNA, \( K_d = 3.1 \pm 0.1 \times 10^{-9} \text{ M}_{\text{tet}} \); conDNA, 
\( K_d = 1.6 \pm 0.1 \times 10^{-9} \text{ M}_{\text{tet}} \)). Hill coefficients were similar for both proteins 
(~1.6). The slightly increased affinity S392E displays for 40DNA over 
conDNA may correlate with the higher affinity seen for S392E-NS2 binding. 
If so, the increase would not be expected for the wild-type protein whose 
affinity for nonspecific DNA is greatly reduced in comparison to specific 
DNA binding; indeed, a decrease relative to conDNA is observed. The 
increase in S392E nonspecific DNA binding may therefore contribute to a 
change in the way this protein recognizes DNA, resulting in a higher specific 
DNA affinity for longer sequences.

*Glutaraldehyde Cross-Linking of S392E*

The ability of the S392E protein to form tetramers was assessed by 
chemical crosslinking using low concentrations of glutaraldehyde (0.01%). 
S392E samples incubated in the presence of glutaraldehyde were analyzed 
after electrophoresis on a SDS-polyacrylamide gel (Figure 4.11). Named for 
its apparent molecular weight on this type of gel, p53 is actually ~47,000 Da 
as a monomer and ~188,000 Da as a tetramer. Monomeric S392E p53 was 
visible in the absence of crosslinker but no band was detected just under
Figure 4.11 Glutaraldehyde crosslinking of S392E protein. Protein was incubated at either room temperature (lanes 2, 3, and 5) or 50 °C (lanes 4 and 6) in the presence (lanes 3-6) or absence (lane 2) of 0.01% glutaraldehyde. Reactions were allowed to progress for 5 min (lanes 3 and 5) or 20 min (lanes 4 and 6) before they were stopped by boiling. Samples were then loaded onto an SDS-PAGE, and gels were silver stained. Monomeric p53 is visible in lane 2 (m). The formation of a higher order oligomer is apparent in the last three lanes (asterisk). No tetrameric form (~188,000 Da) was detected. Approximate molecular weights of protein standards in lane 1 are expressed in kDa.
200,000 Da in the presence of crosslinker such as can be seen for the wild-type protein (Figure 3.11). Instead, a band was detected above the 200,000 Da marker, just below the stacking gel. Subsequent experiments attempting to drive this band further into the resolving gel were unsuccessful, therefore preventing an accurate molecular weight determination of this oligomeric complex.

Analytical Ultracentrifugation of p53 Proteins

Both sedimentation velocity and sedimentation equilibrium methods were used to determine the mass of wild-type and S392E proteins. The use of analytical ultracentrifugation to determine mass values also allows interpretation of sample heterogeneity as it relates to assembly states, often allowing a more detailed analysis of the kind and prevalence of specific oligomeric species in both samples.

Results obtained from both sedimentation velocity experiments and sedimentation equilibrium experiments suggest the presence of multiple oligomeric species in both the wild-type and S392E samples. Although glutaraldehyde crosslinking experiments did not allow interpretation of oligomeric state for the S392E protein, analytical ultracentrifugation
experiments demonstrate that both proteins behave similarly.

Sedimentation equilibrium results shown in Figure 4.12 reveal a similar pattern for both proteins. These data are best fit to a tetramer, but are complicated by the formation of higher order oligomers. S392E displays a slightly increased tendency to form higher ordered species, but no quantitative analysis is possible. Despite the complications due to larger complexes, the data cannot be well described by any model that includes monomeric or dimeric species, confirming that the majority of p53 protein is present as tetramers or higher order oligomers.

DISCUSSION

Numerous DNA binding experiments have demonstrated the ability of the C-terminus to regulate sequence-specific DNA binding at the core domain. Deletion of the last 30 amino acids, incubation with C-terminal antibody, Ab421 or C-terminal peptides, as well as acetylation and serine 392 phosphorylation at the C-terminus have all been shown to activate p53 function (80, 135, 161, 162, 204, 215, 216), thereby marking the C-terminus as a negative regulator of core domain specific DNA binding. However, this
Figure 4.12 Analytical ultracentrifugation of wild-type and S392E proteins. Sedimentation equilibrium experiments conducted at 7,000 rpm at 22 °C are shown for each protein and are representative of all data collected. Increased absorbance at higher radial positions monitors enhanced protein concentration along the gradient of centrifugal force. The difference in X-axis position between the two protein data sets is dependent on radial position in the cell and is defined by sample volume.
view of the C-terminus is complicated by its own ability to bind nonspecific DNA (89). Further, C-terminal nonspecific DNA binding has been demonstrated by ourselves and others to inhibit sequence-specific DNA binding by the core domain (171, 172, 214). We have demonstrated the ability of the wild-type "latent" p53 protein to bind specific DNA with high affinity in the absence of nonspecific DNA used prevalently in other assays (Chapter 3). The S392E mutation, deduced to mimic phosphorylation and to increase activity of the modified protein (80, 161-164, 203, 204), provides the opportunity to explore the effects of "activation" on the structure and function of this tumor suppressor protein. Our studies were conducted in vitro on wild-type and S392E proteins purified from E. coli, allowing a direct correlation of a single mutation to changes in both the structure and function of p53 without the complication of other posttranslational modifications.

S392E Displays Elevated Structural Thermal Stability

As temperature is increased, the S392E protein maintains significantly more secondary structure than the wild-type protein, as monitored by circular dichroism spectroscopy. Enhanced stability is seen in both the β-
sheet and α-helical structure signals, with minimal loss of β-sheet signal
to temperatures as high as 100 °C. As demonstrated in Chapter 3, X-ray and
NMR structures as well as structure prediction algorithms indicate that the
β-sheet CD signal corresponds to the core domain of p53. Of significant
interest, a serine to glutamate mutation at the penultimate residue of the
protein is capable of exerting substantial effects on core domain structural
stability, though the mechanism of this effect is not completely understood.

Substitution of the CKII serine with glutamic acid also appears to
increase the content and thermal stability of α-helical structure in p53.
Regions of p53 containing α-helical structure include both N- and C-termini
as well as a portion of the core domain, precluding the identification of the
specific region affected. Structures of wild-type p53 have indicated that
portions of both the N- and C-termini are capable of forming helical structure
in the presence of other peptides (66, 120), and effects of “activating” p53,
mimicked by the S392E substitution, may include stabilizing such structures.

Increased thermal stability for the S392E protein can also be observed
by reactivity experiments using the monoclonal conformational antibody,
Ab1620. Although reactivity to Ab1620 has been used previously to predict
the presence or absence of DNA binding function, we have demonstrated
that Ab1620 reactivity does not correlate absolutely with function, particularly at high temperatures (Chapter 3). However, experiments with wild-type p53 demonstrate an ability of the antibody to detect small conformational changes in the DNA binding core domain that correlate with a slight decrease in DNA affinity observed between 37-42 °C. For the S392E protein, a decrease in Ab1620 reactivity is also observed between these temperatures; however, the magnitude of decrease at 42 °C is diminished compared to wild-type p53. Levels of reactivity at 50 and 65 °C are similar for both proteins. Thus, increased structural stability can be observed for S392E both by a decrease in thermal denaturation and an increase in Ab1620 reactivity at 42 °C. Differences in the structural stability of S392E compared to wild-type p53 could affect a number of in vivo processes, including MDM2 binding (and thus protein degradation) or DNA binding interactions, either of which could contribute to the observed "activated" phenotype (136, 203, 212).
Increased Thermal Tolerance and Lack of "Activation" for S392E-ConDNA Binding

The increased thermal stability of S392E is mirrored by a decreased functional sensitivity to elevated temperature. Apparent binding constants decrease only ~2-fold from 0-50 °C, compared to a ~5-6-fold decrease measured for the wild-type protein. Strikingly, an increase in overall affinity for specific DNA is not observed for this "activated" mutant, with apparent S392E $K_d$ values near or above wild-type levels at most temperatures. At 50 °C, however, S392E-conDNA affinity surpasses wild-type-conDNA values, presumably due to the greater thermal sensitivity of the wild-type protein. An examination of CD signal within this temperature range to correlate differential function with observed structure reveals minimal structural changes for either protein. Structural differences begin to emerge at 50 °C, where a loss of wild-type, but not S392E, β-sheet signal can be detected. Since the β-sheet signal has been shown to correspond to the core DNA binding domain, enhanced stability in this region may contribute to the ability of the S392E protein to maintain higher DNA affinities at elevated temperatures.
Thermodynamic Comparisons Underscore Altered DNA Interactions

A comparison of specific DNA binding thermodynamics reinforces the subtle differences between the two proteins. Large, negative heat capacity ($\Delta C_p$) changes upon protein-ligand binding are not explained effectively by a lock-and-key fit between the two interacting species (189, 201). Instead, an induced-fit mechanism, first proposed by Koshland for enzyme-substrate interactions, in which structural rearrangement of one or both species is linked to binding, is a more likely model (217). One such protein-ligand interaction that exhibits a characteristically large negative heat capacity change is site-specific DNA binding (189, 201). However, protein binding to DNA in a nonspecific manner generally results in a small or undetectable $\Delta C_p$ (201). A 2-fold decrease in the change in heat capacity upon S392E-conDNA binding is observed compared to the wild-type interaction (S392E, $\sim -0.28$ kcal/mol•K; WT, $\sim -0.50$ kcal/mol•K).

Therefore, the absence of a large negative $\Delta C_p$ for S392E-conDNA binding indicates a thermally stable, rigid association, either due to a decreased interaction specificity or a conformation change that provides a unique lock-and-key binding surface. Although S392E-conDNA binding is not truly "activated" (increased affinity), its DNA binding affinity is maintained at
elevated temperatures. The structural stability evident in the CD spectra at elevated temperatures appears to have significant effects on in vitro DNA binding function and may account for the altered in vivo behavior observed for these “activated” proteins (136, 203, 212).

*Increased Nonspecific DNA Binding Affinity has Potentially Advantageous Effects*

Thermodynamic analysis of S392E-conDNA binding suggests a rigid association between S392E and conDNA not unlike typical nonspecific DNA interactions. Of interest, DNA binding levels of the mutant protein to NS2 are significantly increased over wild-type values. Whereas wild-type-NS2 affinity is too weak to detect binding saturation experimentally, under the same conditions S392E-NS2 affinity reaches saturation at all temperatures investigated. Additionally, at elevated temperatures, nonspecific S392E binding affinities closely approach (~2-fold difference) specific S392E binding affinities. This result suggests an altered ability of the activated protein to distinguish between p53 response elements and random nonspecific DNA and does not intuitively correlate with the concept of a “guardian of the genome” searching out p53REs to initiate apoptosis or
cell cycle arrest. However, cooperativity appears to be greater in the S392E mutant than in the wild-type protein, illustrating an additional difference in the way these proteins sense and respond to DNA.

A possible in vivo advantage for this increased nonspecific binding is demonstrated by the increased affinity of the S392E protein for a 40 base pair DNA containing the conDNA 20mer sequence. If S392E specific DNA binding is enhanced by its increased nonspecific DNA binding activity or altered DNA recognition, these changes may result in increased affinity for p53REs as well as nonspecific sequences.

**Competition Experiments Suggest New DNA Binding Model**

Use of a monoclonal antibody, Ab421, specific for an epitope in the C-terminus, in competition assays with DNA provides a mechanism to distinguish between binding contributions from the core and C-terminal domains. The ability of both wild-type and S392E to retain conDNA binding in the presence of Ab421 confirms that the core domain is responsible for specific DNA binding for both proteins. The presence of Ab421 in these assays is sufficient to negate the inhibitory effects of nonspecific DNA, identifying the C-terminus as the site of nonspecific
interactions. Competition between the two DNAs at the core domain is not evident, since decreased conDNA binding is not observed in the presence of Ab421 and excess NS2. We further conclude that the absence of competition between NS2 and conDNA at the core domain of S392E indicates that this domain is not responsible for the observed increased affinity for nonspecific DNA. Therefore, the C-terminus and not the core domain appears to display an enhanced affinity for NS2 over that observed for the wild-type protein.

Diminished competition between the two DNAs for S392E binding is also seen in the absence of Ab421. The addition of NS2 to the S392E-conDNA complex in the absence of C-terminal antibody results in a less pronounced release of conDNA than that seen for the wild-type protein. We and others have shown that in the presence of nonspecific DNA, “latent” p53 is unable to bind specific DNA, yet many studies have demonstrated that activated mutants exhibit binding under these conditions. The model proposed here to account for these results is that S392E and other “activated” p53 mutants bind to nonspecific DNA at the C-terminus and specific DNA at the core domain simultaneously. Mutants would therefore not be inhibited by nonspecific DNA commonly added to DNA binding
assays and would appear “activated”. Under the same conditions, the
wild-type protein would not be free to bind specific DNA due to the
inhibitory presence of nonspecific DNA bound at the C-terminus. This
model predicts increased retention of conDNA by S392E in the presence of
NS2, since both DNAs can be retained by the “activated” protein.

The ability of p53 to bind DNA at two distinct domains presents an
opportunity for in vivo functional regulation. The discovery of significant
differences between wild-type and “activated” mutant protein structure,
DNA recognition, and affinity for both nonspecific and specific DNA
provides early elements of understanding the differences in these proteins
and the effects of “activation”. Still to be answered are questions addressing
the regulation of both specific and nonspecific DNA binding and effects of
additional physiologically relevant protein modifications. Clearly, the next
step in our understanding of p53 activation will require the ability to
integrate the function of the protein in vitro with the performance of the
same protein in vivo.
CHAPTER 5

TRYPTOPHAN FLUORESCENCE AND THERMODYNAMIC

STABILITY OF P53 PROTEINS
INTRODUCTION

Proteins acquire three-dimensional structure \textit{in vivo} based on structural determinants contained in their linear sequence. The ability to assess these determinants and the stability of the resulting fold is essential to understand the protein structure-function relationship. Experimentally, information about protein folding and stability can be accessed through quantitative examinations of protein unfolding. Commonly, mutational studies are used to alter residues in a protein and compare the stability and structure of the resulting mutants to the wild-type protein, allowing interpretations to be made about the contributions of single residues to the stability of an entire protein. This type of comparison allows a wealth of information to be gained from both stabilizing and destabilizing mutations.

Perturbations of structure useful in protein unfolding include temperature and chemical denaturants such as urea and guanidine hydrochloride (GuHCl). Because chemical and thermal unfolding often occur via unique unfolding pathways and intermediates, both methods are used to determine protein stability. Thermal denaturation can provide experimental advantages, \textit{e.g.}, DNA binding can be monitored as a function of temperature but not in the presence of a denaturant, but unfolding at
elevated temperatures is rarely reversible. As discussed in Chapters 3 and 4, thermal denaturation of p53 is irreversible and therefore cannot be used to determine thermodynamic parameters of unfolding for wild-type and S392E proteins. The ability to make quantitative comparisons of protein stability requires that the system exhibit reversibility and be monitored under equilibrium conditions. Chemical denaturation is often reversible, and the free energy change upon protein folding in the presence of denaturants, $\Delta G^{D}_{D-N}$, is approximately linearly related to the concentration of denaturant, allowing the free energy change of folding the protein in the absence of denaturant, $\Delta G^{H_2O}_{D-N}$, to be determined (reviewed in 18I). The subscript on $\Delta G$ indicates the transition being represented, in this case from denatured (D) to native protein (N) and can also be represented as a transition from unfolded (U) to folded (F) structure; however, care must be taken to recall that “unfolded” proteins are rarely without structure.

Spectroscopic properties of a protein, such as changes in circular dichroism or fluorescence signal, can be used to monitor protein folding/unfolding as a function of denaturant concentration (reviewed in 182, 218). Steady state fluorescence spectroscopy monitors protein folding by assessing the environment around aromatic amino acid side-chains.
Tryptophans are the dominant protein fluorophore at wavelengths from 310-370 nm, are highly sensitive to solvent polarity, and occur infrequently (~1%) in protein sequences (reviewed in 187). As such, tryptophan residues, either naturally occurring or added by mutagenesis, are useful tools to monitor protein unfolding. A tryptophan exposed to a polar solvent environment exhibits a maximum emission ($\lambda_{\text{max}}$) at ~350 nm. The more apolar the environment, the more blue-shifted (to lower wavelengths) the $\lambda_{\text{max}}$ becomes. It is not unusual for this blue-shift to also be accompanied by an increase in fluorescence intensity. As proteins unfold, more of the hydrophobic core will be exposed to solvent. The presence of tryptophan residues in or near these buried regions reports this unfolding – typically detected by a red-shift in fluorescence signal and decrease in signal intensity (Figure 5.1).

p53 has four tryptophans, located in the N-terminal half of the protein (Figure 5.2). Trp 146 is located in the N-terminal portion of the DNA binding domain, and its fluorescence has been used by the Fersht laboratory to monitor the unfolding of the p53 core domain (99). Fluorescence experiments demonstrate that the native core domain of p53 exhibits a very weak fluorescence signal. Fluorescence from Trp 146 is increased upon
Figure 5.1 **Typical tryptophan fluorescence spectra.** A tryptophan exposed to solvent exhibits a fluorescence maximum ($\lambda_{\text{max}}$) at ~350 nm. A $\lambda_{\text{max}} < 350$ nm indicates the presence of an apolar environment, commonly found in the interior of proteins. Upon protein unfolding, the tryptophan can become exposed, and its $\lambda_{\text{max}}$ will become red-shifted to higher wavelengths. Due to solvent quenching, an exposed tryptophan will often have a lower quantum yield than a tryptophan buried in a hydrophobic protein pocket (187).
Figure 5.2  Location of tryptophan residues on the linear amino acid sequence of p53. Vertical bars represent tryptophan locations and corresponding amino acid residue numbers. Trp146 is the only tryptophan located outside of the N-terminal activation domain, in conserved region II in the DNA binding core domain.
addition of the chemical denaturant urea, with a $\lambda_{\text{max}}$ at ~350 nm.

Bullock et al. (1997) report that chemically-induced unfolding of the core domain of p53 is reversible, and concentrations of ~2.6 M urea are sufficient to unfold 50% of the core domain at 25 °C.

The lack of fluorescence signal in the native core domain of p53 is reported to be due to fluorescence quenching (99). Quenching describes any process that decreases the intensity of a fluorophore and can occur through both static and collisional interactions (187). Static quenching results from the formation of a nonfluorescent complex between a fluorophore and a quencher. Collisional quenching is dependent on the rate of diffusion of the quencher to the fluorophore and can be correlated to the accessibility of the fluorescent moiety. The location and accessibility of tryptophan residues can therefore be used to interpret protein conformation, particularly as a function of thermodynamically relevant processes, such as ligand binding and protein denaturation.

This study of p53 protein folding reveals that steady state tryptophan fluorescence cannot be used to monitor denaturation in the full-length protein in contrast to results reported for the DNA binding domain (99). No change in fluorescence is detectable over a wide range of either urea or
guanidine hydrochloride concentrations. Further investigation of p53 fluorescence using potassium iodide as a quenching agent demonstrates no measurable change in the tryptophan fluorescence, suggesting they are already substantially quenched in the native structure. An examination of X-ray structures (66, 96) containing two of the four tryptophan residues of p53 reveals environments consistent with quenched fluorophores. Because this tryptophan quenching is not relieved during protein unfolding, it is not possible to use full-length p53 protein fluorescence to monitor denaturation. However, under similar denaturant conditions, circular dichroism spectroscopy demonstrates significant protein unfolding for both the wild-type and S392E proteins, with increased apparent structure loss compared to that detected during thermal denaturation. Of interest, specific DNA and antibody binding result in increased fluorescence intensity for S392E but not wild-type p53, highlighting a potentially useful tool for p53 ligand binding analysis.
RESULTS

Tryptophan Fluorescence and Chemical Denaturation

p53 core domain thermal stability, as reported from the Fersht laboratory, is dramatically decreased in comparison to results described in Chapter 3 for the full-length protein (99, 214). Since thermal denaturation is not reversible for either the intact protein or the core domain, the ability to make quantitative evaluations of the thermodynamic stability of either protein is not possible. However, Bullock et al. (1997) report the ability to monitor reversible denaturation of the core DNA binding domain (DBD) by fluorescence as a function of urea denaturation. Our attempts to monitor full-length p53 fluorescence changes as a function of denaturant concentration proved much less informative.

At concentrations as high as 6 M urea (Figure 5.3, Panel A), no significant change in fluorescence was observed for the full-length wild-type protein. Experiments conducted with up to 5 M guanidine hydrochloride (GuHCl), a stronger denaturant than urea, demonstrated similar results for both the wild-type and S392E proteins (Figure 5.3, Panel B), though ~2.6 M urea was sufficient to unfold 50% of the core domain peptide (99). Fluorescence was monitored for protein-denaturant samples for up to 24
Figure 5.3  p53 tryptophan fluorescence as a function of denaturant.  
A. At concentrations as high as 6 M urea, no change is seen in the 
fluorescence spectra of wild-type p53 monitored at either 340 nm 
(circles) or 355 nm (triangles). Samples were incubated with urea for 2 
hrs (red), 4 hrs (green), or 24 hrs (blue) before spectra were collected.  B. 
Experiments conducted with guanidine hydrochloride yielded similar 
results for both wild-type (triangles) and S392E (circles). Fluorescence is 
defined relative to a protein sample in the absence of denaturant.
hours to exclude the possibility that slow unfolding kinetics were responsible for the unchanged fluorescence spectra.

Two possible theories can explain the absence of any significant change in p53 fluorescence signal as a function of chemical denaturation. Either no significant change in tryptophan environment takes place in the intact protein, i.e., full-length p53 cannot be unfolded by high concentrations of urea or GuHCl, or there is a change in structure that the tryptophans do not report.

Circular Dichroism as a Function of Chemical Denaturant

Use of wild-type p53 samples incubated in ~7 M GuHCl in previous circular dichroism experiments suggested that unfolding of p53 was possible by chemical denaturation (Chapter 3). Since fluorescence spectroscopy proved uninformative as an indicator of p53 unfolding, circular dichroism was used to monitor structural changes. To determine the destabilizing effects of chemical denaturation on the structure of the intact protein, CD spectra were collected as a function of both urea and GuHCl concentrations.

Significant denaturation of either wild-type or S392E proteins was not observed by CD spectroscopy in the presence of urea, with approximately
80% of the starting structure signal persisting to 3.5 M urea (data not shown). Higher concentrations of urea were precluded by protein concentration and experimental design. GuHCl, however, was sufficient to generate partial unfolding of both proteins, confirming that the lack of fluorescence change as a function of denaturant cannot be explained by the absence of structural changes.

GuHCl-mediated denaturation of wild-type and S392E was monitored as a loss of structural signal at 218 nm from 0-4.8 M GuHCl. At 4.8 M GuHCl, neither protein appeared to be completely unfolded (detected by the absence of a significant lower plateau, Figure 5.4), but buffer conditions and protein concentrations prevented the use of higher denaturant concentrations. A 4.8 M GuHCl, approximately 25% of the starting structure signal remained, significantly less than results from thermal denaturation experiments at the highest temperature, illustrating increased protein destabilization by GuHCl denaturation.

Monitoring samples over many hours revealed that equilibrium conditions were reached quickly, on the order of minutes. Renaturation experiments were conducted to verify the reversibility of the observed transitions, and results were identical to those from denaturation experiments
Figure 5.4 Guanidine hydrochloride denaturation of S392E and wild-type full-length p53 proteins. Structural signal was monitored at 218 nm at 22 °C and reported as a fraction of starting signal (R) in the absence of denaturant. Data from three independent experiments for S392E (black) and wild-type (red) proteins. Data from renaturation experiments of both proteins were similar to that collected from denaturation experiments and are also included. Data were fit (solid line) to Equation 2.5 in an attempt to determine the thermodynamic stability of each protein. Higher concentrations of GuHCl to achieve a lower plateau necessary for thermodynamic analysis could not be obtained due to experimental constraints.
over the entire range investigated. Presuming that the transition monitored corresponded to monomer unfolding only, both renaturation and denaturation data were fit according to Equation 2.5 to determine the thermodynamic stability of each protein and the change in Gibbs Free Energy ($\Delta G_{\text{D-N}}^{\text{H}_2\text{O}}$) assuming a two state transition (181, 182). The estimated value of $\Delta G$ for S392E is slightly larger than that estimated for the wild-type protein at 22 °C (WT, -4.1 ± 0.8 kcal/mol; S392E, -5.1 ± 1.2 kcal/mol). The small apparent differences between wild-type and S392E cannot be further analyzed given the relatively high error values of the measurements, an inherent consequence of the lack of well-defined lower plateaus and the unknown nature of the monitored transitions. However, differences in the data for S392E vs wild-type do suggest the possibility of structural alterations in the mutant protein.

*Potassium Iodide Quenching of p53 Tryptophan Fluorescence*

p53 tryptophan accessibility was probed by potassium iodide quenching experiments to determine whether the fluorescence of any of the four tryptophans of p53 could be decreased by collisional quenching. Iodide is a potent fluorescence quencher whose charge and large size prevents it
from approaching tryptophans located within the hydrophobic core of a protein. Iodide quenching can therefore be correlated to the accessibility of the residues, providing additional information about their environment (187).

Iodide quenching effectiveness is measured as the Stern-Volmer quenching constant, \( K_{sv} \) on a scale of \( \sim 0-1 \), where a \( K_{sv} \leq 0.2 \) represents essentially no quenching (187). The concentration of potassium iodide was varied from 0-0.2 M and the quenching of tryptophan fluorescence was measured. Similar experiments were conducted with the addition of buffer to the protein sample to correct for dilution and photobleaching effects (Equation 2.6). The slope of a plot of \( F_0/F \) (fluorescence signal in the absence of quencher /fluorescence signal in the presence of quencher) versus quencher concentration yields \( K_{sv} \) for the interaction (Equation 2.7, Figure 5.5). Iodide quenching of wild-type p53 yields a \( K_{sv} = 0.15 \pm 0.11 \text{ M}^{-1} \), indicating that none of the four tryptophans can be quenched effectively by iodide. The observation that four tryptophans (sixteen in the tetramer) are structurally inaccessible to iodide is of particular interest, since three of the four tryptophan residues are located in the N-terminus of p53, a portion
Figure 5.5 Stern-Volmer plot of potassium iodide quenching. Fluorescence of a wild-type p53 protein sample was monitored at 336 nm upon addition of small aliquots of potassium iodide. KI is known to quench tryptophan residues and, due to its a relatively large size, will access only those residues exposed on the surface of the protein. The ability of KI to quench the four tryptophans of p53 can be measured by a plot of the change in fluorescence as a function of quencher concentration. The slope ($K_{sv}$) of the linear fit (solid line) of the resulting data (triangles) describes the ability of KI to quench the tryptophans (Equation 2.7). $K_{sv}$ for wild-type p53 is $0.15 \pm 0.1$. 
of the protein that is reported to be loosely structured (66, 93, 95).

*Comparison of p53 Fluorescence to a Tryptophan Standard*

To discern whether the p53 tryptophans are intrinsically quenched in the protein structure and therefore unable to be additionally affected by collisional quenching, or buried and therefore inaccessible, a fluorescence standard was employed. NATA (N-acetyl-L-tryptophanamide) is a modified tryptophan moiety whose fluorescence in water corresponds approximately to that of a fully exposed tryptophan in a protein ($\lambda_{\text{max}}$ at 350 nm). As a standard, NATA is useful because it lacks the complicated environment that is able to affect tryptophan fluorescence in proteins. Comparison of p53 tryptophan fluorescence to NATA fluorescence was undertaken at equal molar tryptophan concentrations to allow simple qualitative comparisons. If the p53 tryptophans are not quenched, their signal should be of similar intensity as the standard. Figure 5.6 illustrates that NATA fluorescence is significantly greater than the fluorescence intensity of either p53 protein (per mole of tryptophan), indicating that significant quenching occurs in the native protein structure of both wild-type and S392E. The location of
Figure 5.6 Fluorescence spectra of wild-type and S392E vs. NATA.
Fluorescence spectra were collected for wild-type (blue), S392E (green), and NATA (red) at the same molar tryptophan concentration. N-Acetyl-L-tryptophanamide (NATA) is a commonly used tryptophan fluorescence standard that exhibits a $\lambda_{\text{max}}$ characteristic of a solvent-exposed tryptophan (350 nm). Samples were excited at 285 nm, and emission spectra were collected from 310-370 nm at 2 nm/sec.
the $\lambda_{\text{max}}$ of both p53 proteins (~336 nm) is significantly blue-shifted compared to the $\lambda_{\text{max}}$ of NATA, suggesting that the minimal unquenched fluorescence may derive from tryptophan residue(s) in an apolar environment.

*Fluorescence As a Monitor of Ligand Binding*

Changes in fluorescence intensity of both wild-type and S392E proteins were examined in the presence of DNA and core domain antibody, Ab1620. Both 20mer (conDNA) and 40mer DNA (40DNA) were added at concentrations equimolar to the proteins and were also examined in the presence of Ab1620. Similar experiments were also undertaken in the absence of p53 protein to allow appropriate corrections for additives to be made. Fluorescence intensity results were buffer-corrected, and all intensities are reported relative to protein fluorescence in the absence of additives (Figure 5.7). The addition of conDNA resulted in an apparent increase in S392E but not wild-type fluorescence intensity. A similar, but less intense result was seen upon the addition of 40mer DNA. The addition of Ab1620 had a significant effect on the S392E-DNA complexes, increasing fluorescence intensity nearly 2-fold for the S392E-conDNA
Figure 5.7 Effects of Ab1620 and DNA on p53 fluorescence. Fluorescence of WT and S392E protein samples was monitored at 336 nm upon addition of conDNA, 40DNA, or either DNA in the presence of Ab1620. Fluorescence of corresponding control samples (without protein) was subtracted from each sample before data were corrected to reflect the fraction of protein fluorescence in the absence of additives (yellow), defined as 1. Blue bars represent the corrected fluorescence of samples containing protein with DNA (as specified); green bars represent protein and DNA with Ab1620.
sample. Effects on the wild-type protein were much less significant, with a slight increase detected only for the wild-type-40DNA sample in the presence of Ab1620.

The ability of additives that interact with the core domain to alter the fluorescence intensity of p53 samples suggests that binding is able to reduce internal quenching effects for at least one tryptophan. This result could be accomplished by either the location of one or more tryptophan residues near the ligand binding site or a change in local environment that is propagated via a conformational change in response to ligand binding.

DISCUSSION

Determinants for protein structure are contained within the primary amino acid sequence of a protein. Though secondary structure algorithms can provide information on major structural elements from primary sequence, the effects of single amino acids on the structure and stability of a protein are not easily predicted. In recent years, determining the stability of p53 protein has gained importance from studies that have demonstrated the increased thermodynamic stability for DNA binding domains from many p53 hotspot mutants (99, 102, 184). These results suggest p53 mutants
commonly found in human tumors have a structural advantage over the wild-type protein. These studies have demonstrated that thermodynamic protein stability is a crucial component of protein function and can contribute to our insight into *in vivo* effects. The ability to attack cancer cells that lack functional p53 protein and subsequently exploit protein stability for use in cancer therapies requires a thorough understanding of the full-length wild-type protein stability.

We have examined both the full-length wild-type p53 protein and the "activated" S392E protein. We have previously demonstrated that S392E possesses significant structural alterations from the wild-type protein, which correlate with increased thermal stability of both structure and function in this mutant that mimics a common *in vivo* p53 modification.

*Chemical Denaturation Has No Effect on p53 Fluorescence*

Although fluorescence spectroscopy was used previously to monitor p53 DNA binding domain unfolding in the presence of urea (99), we demonstrate that similar experiments are not possible for the full-length protein. Between experiments described by the Fersht laboratory and those described herein, two significant differences are found. The DNA binding
domain contains only one tryptophan, Trp 146, whereas the full-length protein contains four, complicating a comparison of observed fluorescence. The second difference lies in the unknown structural arrangement of the tetrameric protein. Because tryptophans are sensitive to the polarity of their environment, the added interactions of the N- and C-termini within the full-length protein as well as those from other monomers may generate a significantly altered environment for Trp 146 from that found in the DBD alone. Bullock et al. (1997) also report observed tyrosine fluorescence of the native DNA binding domain (~310 nm) that is not detected in the native structure of either full-length protein, further illustrating the significant differences in the fluorescence profiles described by these two studies.

Guanidine Hydrochloride, but not Urea, Denatures Full-length Wild-type p53 and S392E

Denaturation experiments monitored by circular dichroism reveal that neither full-length wild-type p53 protein nor S392E is substantially unfolded by urea, in contrast to the isolated core domain (99). However, both proteins can be significantly unfolded (loss of ~75% signal) by the stronger denaturant, guanidine hydrochloride (GuHCl). The extent of signal loss
observed in the presence of GuHCl is much greater than that monitored for thermal denaturation, confirming that even 100 °C is not sufficient to unfold either p53 protein. Studies of the core domain peptide have demonstrated the presence of 1 mole Zn$^{2+}$ per mole of DNA binding domain, even under denaturing conditions (99), suggesting that the “unfolded” full-length p53 protein would likely maintain sufficient structure to coordinate the crucial Zn$^{2+}$. Renaturation of p53 is presumably dependent on the ability of p53 to maintain Zn$^{2+}$ coordination.

Denaturation of full-length p53 proteins with GuHCl is reversible and a relatively rapid kinetic process, complete within 1-2 min. The denaturation profiles suggest similar stabilities for the two proteins, although S392E appears slightly more stable to guanidine hydrochloride. A thorough thermodynamic analysis of unfolding was not possible due to the absence of clearly defined pre- and post-transition plateaus and the unknown nature of the transitions. However, presuming the transition corresponds to full-length monomer unfolding, estimated $\Delta G$ values, ($\Delta G_{D\to N}^{H_2O} = 4-5$ kcal/mol), are similar to those reported for the DNA binding core domain alone (99). Whereas 2.6 M urea was sufficient to unfold 50% of the core domain this concentration of denaturant did not elicit significant changes for either of the
full-length proteins, which required ~3 M GuHCl to achieve substantial unfolding.

*p53 Tryptophans are Resistant to Collisional Quenching*

Concentrations of GuHCl that are sufficient to unfold p53 proteins as monitored by CD do not result in any detectable change in fluorescence signal. Because information about tryptophan accessibility can help interpret data related to protein structure, efforts to determine why the environments of the four tryptophans of p53 do not reflect the observed structural changes during protein unfolding were undertaken.

Fluorescence involves the absorption of light (energy) to an excited state, which is followed by relaxation to lower levels within the excited state and a subsequent return to the ground state through the emission of a photon. If, during the time that a fluorophore is excited, alternate pathways of energy loss are dominant, the excited electron of the fluorophore will return to the ground state without the emission of light. When an alternate pathway for relaxation is provided via energy transfer to a small molecule that diffuses into the tryptophan environment, collisional quenching occurs (187, 218).
Because of its charge and large size, iodide is a commonly used fluorescence quencher that is only able to access residues exposed or partially exposed on the surface of a protein. Therefore, information about the accessibility of tryptophan residues can be gained from a study of iodide quenching. For both the wild-type and S392E proteins, iodide was unable to quench the observed tryptophan fluorescence. Complications of fluorescence-quenching data can occur in multi-tryptophan proteins due to the inability to determine which residue is responsible for the fluorescence signal. In this case, the lack of quenching simplifies this interpretation. The absence of significant quenching in p53 proteins could arise from either inaccessibility of tryptophan residues to the quencher or from intrinsic quenching of the tryptophan residues. Because three of the four tryptophans of p53 are located in the loosely structured N-terminal domain, the possibility of intrinsic, static quenching was investigated.

Significant Tryptophan Quenching is Found in the Native Structure

Bullock et al. (1997) report that tryptophan 146 is quenched in the native protein structure, but this quenching is relieved upon protein unfolding. However, experiments with the full-length protein reveal no
change in fluorescence under conditions that are sufficient for protein unfolding monitored by circular dichroism. A comparison of p53 fluorescence intensity to that of a tryptophan standard revealed that significant fluorescence quenching is present in the native p53 structure. Because p53 fluorescence does not change as a function of denaturant, p53 tryptophans must be continually quenched even as the protein denatures. Tryptophan quenching in a denatured protein structure is not unusual, and the archetypal myoglobin is one of the best known examples of a protein whose fluorescence signal is quenched in both the native and unfolded states (219).

p53 Tryptophan Fluorescence Reports Ligand Binding

Because both tryptophan fluorescence and quenching are environment-dependent, fluorescence spectroscopy can often be used to monitor ligand binding. Changes in fluorescence spectra can arise upon complex formation, including DNA and small molecule binding if the ligand either binds near a fluorescent residue or evokes a conformational change that alters the environment around a tryptophan. Although the tryptophans of p53 are quenched in the native conformation, they could still be capable
of reporting complex formation, particularly if quenching is relieved.

Ligand binding could result in a conformational change that removes one or more of the four tryptophans from its quenched environment or alternatively, the ligand could shield a fluorophore from its quencher. In both cases, fluorescence intensity could be expected to increase.

The change in fluorescence upon DNA binding to S392E and not to the wild-type protein is of particular interest, as is the identification of which of the four tryptophans is responsible for the observed phenotype. S392E but not wild-type fluorescence increases upon the addition of either DNA or core domain antibody. Tryptophan 146 is located in the DNA binding core domain (Figure 5.8) in a β-sheet corresponding to conserved region II.

Although this region does not contact DNA, it is located C-terminal to Loop 1 (Figure 1.8) which makes direct contacts to the major groove of specific DNA. It is possible that DNA binding by Loop 1 alters the environment of Trp 146, but it could also alter the environments of any of the other three tryptophans since their location with respect to the DNA binding surface is not known.
**Figure 5.8 Environment surrounding tryptophan 146.** **A.** A ribbon diagram of the crystal structure of the DNA binding domain of p53 bound to a half site of consensus DNA (96). The Trp 146 side chain, located in a β-sheet C-terminal to Loop 1, is highlighted in yellow. **B.** A close-up view of the residues surrounding Trp 146 showing van der Waals representations of the side chains (Xtalview).
Similarly, the origin of increased S392E fluorescence in the presence of Ab1620 is difficult to determine since the exact location of this epitope in the DNA binding domain is unknown. Information of the location of the Ab1620 epitope would be useful due to its sensitivity to structural differences between S392E and wild-type p53 proteins. However, identification of the one or more tryptophans that increase their fluorescence intensity upon ligand binding would require further mutational studies.

The fluorescence increase upon the addition of ligands that bind to the core domain suggests that p53 tryptophan fluorescence may report DNA binding or protein conformation. The ability of wild-type and S392E protein fluorescence to differentially report both DNA and antibody binding is but one more piece of evidence indicating conformational and functional differences between the two proteins.

*Structural Evidence for Tryptophan Quenching*

A structural investigation of two of the four tryptophans of p53 is possible with the use of X-ray structures of p53 domains (66, 96). Examination reveals further support for the conclusion that p53 tryptophans are quenched in the native structure. Tryptophan 146 is located in the N-
terminal end of the DNA binding core domain. Residues surrounding tryptophan 146 include arginine 110, aspartate 228, cysteine 229, and glutamine 144 (Figure 5.8). Since charged amino acids are capable of quenching tryptophans, the location of Trp 146 in this charged pocket is consistent with the experimental data.

Trp 23 is in a small peptide that was co-crystallized bound to the MDM2 protein (66). This peptide folds into an α-helix upon binding by MDM2. Residues on the same face of the peptide as tryptophan 23 include phenylalanine 19, leucine 26, and proline 27 (Figure 5.9). These residues constitute a hydrophobic surface that is bound by MDM2. When MDM2 is not bound to p53, these residues are likely to be solvent exposed. Such an environment for Trp 23 would also be consistent with low fluorescence intensity. Further work will be necessary to establish unequivocally the basis for the diminished fluorescence quantum yield for the remaining p53 tryptophans, but their position in the less structured N-terminal domain suggests that solvent exposure may account for their behavior.

Tryptophans of the full-length p53 protein are quenched both in the native and unfolded structures yet are able to report on core domain ligand binding of the “activated” mutant S392E. This novel tool to monitor p53
Figure 5.9 Environment surrounding tryptophan 23. A. A ribbon diagram of the crystal structure of a portion of the N-terminal domain of p53 (yellow) bound to a MDM2 peptide (lavender) (66). The Trp 23 side chain is highlighted in white. B. A close-up view of the residues surrounding Trp 23 demonstrates a hydrophobic MDM2 binding surface on p53. Van der Waals representations are made of the side chains (Xtalview).
ligand binding provides further evidence for significant differences between these two proteins that may in turn lead to increased understanding of the effects of “activating” modifications on p53 structure and function.
CHAPTER 6

CONCLUSIONS
p53 exhibits a low level of function – observed as the lack of apoptosis and cell cycle arrest – in normal, undamaged cells, yet can be activated to promote both these processes upon DNA damage (reviewed in 195). A myriad of cellular effects have been associated with DNA damage, including differential MDM2 degradation and multiple p53 posttranslational events (reviewed in 121, 123). Because of the high percentage of human cancer cells with non-functional p53 protein due to mutation, improper sequestration, or deletion, identifying the mechanisms that generate p53 activation is of significant value (23-25).

The activation of p53 that occurs in vivo upon DNA damage has been correlated to in vitro DNA binding results suggesting that p53 protein is not capable of specific DNA binding without alterations at its C-terminus (54, 135, 161-163). However, we and others have demonstrated that this in vitro activation derives from inhibitory effects of nonspecific DNA present in the in vitro assay conditions (171, 172, 214). We have illustrated that in vitro “latent” p53 is able to bind specific DNA with high affinity – similar to that measured for a mutant that mimics phosphorylation, corresponding to a state previously reported to be “activated” (80, 136, 203, 212). However, the capacity of nonspecific DNA to inhibit wild-type p53 binding to specific
DNA is extremely relevant to *in vivo* p53 protein activity. We propose a model to account for results observed *in vivo* and *in vitro* for "latent" and "activated" p53 proteins (Figure 6.1).

The ability of "latent" p53 to bind DNA has been demonstrated *in vitro* in the absence of nonspecific DNA, but binding is not detectable *in vivo* or in the presence of nonspecific DNA. Although wild-type p53 is able to bind specific DNA in this latent form, we suggest that it is the synergistic effect of two forces that prevent this function *in vivo*. Levels of p53 protein are kept low in undamaged cells through continuous degradation mediated by MDM2 (56-58, 60, 220). Numerous laboratories have demonstrated that the disruption of MDM2-mediated p53 degradation results in an "activated" p53 phenotype, including protein accumulation, DNA binding, and its subsequent downstream effects (134, 165-169). These studies suggest that p53 is simply not available in the cell to bind specific DNA. In fact, in the event that p53 protein begins to accumulate, MDM2 transcription can be increased in an undamaged cell by p53 itself (67).
Figure 6.1 Depiction of in vivo "latent" p53 regulation. p53 protein is continually degraded by MDM2, keeping protein levels diminished in normal cells. Release of degradation has been reported by numerous laboratories to result in "activated" p53 (see text). Nonspecific DNA is able to inhibit sequence-specific DNA binding, preventing "latent" protein from observable specific DNA binding in vivo, though this function is detectable in vitro in the absence of nonspecific DNA. The location of DNA relative to the tetrameric protein is meant to convey different binding sites for the two DNAs, not to depict confirmed orientations.
The ability of nonspecific DNA to inhibit specific DNA binding in the “latent” protein *in vitro* may provide a second level of control (171, 172, 214). Although 200-300 p53 response elements (p53RE) are proposed to exist in the human genome, a large excess of nonspecific DNA is present (156). If, as demonstrated *in vitro*, “latent” p53 protein is unable to bind to specific DNA in the presence of excess nonspecific DNA, latent protein that was able to avoid degradation would not be able to activate or repress transcription at p53RE sites. Others have suggested that although “latent” p53 cannot act as a transcription factor, some of its other less-well understood functions, *e.g.*, 3'-5' exonuclease activity, and/or DNA/RNA strand exchange could be active in this form of p53 protein, coined “noninduced” p53 (221, 222). In this manner, “noninduced” p53 activity/function could be dependent upon nonspecific binding rather than specific binding, consistent with our model.

“Activation” can be elicited by posttranslational modifications of p53 that include phosphorylation of serine 392 (80). Although the phosphorylation mimicked by the S392E substitution does not result in an increased affinity for conDNA compared to the wild-type protein, we have described significant structural and functional differences between these two
proteins. Structurally, S392E displays an observed increase in β-sheet and α-helical structure thermal stability as well as an increased percentage of α-helical structure as monitored by circular dichroism spectroscopy. Increased reactivity at 42 °C to a conformational antibody and increased fluorescence signal upon core domain DNA and antibody binding are also features of this protein. We have also demonstrated a decreased ΔCₚ upon conDNA binding and presented evidence for altered structure by denaturation studies in which S392E displays a significant deviation from wild-type behavior. Effects on the structure of this protein, even those demonstrated to result in core domain changes, surprisingly arise from a single mutation at the C-terminus of the protein. These structural effects indicate inter-domain communication of p53 that is further supported by measurable functional differences.

Experiments assaying DNA binding function presented in this thesis quantitatively describe differences between "latent/noninduced" and "activated" p53 proteins. S392E displays decreased functional sensitivity to temperature as well as increased cooperativity in conDNA specific binding experiments, suggesting altered DNA response. Increased S392E affinity for nonspecific DNA compared to the wild-type protein may account for the
increased affinity of S392E compared to wild-type for a conDNA target containing additional flanking nonspecific DNA sequences. Consistent with this result, S392E also displays an increased ability to maintain specific DNA binding in the presence of nonspecific DNA in a competition assay.

The ability of the "activated" protein to bind specific DNA in the presence of nonspecific competitor, despite increased affinity for the nonspecific target, demonstrates that S392E-specific DNA binding is not as sensitive to the presence of nonspecific DNA. If the S392E protein is inhibited by nonspecific DNA differently than the "latent/noninduced" protein, the addition of increasing amounts of nonspecific DNA to the S392E-conDNA complex should result in the retention of specific DNA to higher concentrations of NS2 than measured for the wild-type protein. These experiments are underway, and preliminary results indeed demonstrate different patterns for the two proteins.

We propose that one of the results of *in vivo* activation of p53 through DNA damage and other pathways may involve differential regulation of specific and nonspecific DNA binding (Figure 6.2). Specifically, our model predicts the ability of "activated" p53 protein (S392E) to bind simultaneously to specific DNA via the core domain and to nonspecific
Figure 6.2 Model of "latent" vs "activated" p53 regulation. *In vitro*, nonspecific DNA is capable of inhibiting specific DNA binding in the "latent" p53 protein (squares). Interestingly, this inhibition is not as strong in the "activated" protein (circles), despite increased affinity for nonspecific DNA. We propose that "activation" of p53 protein is possible both through the relief of MDM2-mediated degradation and by the ability of the modified protein (*i.e.*, S392E, CKII phosphorylation) to bind simultaneously to nonspecific DNA at its C-terminus and specific DNA at its core domain. The dashed line (asterisk) indicates the potential for S392E to form contacts with one DNA containing both specific and nonspecific elements via two separate domains.
DNA by the C-terminus. Thus, increased nonspecific DNA binding by “activated” p53 would serve to aid the protein in the \textit{in vivo} search for specific p53REs and would not prevent specific DNA binding at these sites. Many of our \textit{in vitro} experiments have used two distinct DNA targets to represent specific and nonspecific DNA. However, the increased affinity of S392E for 40DNA (which contains both specific and nonspecific DNA) over conDNA would arise, according to this model, from enhanced interactions of the two domains with separate segments of one DNA target. Experiments to explore this hypothesis are of significant interest to generate a thorough understanding of the effects of protein modification and activation on the \textit{in vivo} DNA binding function of p53. Interpretation of future experiments is enhanced by this quantitative analysis of DNA binding function and protein stability undertaken with both the full-length wild-type “latent/noninduced” p53 protein and an “activated” mutant.
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