Identification of Two Reactive Cysteine Residues in the Tumor Suppressor Protein p53 Using Top-Down FTICR Mass Spectrometry

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Abstract
The tumor suppressor p53 is a redox-regulated transcription factor involved in cell cycle arrest, apoptosis and senescence in response to multiple forms of stress, as well as many other cellular processes such as DNA repair, glycolysis, autophagy, oxidative stress and differentiation. The discovery of cysteine-targeting compounds that cause re-activation of mutant p53 and the death of tumor cells in vivo has emphasized the functional importance of p53 thiols. Using a combination of top-down and middle-down FTICR mass spectrometry, we show that of the 10 Cys residues in the core domain of wild-type p53, Cys182 and Cys277 exhibit a remarkable preference for modification by the alkylation reagent N-ethylmaleimide. The assignment of Cys182 and Cys277 as the two reactive Cys residues was confirmed by site-directed mutagenesis. Further alkylation of p53 beyond Cys182 and Cys277 was found to trigger cooperative modification of the remaining seven Cys residues and protein unfolding. This study highlights the power of top-down FTICR mass spectrometry for analysis of the cysteine reactivity and redox chemistry in multiple cysteine-containing proteins.

Key words: p53, Tumor-suppressor, FTICR mass spectrometry, Top-down fragmentation, Middle-down fragmentation, Cysteine reactivity

Introduction
The tumor-suppressing protein p53 is a transcription factor best known for its key role in mediating cell cycle arrest, apoptosis, or senescence in response to numerous different forms of cellular stress. Over recent years, p53 has also been implicated in the regulation of many other cellular processes including DNA repair, glycolysis, autophagy, oxidative stress and differentiation [1–3]. It has also been shown to exert a pro-apoptotic effect independently of its transcriptional activity [4]. Despite over 30 years of extensive p53 research, comparatively little is known about the specific molecular mechanisms that allow p53 to discriminate between its different functions, and there is particular current interest in determining how p53 differentially activates expression of its target genes [5].

The DNA-binding domain of human p53 contains 10 cysteine residues (Cys124, 135, 141, 176, 182, 229, 238, 242, 275, and 277), nine of which are highly conserved (all but Cys229) [6] and three of which are involved in the coordination of a zinc ion (Cys176, 238, and 242, along with
There is clear and mounting evidence suggesting that redox-regulation of specific Cys residues in p53 plays a crucial role in controlling its activity. The redox-state of p53 cysteine residues influence its capacity to bind to consensus DNA in vitro and in vivo [8–13] and oxidation of endogenous p53 cysteines has been observed in both stressed and unstressed cells [14–16]. Proteins responsible for modulating Cys redox-state, such as thioredoxin (Trx) and redox factor 1 (ref-1), participate in the regulation of p53 activity [17, 18], and multiple signaling pathways exist that link reactive oxygen species (ROS) and reactive nitrogen species (RNS) with p53 [19, 20]. In addition, the recent discovery of cysteine-targeting electrophiles that reactivate mutant p53 in vivo, resulting in apoptosis of tumor cells, emphasizes the functional importance of p53 cysteines [21, 22].

Despite the growing interest in redox-regulated proteins, analysis of multiple Cys-containing proteins remains a challenge. Previous methods used to define the oxidation/modification state of wild-type (wt) p53 Cys residues have not allowed site-specific information on all 10 Cys residues. Here, we show that Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) [23], combined with top-down fragmentation using collision-induced dissociation (CID) [24] and electron capture dissociation (ECD) [25, 26], enables simultaneous and unambiguous definition of the modification state of each Cys residue in the p53 DNA-binding domain. In top-down protein mass spectrometry, the mass-to-charge ratio (m/z) of an intact protein is first measured, followed by isolation and fragmentation of the intact molecule. Analysis of the resulting fragments permits the unambiguous assignment of combinatorial protein modifications [27, 28]. FTICR is the method of choice for top-down MS, due to its superior mass resolving power and mass accuracy, which permits the accurate mass measurement of an intact protein, and is also required to resolve and identify the many multiply-charged product ions that are generated from fragmentation of an intact protein [29, 30].

We have studied the reactivity of p53 cysteine residues towards alkylation with the thiol-derivatizing reagent N-ethylmaleimide (NEM), a reagent which has previously been shown to have a concentration-dependent effect on specific DNA-binding of p53 [31]. Low concentrations of NEM resulted in increased DNA-binding, whereas higher concentrations resulted in decreased DNA-binding. The same trend is observed for alkylation of mutant p53 by the anti-tumor maleimide compound MIRA-1 and its analogues [32]. FTICR mass spectrometry of NEM-modified p53 core domain has revealed that alkylation of the protein proceeds in distinct steps and that two cysteine residues on the p53 surface display enhanced reactivity compared with the remaining Cys residues. We hope that the results from this study will allow more targeted and focused investigations into the unknown redox-mechanisms that are involved in regulating p53 function.

### Experimental

#### Materials

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich. All solvents were purchased from Fisher Scientific and were of LC-MS grade purity.

#### Recombinant Expression and Purification of p53 Core Domain

Wild-type human p53 core domain (a.a. residues 94-312) was expressed from the vector pRSET(A) and purified as previously described [33, 34]. Briefly, *E. coli* C41 (DE3) cells, harboring the pRSET(A) plasmid were grown to an OD₆₀₀ ~0.6–0.8 at 37°C before the temperature was reduced to 22°C, and overnight expression was induced with 1 mM isopropyl β-D-thiogalactoside. Cells were lysed using BugBuster Protein Extraction Reagent (Novagen) and protein was purified using a HiTrap SP XL column (GE Healthcare) on an ÄKTA FPLC system. p53 concentration was determined from A₂₈₀nm using the extinction coefficient ε=17,130 M⁻¹ cm⁻¹ [35]. Purified protein was stored at −80°C in the presence of 10% glycerol.

#### Site-Directed Mutagenesis

Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. p53 Cys mutants were expressed and purified as described above.

#### Cysteine Alkylation

All alkylation experiments were performed in 25 mM sodium phosphate (pH 7.2), 100 mM KCl, 0.2 mM EDTA, and 20% glycerol. Buffer exchange was performed using PD MiniTrap G-25 columns (GE Healthcare). Wild-type or mutant p53 core domain (35 μM) was incubated with 0, 0.05, 0.1, 1, 2, or 5 mM N-ethylmaleimide (NEM) for 15 min at 22°C or 37°C. At 22°C, cysteine alkylation was quenched by the addition of dithiothreitol (DTT) to a final concentration of 25 mM. At 37°C, alkylation was quenched by trichloroacetic acid (TCA) precipitation and protein was re-suspended in 6 M guanidine hydrochloride, 25 mM sodium phosphate (pH 7.2), and 25 mM DTT. For time course experiments, 1 mM or 0.5 mM NEM was incubated with wild-type or mutant p53 core domain (35 μM) at 22°C or 37°C, respectively. Aliquots were removed at various time points and quenched with DTT or TCA precipitation. Quenched samples were analyzed by on-line liquid chromatography FTICR mass spectrometry (LC-FTICR MS).

#### Proteolytic Digestion and Sample Preparation

For middle-down or bottom-up MS, quenched samples of wild-type p53 treated with 0.1 mM NEM were incubated...
with Lys-C (Roche) or trypsin (Promega) for 4 h at 37°C at an enzyme to protein ratio of approximately 1:20 by weight. Following Lys-C digestion, precipitated protein was collected by centrifugation at 13,000 rpm and resuspended in 6 M guanidine hydrochloride, 25 mM sodium phosphate (pH 7.2), and 10 mM DTT. Prior to mass spectrometry, all digested samples were desalted and buffer-exchanged into 50% acetonitrile and 0.6% formic acid using C₁₈ ZipTip pipette tips (Millipore).

**On-line LC-FTICR MS**

On-line liquid chromatography was used as a desalting step prior to mass spectrometry. An Ultimate 3000 HPLC system (Dionex) was equipped with a monolithic PS-DVB (500 μm × 50 mm) reverse-phase analytical column (Dionex) and coupled to a Triversa NanoMate (Advion BioSciences) for nano-electrospray ionization (nESI). Protein (5 pmol) was loaded onto the column (maintained at 60°C) followed by a 10 min linear gradient from 2% to 70% acetonitrile (flow rate 20 μL/min). Prior to top-down MS of 2NEM-p53, LC eluent containing intact NEM-modified p53 core domain was manually collected and directly infused into the mass spectrometer.

**FTICR Mass Spectrometry**

All mass spectra were recorded on an apex-Qe or solariX FTICR mass spectrometer equipped with a 12 T superconducting magnet (Bruker Daltonics). Direct infusion of protein/peptide samples (typical concentration ~10 μM) was achieved using the Triversa NanoMate for denatured spectra, or a solariX ESI source (Bruker Daltonics) for native protein spectra. Native spectra were recorded from 50 mM ammonium acetate (pH 7) and 10% methanol. Transient data size was 512 Kword for each acquisition. Ions were detected over 550–3800 m/z for denatured conditions (transient length 0.8 s) and 800–4000 m/z for native conditions (transient length 1.4 s).

**Tandem FTICR Mass Spectrometry**

MS/MS was performed on an apex-Qe FTICR mass spectrometer equipped with a 12 T superconducting magnet. Specific ion species were isolated using the mass resolving quadrupole and MS/MS was performed using collision-induced dissociation (CID) or electron capture dissociation (ECD). Typical collision energy values for top-down and middle-down MS were between ~10 and ~22 eV. The collision energy used to fragment the tryptic peptide VCACPGR was ~29 eV. For ECD fragmentation, 1.8 A was applied to the hollow dispenser cathode (HeatWave Technologies) and pulse lengths were between 8 and 18 ms; 100–500 transients were averaged for fragmentation spectra.

**Data Analysis**

All spectra were externally calibrated using ES tuning mix (Agilent Technologies) and analyzed using DataAnalysis software (Bruker Daltonics). Neutral spectra were created using Maximum Entropy deconvolution (Bruker Daltonics). Isotope distributions of specific charge states were predicted from theoretical empirical formulas. These were overlaid upon the recorded experimental data as scatter plots, with the theoretical apex of each isotope peak designated by a circle. Relative ratios of alkylated forms of p53 were calculated from peak heights [36]. The SNAP 2.0 algorithm was used for automated peak picking of top-down and middle-down fragmentation data and the resulting mass lists were searched against the relevant primary sequences using ProSight PTM [37] or Biotools (Bruker Daltonics) software. Error tolerances were 10 ppm.

**Results and Discussion**

**Concentration and Time-Dependence of p53 Alkylation**

Accurate mass measurement of p53 core domain prior to alkylation confirmed that each of the 10 cysteine residues was in the reduced form (Cys-SH; Fig. 1). In addition, analysis of the protein under native MS conditions (i.e., neutral pH and ~10% organic solvent to aid the desolvation process) revealed the presence of one Zn²⁺ ion per protein molecule (Fig. 1). This observation is consistent with the known metal binding properties of p53 and indicates correct folding of the p53 DNA-binding domain [38, 39]. p53 core domain (35 μM) was incubated with increasing concentrations of NEM for 15 min at 22°C. The reaction was quenched by the addition of dithiothreitol (DTT) and the degree of thiol alkylation was monitored by FTICR MS. The resulting deconvoluted neutral mass spectra (Fig. 2a) clearly show that p53 is predominantly modified with two NEM groups (2NEM-p53) at low concentrations of alkylating reagent (NEM<1 mM). At higher concentrations (NEM>1 mM), p53 was modified with ten NEM groups, i.e., all Cys residues were alkylated. Complete conversion to 10NEM-p53 was observed with 5 mM NEM (see Supplementary Figure S1 for accurate mass measurement of modified forms of p53). It was notable that only relatively small amounts of species with intermediate stages of alkylation were detected.

To monitor the time-dependence of alkylation, p53 was incubated with 1 mM NEM and the reaction was quenched with DTT at various time points followed by FTICR MS. Rapid alkylation of two cysteine residues was apparent; after 5 s the neutral mass spectra (Fig. 2b) showed that ca. 32% of p53 was monoalkylated and 63% dialkylated. The proportion of 2NEM-p53 increased to ca. 85% after 15 s and ca. 100% by 1 min. Alkylation of a third Cys residue (i.e., formation of 3NEM-p53) was not apparent until the 5-min
time point, after which direct conversion of 3NEM-p53 to 10NEM-p53 was observed.

p53 Alkylation at Physiological Temperature

The p53 core domain is known to have a low thermodynamic stability [40] and to unfold at body temperature [41]. Therefore, concentration and time dependence of p53 alkylation was investigated at 37°C (Supplementary Figure S2). A similar pattern of p53 alkylation was observed, namely, initial alkylation of two Cys residues followed by direct conversion of 3NEM-p53 to 10NEM-p53 upon increasing NEM concentration or time. At 37°C, complete conversion to 10NEM-p53 was observed with 0.5 mM NEM, and ca. 80% of p53 had been fully alkylated by the 5-min time point.

Identification of Reactive Cysteine Residues

In order to identify the two preferentially alkylated Cys residues using top-down FTICR mass spectrometry, individual charge states (26+, 29+, 31+ and 32+) of the 2NEM-p53 species were sequentially isolated and fragmented using CID or ECD (Fig. 3). The resulting fragment masses were searched against theoretical fragment mass lists for each permutation of Cys-NEM (assigned product ions are listed in Supplementary Tables S1 and S2). Taken together, top-down fragmentation data resulted in 51% sequence coverage, and allowed assignment of the oxidation state of eight out of the 10 cysteine residues within p53 (Fig. 3c). No fragment ions were observed between Cys275 and Cys277 and, thus, the state of these two residues could not be distinguished unambiguously. Top-down FTICR MS of 2NEM-p53 indicated that Cys182 was alkylated and the remaining NEM group was located on either Cys275 or Cys277.

In order to increase sequence coverage over the internal region of the p53 core domain and to obtain a diagnostic fragment between Cys275 and Cys277, 2NEM-p53 was cleaved with Lys-C protease. Such enzymatic or chemical cleavage of proteins into large peptides prior to tandem mass spectrometry provides a useful strategy (termed middle-down mass spectrometry) for increasing sequence coverage compared with fragmentation of the intact protein [42]. Lys-C cleavage resulted in a 127 amino-acid peptide, from Gln165 to Lys291, containing the seven cysteine residues

Figure 1. Accurate mass measurement of p53 core domain by FTICR MS. (a) nESI mass spectrum of p53 recorded under denaturing conditions. (b) 24+ charge state of denatured p53. The experimental isotope distribution is consistent with the theoretical isotope distribution of apo-p53 core domain with each cysteine residue in the reduced form (empirical formula [C1055H1697N321O324S16]24+; Δ; 5 ppm mass measurement error (MME); 80,000 resolving power (RP) at m/z 1023.9). (c) ESI mass spectrum of p53 core domain recorded under native conditions. (d) 9+ charge state of native p53. The experimental isotope distribution is consistent with the theoretical isotope distribution of p53 core domain bound to one Zn²⁺ ion (empirical formula [C1055H1680N321O324S16Zn]9+; Δ; 1 ppm MME; 135,000 RP at m/z 2735.9)
from Cys176 to Cys277. Incubation of 2NEM-p53 with Lys-C resulted in a protein precipitate. This was resuspended in 6 M guanidine hydrochloride, desalted, and analyzed by FTICR mass spectrometry (Supplementary Figure S3).

Individual charge states (13+, 14+, 18+, 19+, 20+, and 21+) of the 127 amino-acid peptide modified with two NEM groups (2NEM-p53Lys-C) were sequentially isolated and fragmented using CID or ECD (Fig. 4; assigned product ions are listed in Tables S3 and S4). Fragmentation of 2NEM-p53Lys-C resulted in an increase in sequence coverage from 39% (achieved for the region Gln165 to Lys291 in intact 2NEM-p53) to 67%, and allowed the oxidation state of each of the seven cysteine residues to be determined (Fig. 4c). Increased fragmentation between Cys176 and Cys182 confirmed the previous assignment of a NEM group on Cys182. Middle-down MS also resulted in a fragment ion between Cys275 and Cys277 (y16; Figure S3), which supported assignment of the second NEM group on Cys277. This assignment was confirmed by bottom-up MS using a tryptic digest of 2NEM-p53. The peptide mixture was directly infused into the mass spectrometer without prior separation. Five out of the seven cysteine-containing tryptic peptides were detected (Supplementary Figure S4). Hence, use of the bottom-up approach alone does not allow the oxidation state of all ten cysteine residues in p53 to be determined. However, the peptide containing Cys275 and Cys277 (VCACPGR) was detected and its mass-to-charge ratio (m/z) corresponded to the addition of one NEM group (Figure S4). Fragmentation of this peptide by CID confirmed the assignment of the NEM group on Cys277 (Figure S4 and Table S5).

**Alkylation of p53 Cysteine Mutants**

In order to test the FTICR MS fragmentation results showing that Cys182 and Cys277 are preferentially alkylated in p53, alkylation experiments were repeated with the p53 cysteine-to-serine mutants C182S, C277S, and the double mutant C182/277S. Analysis of the mutants under native MS conditions confirmed that each variant was bound to one Zn²⁺ ion, thus indicating that the mutations did not have detrimental effects on p53 tertiary structure (Supplementary Figure S5).

The NEM titration and time course experiment were carried out for each mutant at 22°C (Fig. 5). As expected, for C182S and C277S only one cysteine residue was preferentially alkylated, and for the double mutant C182/277S, no preferential alkylation was observed. In both single mutants, direct conversion to 9NEM-p53 was observed upon alkylation of a second Cys residue, and in the double mutant, direct conversion to 8NEM-p53 was observed upon alkylation of one Cys residue. For each mutant, the formation of fully alkylated species became notable at 1 mM NEM (at 15 min), similar to wild-type p53.

**Preferential Alkylation of Cys182 and Cys277**

Mass spectrometry of p53 core domain incubated with the alkylating reagent NEM under various conditions revealed that alkylation of p53 cysteine residues proceeds in distinct steps. Initially, two cysteine residues are rapidly modified to form a stable dialkylated species. An increase in NEM
concentration or reaction time leads to alkylation of a third Cys residue, which initiates rapid alkylation of the remaining seven Cys residues. Alkylation of wild-type p53 was repeated at 37°C and the same trend was observed, albeit at a faster rate. The use of top-down and middle-down FTICR MS allowed the unambiguous identification of Cys182 and Cys277 as the sites of preferential alkylation. These assignments were confirmed by alkylation and MS analysis of the p53 mutants C182S, C277S, and C182/277S.

Solvent accessibility values for each of the 10 thiol groups in p53 are listed in Table 1. These were determined from the X-ray crystal structure of the p53 core domain (PDB ID 2OCJ) using Accelrys Discovery Studio 2.5 software [43]. Cys182 and Cys277 are the most accessible residues, accounting for their preferential reactivity with alkylating reagent. The sulfur atoms in Cys182 and Cys277 are significantly more accessible than the next most exposed sulfur atom, that of Cys229 (accessibility values are 39.34 and 43.34 for Cys182 and Cys277 respectively, and 14.82 for Cys229). It can clearly be seen in the surface contour representation of the crystal structure of p53, shown in Fig. 6, that Cys182 and Cys277 are the most accessible cysteine residues on the protein surface.
During the preparation of this manuscript, X-ray crystallography data was reported by Kaar et al. [44], which is in agreement with the p53 FTICR mass spectrometry results presented here. Stabilised p53 core domain (T-p53C; an engineered p53 variant that contains the four mutations M133L/V203A/N239Y/N268D and displays increased thermostability compared with the wt protein [45]) containing the cancer-derived mutation Y220C was treated with an electrophilic compound, 3-benzoylacrylic acid, and the resulting crystal structure revealed pronounced electron density surrounding cysteine residues 182, 229, and 277. However, the density was not sufficient to model the

Figure 5. Reaction of N-ethylmaleimide with p53 core domain mutants C182S, C277S, and C182/277S. (a) NEM Titrations. (b) Alkylation over time. Reaction conditions were the same as described for wild-type p53. Deconvoluted neutral mass spectra are shown.
modifications unambiguously. Kaar et al. [44] went on to use a bottom-up MS approach to investigate the order of cysteine reactivity in T-p53C-Y220C with 3-benzoylacrylic acid. They reported that they were able to map each Cys residue using MALDI-TOF MS and LC-MS/MS. Curiously, they found Cys124 and Cys141 to be the most reactive cysteine residues. This is in conflict with both their X-ray crystallography data and the top-down FTICR MS results described here. Intriguingly, Velu et al. [46] also concluded that Cys141 is the most reactive cysteine residue in p53, after using a bottom-up MS approach to investigate cysteine alkylation. Cys141 has a solvent accessibility value of zero, and the X-ray crystal structure of the p53 core domain shows that it is buried within two sides of the β-sheet sandwich (see Table 1 and Fig. 6). A major draw-back of bottom-up MS is that when analysing peptides originating from more than one protein isoform, the ‘parent’ isoform from which a peptide originated is unknown. Therefore, investigating the order of Cys reactivity using a bottom-up approach requires that an individual isoform of modified protein is completely pure prior to proteolytic digestion. It may be that tryptic peptides originating from p53, which was alkylated beyond Cys182, 229, and 277, were present in the peptide samples analysed by Kaar et al. [44] and Velu et al. [46], and resulted in the misassignment of Cys reactivity.

Interestingly, both Cys182 and Cys277 have previously been implicated in redox-regulation of p53. Cys277 is a DNA-binding residue [7] and oxidation of this residue has been implicated in differential gene recognition [11]. Cys277 has also been identified as a possible substrate for selenomethionine (SeMet)-dependent redox-regulation of p53 [15], and it has been postulated that the redox-state of Cys277 serves as a switch to activate DNA repair machinery [47]. Furthermore, an electrophilic cyclopentenone prostaglandin, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), has been shown to bind to Cys277 in vivo, leading to an increase in p53 stability and reduction of its transcriptional activity [48]. Cys277 is located in the conserved p53 motif BOX-V (amino acid residues 272-288). BOX-V controls the specific activity of p53 by binding to enzymes that mediate either p53 phosphorylation or ubiquitination [49]. Interestingly, redox-regulation of a cysteine residue in the NF-κB related protein, c-Rel, regulates phosphorylation of the protein, which in turn regulates its transcriptional activity [50]. It is entirely plausible that Cys277 plays a similar role in p53.

Cys182 has recently been shown to be particularly susceptible to diamide oxidation in vivo. Held et al. [16] used a differential alkylation approach, along with proteolysis and multiple reaction monitoring (MRM) peptide mass spectrometry, to identify redox sensitive cysteines in endogenous p53. Only seven of the 10 Cys residues could be monitored using this approach (all but Cys229, 238, and 242). Incomplete protein sequence coverage is a problem common to peptide-based proteomic strategies for mapping post-translational modifications, and is one of the principal reasons for the growing recognition of the power of FTICR MS for studies on intact proteins.

### Table 1. Solvent accessibility of thiol groups in the p53 core domain

<table>
<thead>
<tr>
<th>Cysteine</th>
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<td>141</td>
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*Calculated using Accelrys Discovery Studio 2.5 software from the X-ray crystal structure of the p53 core domain (PDB ID 2OCJ)

Figure 6. X-ray crystal structure of human p53 core domain (PDB ID 2OCJ; figures generated using PyMOL). (a) Ribbon diagram highlighting p53 cysteine residues. Sulfur atoms are shown (orange). Cys176, 238, and 242 are involved in the coordination of a zinc ion. (b) Surface contour of the p53 core domain. The thiol groups of Cys176, 182, 229, 242, and 277 are surface-exposed. (c) Alternative orientation of p53 core domain highlighting the anti-parallel β-sheet sandwich and position of Cys229, 124, 135, and 141.
Identification of the Third Site of Alkylation

Since alkylation beyond Cys182 and Cys277 triggers cooperative alkylation of the remaining cysteine residues, we sought to identify the third site of NEM alkylation in wild-type p53 using top-down FTICR MS. After 1 mM NEM treatment for 15 min, individual charge states (23+ and 24+) of 3NEM-p53 were sequentially isolated and fragmented using CID, and the resulting fragment masses were searched against theoretical fragment mass lists for p53 core domain modified with three NEM groups (Supplementary Figure S6; assigned product ions are listed in Table S6). None of the fragment ions was compatible with assignment of a NEM group on Cys124, 135, 141, or 176. However, one fragment ion was observed that supported assignment of the third NEM on Cys229, Cys238, or Cys242 (b169, c76; Figure S6). Since Cys238 and 242 are zinc-binding residues it seems unlikely that either of these is the third site of alkylation. One fragment ion was also observed that supported placement of the third NEM on Cys275 (b141, c5+; Figure S6). Hence, Cys229 and Cys275 are the most likely candidates for the third site of alkylation.

Disruption of Secondary Structure and Protein Unfolding

Modification of a third Cys residue in wild-type p53 (or a second Cys in C182S and C277S, or the first Cys in C182/277S) initiated a cascade of cysteine alkylation resulting in a fully alkylated species. Top-down FTICR MS of 3NEM-p53 revealed that Cys229 and Cys275 are the most likely candidates for the third site of alkylation. Interestingly, X-ray crystallography data has previously indicated that Cys229 is modified by the electrophilic compound 3-benzoylacrylic acid [44]. Both Cys229 and Cys275 are located at the N-terminal and C-terminal ends, respectively, of strands of the anti-parallel β-sheet sandwich. We postulate that alkylation of either of these residues disrupts this stabilizing structural feature and thus allows access of NEM to the cluster of buried cysteines 124, 135, and 141, which are located in between the two sides of the β-sandwich (Fig. 6c). Interestingly, Cys124, 135, and 141 are all located at the beginning or end of β-strands. Alkylation of any one of these cysteines may result in severe disruption of p53 secondary structure and is likely to be responsible for the observed rapid alkylation of the remaining cysteine residues.

Zinc is required for the correct folding and specific DNA-binding activity of p53 [17, 38, 39]. Notably, the observed cascade of cysteine reactivity includes alkylation of all three zinc-binding cysteines, indicating that hyper-alkylation of p53 causes extensive unfolding of the core domain. This has implications for the reactivation of mutant p53 with cysteine-targeting compounds such as PRIMA-1 and MIRA-1 [21].

Conclusion

Selective modification of cysteine residues is a fundamental requirement in cellular redox-signaling [51]. Here, we have clearly and unambiguously shown that Cys182 and Cys277 in p53 are selectively alkylated by N-ethylmaleimide and are thus the most likely candidates for controlling p53 activity via redox-regulation. The p53 core domain has a low thermodynamic stability [40] and is known to unfold at body temperature [41]. However, we have shown that selective alkylation of p53 occurs at 37°C, providing further support for the role of Cys182 and Cys277 in p53 redox-regulation. These results demonstrate the power of FTICR mass spectrometry for the study of multiple Cys-containing proteins.

Acknowledgments

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