Effect of p53 Protein Redox States on Binding to Supercoiled and Linear DNA*

(Received for publication, February 4, 1999, and in revised form, June 3, 1999)

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The binding of p53 to its DNA consensus sequence is modulated by the redox state of the protein in vitro. We have shown previously that reduced wild-type p53 binds strongly to supercoiled DNA (scDNA) regardless of the presence or absence of p53CON. Here we compare the effects of oxidation of p53 by azodicarboxylic acid bis[dimethylamide] (diamide) and other agents on p53 binding to p53CON and to scDNA. Oxidation decreases the binding of p53 to scDNA; however, under conditions where binding to p53CON in a DNA fragment is completely abolished, some residual binding to scDNA is still observed. Increasing the concentration of oxidized p53 confers minimal changes in p53 binding to both scDNA and p53CON. Reduction of the oxidized protein by dithiothreitol neither restores its binding to DNA nor to p53CON in DNA fragments. In the presence of excess zinc ions, oxidation of p53 is, however, reversible. We conclude that the irreversibility of p53 oxidation is due, at least in part, to the removal of intrinsic zinc from its position in the DNA binding domain accompanied by a conformational change of the p53 molecule after oxidation of the three cysteines to which the zinc ion is coordinated in the reduced protein.

In the last several years of this decade, p53 protein has become one of the most important molecules in the field of contemporary cancer research (1–6). Mutated p53 was found in about 50% of human malignancies. The majority of these mutations maps to the p53 core domain, which is responsible for sequence-specific binding to the p53 consensus sequence 5'-RRRC(A/T)(T/A)GYYY-3' (where R is a purine nucleoside, and Y is a pyrimidine nucleoside) separated by 0–13 base pairs. p53 is a transcription factor involved in maintaining the integrity of the genome. p53 responds to DNA damage by promoting cell cycle arrest in G1 phase (2, 3, 7) and in some cases by inducing apoptosis. The diverse functions of p53 are connected with its ability to bind DNA. The activity of the DNA binding domain in the full-length protein is modulated by other domains in the protein. Deletion of the C terminus produces a p53 molecule that is considered to be constitutively active for sequence-specific DNA binding (8, 9). The role of the basic C terminus of p53 (amino acids 363–393) in regulating DNA binding has been the subject of several studies. It has been shown that binding of the monoclonal antibody PAh21 (amino acids 372–382) (8, 10, 11), phosphorylation (e.g. serine 392) (12–15), or interaction with single-stranded DNA (16) results in activation of DNA binding. The addition of short peptides spanning the critical basic residues in the C terminus (9, 10, 17) or of the entire C terminus stimulates p53 DNA binding. Recently it has been shown (18) that the redox/repair protein Ref-1 is a strong activator of p53 for DNA binding both in vitro and in vivo. Altogether, the C terminus of p53 may play a role in negative regulation of sequence-specific DNA binding by the protein central domain. Negative regulation of p53 also occurs by binding of the oncoprotein mdm2 to the transactivation domain of p53 (19, 20).

The crystal structure of the core domain bound to DNA identifies a zinc ion coordinated by amino acids Cys-176, His-179, Cys-238, and Cys-242 in human p53 (21–23). Cation of Zn2+ ion by 1,10-phenanthroline abolished sequence-specific DNA binding by p53 (24–26). This binding was also influenced by the redox state of p53; oxidation yielded a form of p53 that did not bind to p53CON. On the other hand, oxidized and reduced protein forms showed no difference in the nonspecific DNA binding and in binding to mismatched DNA in gel mobility shift assays (26–30). Hydrogen peroxide treatment of cells resulted in a decrease in the ability of p53 to bind to p53CON and to transactivate target genes in vivo (28).

We have previously shown that wild-type human p53 protein (expressed in insect cells) is strongly bound to scDNA1 at native superhelix density (31) both in the presence and absence of p53CON in the DNA molecule. Binding of p53 to scDNA resulted in full or partial relaxation of DNA as demonstrated by scanning force microscopy images. In competition assays p53 showed a preference for scDNA as compared with linear double-stranded DNA containing p53CON. On the other hand, thermally denatured DNA was efficient in competing for the binding of p53 to scDNA. These results suggested that interactions involving both the core domain and the C-terminal domain may regulate the binding of p53 to scDNA.

In this paper, we compare the effect of p53 oxidation on binding of the protein to scDNA and to p53CON in linear DNA fragments. We show that oxidation strongly decreases but does not completely abolish the ability of p53 to bind to scDNA under conditions when binding to p53CON is completely abolished. We also show that oxidation of p53 by diamide, which has been considered to be an irreversible process, may become reversible under certain conditions.

* This work was supported by GACR Grant 204/97/K084, by a Volkswagen Stiftung (to E. P. and Professor T. M. Jovin), and by GA ASCR Grant A5004803 (to E. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶ Partially supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic. Grants 3477-3 and 4783-3 and GACR Grant 312/99/1550.
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1 The abbreviations used are: scDNA, supercoiled DNA; ssDNA, single-stranded DNA; p53CON, p53 consensus sequence; diamide, azodicarboxylic acid bis(dimethylamide); DTTH, 1,4-dithio-threitol; p53red, oxidized p53.
**Experimental Procedures**

Supercoiled DNA—pBSK, and pPGM1 (a derivative of pBSK), containing 20-mer p53CON (AGACATGCCTAGACATGCCT) were prepared as described (31). Fragments of the same plasmids generated containing 20-mer p53CON (AGACATGCCTAGACATGCCT) were prepared as described (31). The p53 concentration was determined with the protein dye binding procedure according to Bradford, with bovine serum albumin as a standard (33).

Oxidation of p53 Protein—Free p53 protein or p53-DNA complexes (prepared as described below) were treated with diamide (Sigma) or other reagents (concentrations and other details indicated in figure legends) in 50 mM KCl, 5 mM Tris-Cl, 0.5 mM EDTA, 0.01% Triton X-100 (pH 7.6) at 0 °C for 30 min. The reduced form of p53 was incubated with 2 or 5 mM DTT (Sigma) in parallel experiments.

DNA Binding Assay—DNA (400 ng of whole plasmid or 70 ng of isolated fragments) was added to the pretreated p53 samples to a final volume of 20 μl at p53/DNA molar ratios between 1 and 15 (calculated from the concentrations of DNA molecules and p53 tetramers) and incubated for 30 min on ice followed by electrophoresis on 1% agarose (Bio-Rad) gel in 30 mM Tris, 30 mM H3BO3. 0.7 mM EDTA buffer (pH 8.0) at 120 V and 4 °C for 4–4 h. Gels were stained with ethidium bromide and photographed.

Immunoblotting Analysis—Gels were blotted onto nitrocellulose transfer membrane Protran R (Schleicher & Schuell) or nylon membrane Hybond N+ (Amersham Pharmacia Biotech) in 3 mM NaCl, 0.3 mM sodium citrate (pH 7.0) under 80 millipascals on a vacuum blotting system (Amersham Pharmacia Biotech). The membrane was blocked by 5% milk in phosphate-buffered saline. p53 was detected with primary monoclonal antibody DO-1 (supernatant diluted 1:5–1:15) and secondary antibody anti-mouse IgG (alkaline phosphatase conjugate, Sigma) diluted 1:1000. p53 bands were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium chloride (Sigma).

Voltammetric Measurements—p53 was measured using an EG&G PAR 283A potentiostat/galvanostat connected to a Metrohm 663 VA stand electrode in hanging mercury drop electrode mode. The following settings were used for the differential pulse voltammetric measurements: accumulation time 90 s, accumulation potential −0.6 V, scan rate 10 mV s⁻¹, step potential 5 mV, pulse amplitude 50 mV.

Influence of Zinc Ions on p53 Oxidation—p53 was incubated with zinc ions at a given concentration (up to 0.1 mM) for 20 min on ice in 5 mM Tris, 0.5 mM EDTA, 0.01% Triton X-100. Diamide was added, and the samples were incubated for another 20 min, followed by a 20 min incubation in a 2-fold molar excess (related to diamide) of DTT. In some experiments, the order or concentrations of the above reagents was altered. Finally, the excess of Zn²⁺ ions was removed by 1 mM EDTA. After 20 min, DNA was added, and the p53-DNA complexes were allowed to form for 30 min on ice. For other details, see Figs. 4 and 5. Since Zn²⁺ and diamide were present in reaction mixtures, we examined the possibility of interactions between these two compounds by voltammetry and obtained negative results. Diamide was used in a 5- to 50-fold excess over zinc, and no significant effect of diamide concentration was observed; the diamide-zinc interaction was, thus, not further considered.

**Results**

Binding of p53 to scDNA Is Inhibited by Oxidation of the Protein with Diamide—Complexes of p53 with closed circular duplex DNA yielded a series of discrete bands on agarose gel (Fig. 1A, lane 2) (31). The complex of pBSK₂⁺, scDNA with reduced p53 produced at p53/DNA = 5 retarded bands 1–4 (Fig. 1A, lane 2) with maximal intensity in bands 1 and 2 containing 15 and 17% of total monomeric scDNA, as estimated...
from densitometric tracing, and the fastest band 0 of the free (protein-unbound) scDNA corresponding only to 14% of total monomeric scDNA in the DNA sample free of p53.

We used diamide to oxidize the sulfhydryl groups in the p53 protein (27) and found that its effect on p53 binding to scDNA did not depend on concentration between 0.2 and 5 mM diamide. Incubation of oxidized p53 with scDNA yielded only two retarded bands, a well developed band 1 and a weak, smeared band 2 (Fig. 1A, lane 4). Band 0 corresponded to 42% of total monomeric covalently closed circular DNA; this band was much more intense than the sc band obtained with reduced p53 (lane 2).

p53 protein was blotted and detected on a membrane using the antibody DO-1 (Fig. 1B), which maps to amino acids 20–25 of the N-terminal domain of p53 (34, 35). A superimposition of the immunoblot with the ethidium-stained gel (Fig. 1C) showed four bands corresponding to ethidium-stained bands 1–4 obtained with reduced p53 and an additional (slowest) band 5 detectable on the blot but not on the gel. No band on the blot corresponded to band 0 on the gel (Fig. 1B), in agreement with our assumption that band 0 is due to scDNA free of p53 protein (31). No free p53 was detected on the blot, suggesting that almost all reduced p53 protein was bound to DNA. On the other hand, an appreciable amount of the free p53 was detected with oxidized protein (Fig. 1B, lane 4). The band of p53 on the blot matching the ethidium-stained band 1 was more intense, whereas band 2 was weaker (Fig. 1, A–C, lane 4) than the respective bands of reduced p53 (lane 2); a weak band 3 was observed on the blot (Fig. 1B, lane 3) but not on the gel (Fig. 1A, lane 3). These results suggest that oxidation of p53 with diamide results in a decrease in its affinity to scDNA but does not completely abolish this binding.

Increasing the Concentration of Oxidized p53 Does Not Enhance p53 Binding to scDNA—Parks et al. (28) showed that increasing the concentration of reduced murine p53 resulted in enhanced protection of p53CON sequence, whereas increasing the concentration of oxidized p53 conferred minimal protection to p53CON in the DNase I footprinting of supercoiled DNA. Considering our finding (31) that (reduced) p53 binds preferentially to scDNA, their results (28) can be taken as evidence for the ability of reduced p53 to bind to p53CON in scDNA and of a decrease in this ability in p53ox.

We studied the effect of increasing the concentration of p53ox on its binding to pBSK−; i.e. to scDNA not containing p53CON (Fig. 1, A–C). With reduced p53, increasing the p53/DNA ratio from 5 to 10 resulted in a greater retardation of the bands and appearance of an intense band migrating more slowly than the band of relaxed DNA on the ethidium-stained gel (Fig. 1A, lanes 2 and 3). At p53/DNA = 10, a marked increase in intensities of the well resolved bands was observed on the blot (Fig. 1, inset in panel B, lane 3); these bands matched well with the respective ethidium-stained bands (Fig. 1C, lanes 2 and 3). In addition to a strong band 5, a weaker band 6 was observed on the blot that was not detectable by the ethidium staining (Fig. 1A, lane 3).

Increasing of p53ox/DNA from 5 to 10 also produced greater retardation of the DNA bands, but the bands were smeared both on the ethidium-stained gel and on the blot (Fig. 1, A–C, lanes 4 and 5). At p53ox/DNA = 15, the smear strongly prevailed over hardly detectable bands (Fig. 1, A–C, lane 6). The amount of free p53 observed on the blot (Fig. 1, B and C, lanes 4–6) increased with increasing p53ox/DNA; no free p53 was detected on the blot of reduced p53 (Fig. 1, B and C, lanes 2 and 3).

A similar experiment was performed using the isolated 474-base pair DNA fragment containing p53CON (instead of scDNA) in the presence of a 7-fold weight excess of calf thymus DNA (to reduce nonspecific binding of p53 to the fragment at high protein/DNA ratios). With reduced p53, we obtained a retarded band R at p53/DNA = 5 (Fig. 1, D and E). With oxidized protein at p53ox/DNA = 5, no band R appeared, and the band of the (protein-free) 474-bp fragment was much stronger than that obtained with reduced p53. Increasing the p53ox/DNA up to 15 did not produce any retarded band. These results suggest that increasing the concentration of p53ox does not improve the ability of the protein to bind to p53CON (Fig. 1, D and E) (in agreement with Parks et al. (28)) and to scDNA not containing p53CON (Fig. 1, A–C). At lower p53/DNA ratios, differences between the effect of p53 oxidation on binding to scDNA on one hand and to p53CON in linear DNA fragment on the other hand was observed; at p53/DNA = 2.5 or 1, p53ox produced at least one retarded band of p53-DNA complex, whereas under the same conditions p53ox did not bind to p53CON (data not shown). This suggests that the binding of p53 to scDNA may be less affected by p53 oxidation than binding to p53CON in a linear DNA fragment.

p53 Protein Bound to scDNA Is Partially Protected against Oxidation—It has been shown (27, 28) that p53 oxidized on air is unable to bind p53CON in a DNA 21-mer, but the DNA binding activity of the protein can be restored by the addition of DTT. However, oxidation of p53 with diamide prevented the binding activity of the protein can be restored by the addition of DTT. Oxidation of p53 with diamide prevented the DNA binding even in the presence of 5 mM DTT. Preformed complexes of p53 with p53CON-containing DNA oligomers were partially resistant to diamide at concentrations sufficient to oxidize the free protein and cancel its DNA binding activity (27). Using scDNA of pBSK− (not containing p53CON), we obtained similar results. Our experiments also indicated that the presence of an excess of DTT protected p53 against oxidation with diamide. On the contrary, binding of diamide-oxidized p53 was not improved by the addition of DTT. The effects of diamide on free protein and on p53 bound in a complex with scDNA were compared (Fig. 2). The diamide treatment of p53-DNA complexes produced a smaller effect than the same treatment of free p53, as detected both on the ethidium-stained gel (Fig. 2A) and on the immunoblot (Fig. 2, B and C). As compared with diamide-untreated DNA–p53 complexes (Fig. 2, lane 2), the bands in diamide-treated samples were more diffuse and smeared (Fig. 2, lane 4); such a pattern suggests a reduced stability of the diamide-treated complex,
pretreated protein simultaneously. p53/scDNA produced partial inhibition of the p53 binding, whereas H$_2$O$_2$ and other oxidants (hydrogen peroxide, osmium tetroxide, potassium permanganate, and sodium hypochlorite at 1 mM concentrations) were also tested for their influence on p53-scDNA binding. Compared with reduced p53, the oxidized protein produced a smaller number of retarded bands on binding to scDNAs detected both on an ethidium-stained gel (Fig. 3A) and on the blot (Fig. 3B, lanes 2, 4, 7, and 9). On the addition of thermally denatured calf thymus DNA (mass ratio ssDNA/ssDNA = 0.5), these bands were completely eliminated (lanes 5 and 10). Reduced p53 yielded the same result with pBSK, scDNA (lane 3), whereas pPGM1 retarded band 1 on the ethidium-stained gel (Fig. 3A) and on the blot was resistant to competition of ssDNA (Fig. 3B, lane 8), in agreement with the assumption that band 1 is due to binding of reduced p53 to p53CON (31). Binding of p53 to ssDNA was clearly observed on the blot (Fig. 3), suggesting that both the reduced and oxidized forms of p53 bind to ssDNA. When denatured pBSK, scDNA was used as the competitor, similar results were obtained (not shown). In some experiments involving p53 binding to DNA oligomers (e.g., 28), the supercoiled DNA was used as competitor. The results contained in this and in our previous papers (31, 33) showing specific binding properties of scDNAs should be therefore considered prior to the use of scDNA as a competitor; it cannot be excluded that reinterpretation of some earlier data will be necessary.

**Zinc Ions Protect p53 Protein against Irreversible Oxidation with Diamide**—In agreement with previous data (27), loss of DNA binding activity of baculovirus-expressed full-length human wild-type p53 due to its oxidation with diamide (concentration range between 0.2 and 10 mM) was irreversible and was not reverted by the addition of an excess of DTT. This conclusion could be made from the results obtained with sc pBSK(-) DNA (Fig. 4, lane 4) as well as with a linear p53CON-containing DNA fragment (Fig. 5, lane 4). This irreversibility might be due (a) to a deeper oxidation of sulfhydryl groups (as opposed to formation of disulfidic bonds), (b) to a release of zinc ion from the DNA binding domain resulting from oxidation of the three sulphydril groups to which the metal ion is coordinated, and (c) to an irreversible change in protein conformation due to protein oxidation (possibly related to b). To test possibility a, we examined p53 treated with 1 mM diamide, performic acid, KMnO$_4$, or OsO$_4$ by voltammetry with a mercury electrode in a solution containing cobalt ions (36–39). This voltammetric/polarographic method is known to provide a sensitive test for the presence of cysteine/cystine residues in proteins (Refs. 38–40 and references therein). Cystine and cysteine residues yield the same voltammetric effect, because the disulfidic groups are reduced at the electrode to sulfhydryl groups at less negative potentials than the potential of the measured catalytic current produced by the cysteine residues.

Oxidation of p53 with 5 mM diamide yielded a voltammetric curve identical to that obtained with p53 before its oxidation (Fig. 7), suggesting that the oxidation of cysteine with diamide did not proceed further than to a disulfidic group. Treatment of p53 with performic acid (known to oxidize sulfhydryl groups to sulfonic acid) with potassium permanganate (Fig. 7) and with osmium tetroxide (not shown) resulted in strong depression of the catalytic currents, suggesting deeper oxidation of sulfur in the protein. Similar results were also obtained with bovine serum albumin used as a control (not shown).

To test possibilities b and c we assumed that the irreversible loss of DNA binding might be prevented by the presence of abundant zinc ions in solution during the oxidation or reduction steps. When 20–100 μM zinc was added to p53 before incubation with 1 mM diamide (followed by the addition of DTT and EDTA), the irreversible effect of diamide both in scDNA (Fig. 4, lane 9) and in the p53CON-containing fragment (Fig. 5, lane 9) was partially prevented. The addition of zinc to the oxidized protein (after diamide and before DTT, Fig. 4, lane 8, and Fig. 5, lane 8) resulted in a further decrease of binding and in weakening of the p53 signal on the blots. The addition of 20–400 μM zinc after DTT (Fig. 4, lanes 9–10; Fig. 5, lane 9) had no effect. Nonspecific binding of p53 to linear fragments of DNA was prevented by higher concentrations of zinc ions (not shown). This indicates that p53 binding to DNA is prevented by zinc ions before and after its oxidation, which is in agreement with our previous results (32, 33) showing that zinc ions stabilize the DNA binding activity of baculovirus-expressed full-length human p53 due to its oxidation with diamide (concentration range between 0.2 and 10 mM) was irreversible and was not reverted by the addition of an excess of DTT. This conclusion could be made from the results obtained with sc pBSK(-) DNA (Fig. 4, lane 4) as well as with a linear p53CON-containing DNA fragment (Fig. 5, lane 4). This irreversibility might be due (a) to a deeper oxidation of sulfhydryl groups (as opposed to formation of disulfidic bonds), (b) to a release of zinc ion from the DNA binding domain resulting from oxidation of the three sulphydril groups to which the metal ion is coordinated, and (c) to an irreversible change in protein conformation due to protein oxidation (possibly related to b). To test possibility a, we examined p53 treated with 1 mM diamide, performic acid, KMnO$_4$, or OsO$_4$ by voltammetry with a mercury electrode in a solution containing cobalt ions (36–39). This voltammetric/polarographic method is known to provide a sensitive test for the presence of cysteine/cystine residues in proteins (Refs. 38–40 and references therein). Cystine and cysteine residues yield the same voltammetric effect, because the disulfidic groups are reduced at the electrode to sulfhydryl groups at less negative potentials than the potential of the measured catalytic current produced by the cysteine residues. Oxidation of p53 with 5 mM diamide yielded a voltammetric curve identical to that obtained with p53 before its oxidation (Fig. 7), suggesting that the oxidation of cysteine with diamide did not proceed further than to a disulfidic group. Treatment of p53 with performic acid (known to oxidize sulfhydryl groups to sulfonic acid) with potassium permanganate (Fig. 7) and with osmium tetroxide (not shown) resulted in strong depression of the catalytic currents, suggesting deeper oxidation of sulfur in the protein. Similar results were also obtained with bovine serum albumin used as a control (not shown).

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p53 DNA binding activity in supercoiled p53CON DNA fragments

presence of Co²⁺ or Ni²⁺ ions, the DO-1 antibody failed to detect p53 on the blot (Fig. 5, lanes 13 and 14). Analogous results were also obtained when DO-11 or DO-14 antibodies (instead of DO-1) were used to detect p53 on the blot (not shown). When 0.5 mM potassium permanganate was used as the oxidizing agent, no zinc protection against the irreversible loss of the sequence-specific DNA binding activity of p53 was observed under the given conditions (data not shown). However, the addition of 1 mM DTT before permanganate treatment was sufficient to protect p53 against oxidation. We believe that the inability of zinc ions to protect p53 against irreversible oxidation by permanganate is due to deep oxidation of sulfhydryl groups as demonstrated by voltammetric analysis (Fig. 7).

Our results suggest that the irreversibility of the effect of diamide oxidation of p53 is due to the release of zinc ion from the DNA binding domain, accompanied by a conformational change. In the absence of zinc, reduction of the disulfide bonds...
by DTT back to sulfhydryl groups cannot restore the original DNA binding activity of p53. Our results do not exclude the possibility that zinc ion changes only its position in some protein molecules and/or remains attached to the histidine residue.

**DISCUSSION**

**Binding of Reduced p53 to scDNA**—A number of papers have been devoted to studies of the specific interactions of p53 with the consensus sequence in DNA fragments and oligonucleotides (Refs. 5, 6, 22, 28, 41, and 42 and references therein). Strong binding of p53 to supercoiled DNA has been described only recently (31), and many aspects of this binding will require further elucidation. In this paper we show that discrete DNA bands on ethidium-stained gels resulting from binding of p53 to scDNA (Figs. 1A, for example) coincide with the bands on immunoblots (Fig. 1B), suggesting that ethidium-stained DNA bands contain p53 and that formation of these bands is due to p53 binding to scDNA. At higher p53/scDNA ratios some bands can be observed both on the gel and on the immunoblot that run more slowly than the relaxed (probably nicked) DNA (Fig. 1, A–C). Such bands cannot be due only to the DNA relaxation observed on atomic force microscopy images (31). Contribution of the number and/or of the oligomerization state (43–45) of the bound p53 molecules to the DNA retardation therefore have to be considered. We cannot exclude that this contribution is more significant than the DNA relaxation itself. In fact our preliminary results suggest that under certain conditions binding of p53 to linear DNA molecules can result in the formation of discrete DNA bands in agarose gels.

**Role of p53 Domains in Binding to scDNA**—Oxidation of p53 with diamide decreases its ability to bind to scDNA (manifested by the appearance of appreciable amounts of free (DNA unbound) p53 on immunoblots, Fig. 1B) and to form sharp bands (Figs. 1). We have previously speculated that p53 binding to scDNA involves both the core and the C-terminal domain (31). Decrease of p53 binding to scDNA due to oxidation of the protein (Fig. 1) and its reversal in the presence of zinc ions followed by DTT reduction (Fig. 4) suggest the involvement of the core domain in binding to scDNA. Cysteine residues (which can be oxidized by diamide) are located in the core domain but not in the C-terminal and N-terminal domains. Our preliminary results obtained with the isolated core domain2 show that the core domain is not sufficient for strong p53 binding to scDNA in the absence of p53CON and for generation of the band ladder in the agarose gel (Fig. 1). Thus, participation of another p53 domain in binding to scDNA appears probable. Strong competition of ssDNA (Fig. 3) identifies the C terminus as a prime candidate. Tight binding of p53ox to single-stranded (denatured) DNA (Fig. 3) is in agreement with the finding that oxidation of p53 does not abolish nonspecific binding to mismatched DNA (28). On the other hand, nonspecific binding of p53 to the 2513-base pair double-stranded DNA fragment is partially inhibited by diamide oxidation (Fig. 5), in agreement with the proposed (46) participation of the core domain in the nonspecific p53 binding.

**Irreversible Loss of DNA Binding Activity and the Effect of Zinc Ions**—In the co-crystal structure (21), two DNA binding loops are tethered to one another and to a small helix via cysteine and histidine residues coordinating a zinc ion. This ion is critical for the DNA binding activity being tightly bound in the DNA binding domain, as documented by its resistance to chelation by DTT (up to 40 mM) and the absence of Zn2+ loss during protein denaturation (1 mol of Zn2+/mol of core domain remains bound even in 5 M urea) (47). We have recently found (33) that binding of zinc ions (present at micromolar concentrations in the absence of chelating agents such as EDTA or high excesses of DTT) to other sites in p53 reversibly inhibits binding of full-length human reduced p53 to p53CON in linear

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DNA fragments as well as to scDNA (not containing p53CON).

The presence of zinc ions during diamide oxidation of p53 prevents the irreversible loss of the ability of p53 to bind to p53CON in linear DNA fragments (Fig. 5) and to scDNA (Fig. 4). This result suggests that the major cause of the irreversibility of loss the DNA binding activity observed in p53ox may be the displacement of the intrinsic zinc from the DNA binding domain.

The mechanism determining the irreversibility of the p53 oxidation might be full or partial removal of the intrinsic zinc from p53, resulting from oxidation of cysteines. When DTT is used for reduction of cystine residues back to cysteines, intrinsic zinc ions, reduced cysteines immediately bind the zinc ion in their vicinity, and the protein assumes its native conformation with one intrinsic zinc ion bound to three cysteine and one histidine residues. An excess of EDTA removes free and loosely bound zinc ions, thereby making the p53 molecule fully competent for sequence-specific DNA binding. This mechanism does not occur if the oxidation agent (such as peroxynitrite) causes further changes in the protein molecule that cannot be restored by the DTT reduction.

In contrast to irreversible oxidation of full-length p53 by diamide reported previously by Hainaut and Milner (27), Hupp et al. (48) observed a reversible loss of DNA binding activity of C-terminal-truncated p53 and of full-length p53 activated with the PAB421 antibody due to diamide oxidation. In agreement with Hupp et al. (48), our preliminary results showed a significant reversibility of the oxidation in isolated p53 core domain and in bacterially expressed full-length p53 activated with the PAB421 antibody due to diamide oxidation. In agreement with Hupp et al. (48), our preliminary results showed a significant reversibility of the oxidation in isolated p53 core domain and in bacterially expressed full-length p53 activated with the PAB421 antibody due to diamide oxidation. In agreement with Hupp et al. (48), our preliminary results showed a significant reversibility of the oxidation in isolated p53 core domain and in bacterially expressed full-length p53 activated with the PAB421 antibody due to diamide oxidation.

Acknowledgments—We are grateful to Dr. M. Sheard, Dr. P. Pečinka, and Dr. J. Fajkus for critically reading of the manuscript and to D. Fridrichová for technical assistance.

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