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Structural characterization of p53 isoforms due to the polymorphism at codon 72 by mass spectrometry and circular dichroism

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ABSTRACT

A common polymorphism at codon 72 of human TP53 gene determines a proline to arginine aminoacidic substitution within the proline-rich domain of p53 protein. The two resulting isoforms (p53P^{72} and p53R^{72}) are different from a biochemical and biological point of view and many reports suggest that they can modulate individual cancer susceptibility and overall survival. In the attempt to explain the observed biological differences, we characterized the two isoforms by mass spectrometry and circular dichroism (CD) to evaluate the possible alteration in the secondary structure of p53 introduced by this polymorphism.

Recombinant human p53R^{72} and p53P^{72} were produced by using *E. coli* expression system then purified by chromatography (affinity chromatography and RP-HPLC), and the whole proteins identified by HPLC–ESI-IT and MALDI-TOF analysis. A bottom-up approach, using both MALDI-TOF and HPLC–ESI-QTOF analysis, was then adopted to obtain the sequence information on the two p53 isoforms. To this purpose, peptide maps were obtained by trypsin proteolysis on the two p53 isoforms. The two isoforms proteolytic digests were separated by LC and subsequent mass spectrometry analysis of both entire and fragmented peptides was performed. In particular, precursor peptide ions obtained by ESI were subjected to collision by the triple quadrupole and TOF separation, allowing us to determine the isoforms aminoacidic peptide sequence by peptide ladder sequencing. Because of the presence of arginine, a selective trypsin proteolytic cleavage at R^{72}, giving rise to two selective shorter peptides, occurred in p53R^{72}, but was missing in the case of p53P^{72} trypsin digest, in which an uncleaved longer peptide was instead identified. Upon primary structure confirmation, the two p53 isoforms were studied by CD in order to investigate the experimental variables, which affect ordered secondary structure adoption. CD analysis indicated that the two isoforms are not structurally different, thus allowing us to exclude that the observed biological differences can be due to a different conformation of the two isoforms introduced by this polymorphism. Furthermore, these studies establish a mass spectrometry method to identify the two isoforms that can be useful for future interactome studies and cancer drug discovery.

1. Introduction

TP53 is a critical tumour suppressor gene that is mutated in about half of all human cancers [1,2] and loss of TP53 predisposes mice and humans to increased morbidity and mortality (reviewed in [3]). p53, the product of TP53 gene, is a key player in a series of biological processes such as apoptosis, cell cycle, cell senescence, DNA repair and glucose and energy metabolism. p53 protein is active as a tetramer, with four identical chains of 393 residues. The N-terminal region consists of an intrinsically disordered transactivation domain (TAD) and a proline-rich domain (PRD). It is followed by the central, folded DNA-binding core domain which is responsible for sequence-specific DNA-binding [4]. The PRD that links the TAD to the DNA-binding domain in human p53 contains five PXXP motifs [5]. Such motifs mediate numerous protein–protein interactions in signal transduction through binding to Src homology 3 [6] and WW domains [7]. The exact role of the PRD is still poorly understood. Nevertheless, a number of studies suggest that this region is essential for a full-blown apoptotic response and inhibition of tumorigenesis [8–11]. Human TP53 gene harbours a common polymorphism at codon 72 (a C to G transversion) that leads to an aminoacidic substitution in PRD. The two isoforms of p53 protein

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(one with an arginine residue at position 72, the other with a proline residue) resulting from this polymorphism are functionally different [12]. In particular, some of us reported that the two p53 isoforms ($p53R^{72}$ and $p53P^{72}$) differ in the capability to induce apoptosis and cell senescence in *ex vivo* experiments [13–15].

We wondered whether these biological differences could be at least in part due to possible alterations of the structure of p53 protein introduced by the polymorphism at codon 72. In order to study such possible alterations, we set up a series of proteomic methods to identify the two isoforms and their structure. After purification and complete p53 isoforms primary structure characterization, circular dichroism (CD) studies were carried out to investigate conformational changes and stability in different media and temperature conditions.

2. Experimental

2.1. Cloning and expression of $p53R^{72}$ and $p53P^{72}$

The two p53 isoforms were amplified by PCR using a cDNA obtained from RT-PCR of total RNA from a heterozygous subject. The primers used were: 5′-CACCATggAggAgCCgCAgTCAgAT-3′ (For) and 5′-TCAgTCTgAgTCAggCCCTTC-3′ (Rev). The PCR products were cloned using pET151 directional TOPO expression kit (Invitrogen) according to manifacturer's protocols. Briefly, both contructs were transformed in One Shot TOP10 chemically competent *E. coli* and plated overnight. Colonies were analysed and positive transformants were selected to proceed with plasmid DNA isolation. The sequences were controlled by sequencing to ensure that no mutations had occurred.

The pET151-p53 encoding plasmids were expressed into *E. coli* strain BL21 Star (DE3) (Invitrogen) and the cells were grown in Luria Broth (LB) medium supplemented with 100 μg/mL Ampicillin (Sigma). Protein overproduction was induced at an OD_{600} of 0.6–0.8 with 0.3 mM IPTG (isopropyl β-D-thiogalactopyranoside) and growth continued for an additional 6 h at 28 °C. Pellets were harvested and frozen at −20 °C.

2.2. Purification of p53R72 and p53P72

2.2.1. Affinity chromatography

The cells, according to hybrid protocol, were resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl) and after a gentle agitation, lysed on ice by sonication. The supernatant containing the soluble protein was obtained by centrifugation at 3000 × *g* for 15 min. Both proteins, in denaturated form, after TCA precipitation were checked on SDS-PAGE and separated on 12% polyacrylamide gel.

To obtain a native p53 a Pro-Bond Purification System (Invitrogen) was used. The supernatant was loaded onto an Ni^{2+}-charged chelating sepharose column and washed. The recombinant protein was finally eluted with native elution buffer (50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0) containing 250 mM imidazole. After estimating the protein concentration by Bradford assay, the recombinant proteins were assessed by SDS-PAGE denaturing samples in SDS loading buffer and separated on 12% polyacrylamide gel.

2.2.2. LC–UV–ESI-MS purification

A mix of proteins obtained by affinity chromatography, as above indicated, was purified by using a Jasco PU-1585 liquid chromatograph (Jasco Corporation, Tokio, Japan) with a Reodyne 7281 injection valve (20 μL sample loop) equipped with heated capillary interface and electrospray ionization (ESI) source, and operating with an ion trap mass spectrometer (LCQ-Duo, ThermoFinnigan, San Jose, CA, USA) and with a Jasco UV-1575 detector (215 nm). ESI system employed a 4.5 kV (positive polarity) spray voltage and a heated capillary temperature and voltage of 180 °C and 43.93 V respectively. Deconvoluted ESI mass spectra of p53 isoforms was obtained by using MagTran 1.0 software. LC preparative purification of recombinant human p53 was performed on a C4 (Jupiter Phenomenex 5 μm, 300 Å, 4.6 mm × 150 mm I.D.) column, assembled with a precolumn SecurityGuard™ HPLC system consisting of a C18 guard cartridge (Phenomenex, 4.0 mm × 3.0 mm I.D.) inserted into its cartridge holder, using a gradient elution from A [water:TFA (100:0.04) (v/v)]/B [acetonitrile:TFA (100:0.04) (v/v)] 80/20 (v/v), to A/B 30/70 (v/v), in 30 min, at a flow rate of 0.4 mL/min; the injection volume was 20 μL. The column was equilibrated with the mobile phase composition of the starting conditions for 10 min before the next injection. Repeated injections of the protein samples were performed and the recombinant human p53 protein was eluted from the column, collected and dried under vacuum. The collected protein isoforms were then dissolved in water and the concentration of these solutions was measured spectrophotometrically by using extinction coefficient at 280 nm (ε_{280}) of 36,900 M^{-1} cm^{-1} calculated on the basis of the amino acid sequence [16–18]. The water solutions of the protein isoforms were then aliquoted out into several eppendorf tubes and dried under vacuum for CD and purity analysis. The purity of the isolated protein was checked under the same chromatographic conditions with LC–UV–ESI-MS analysis and by MALDI-TOF analysis (see method described below).

2.3. Intact protein ($p53R^{72}$ and $p53P^{72}$) MALDI-TOF analysis

MALDI-TOF MS analysis was performed using a Voyager DE Pro (Applied Biosystems, Foster City, CA) equipped with a pulsed N2 laser operating at 337 nm. Whole proteins ($p53R^{72}$ and $p53P^{72}$) positive ion spectra were acquired in linear mode over an m/z range from 10,000 to 80,000 using a 25,000-V accelerating voltage, a 22,500-V grid voltage, and a delay extraction time of 150 ns. The spectrum for each spot was obtained by averaging the result of 120 laser shots. External mass calibration was performed using the single- and double-charged ions of bovine serum albumin (Sigma–Aldrich). The analysis was performed by spotting on the target plate 1 μL of the sample mixed with an equal volume of the matrix solution, 30 mg/mL sinapinic acid in 1:1 (v/v) ACN/H_2O containing 0.1% (v/v) TFA.

2.4. Peptide mapping of $p53R^{72}$ and $p53P^{72}$

2.4.1. $p53R^{72}$ and $p53P^{72}$ in-gel digestion

Protein bands excised from gel with a razor blade were chopped into 1-mm^3 pieces and collected in microcentrifuge tubes. Gel pieces were discoloured in water/acetonitrile (50/50) at 56 °C and then dehydrated in acetonitrile at room temperature for 15 min. Gel pieces were then rehydrated with 10 μL of 10 mM dithiothreitol in 100 mM ammonium bicarbonate buffer and heated at 56 °C for 30 min to reduce disulfides. After dehydration twice in 100 μL of acetonitrile at room temperature for 15 min, gel pieces were incubated with 10 μL of 55 mM iodoacetamide in 100 mM ammonium bicarbonate buffer for 20 min in the dark at room temperature to alkylate cysteine residues. After dehydration with 100 μL of acetonitrile for 15 min and complete drying in a SpeedVac (ThermoSavant), each sample was covered with 15 μL of 20 mM ammonium bicarbonate buffer (pH 8.00) containing sequencing grade modified trypsin 13 ng/μL and incubated for 45 min at 4 °C. Excess of trypsin solution was removed and gel particles were washed with 10 μL of 20 mM ammonium bicarbonate and incubated with 15 μL of washing buffer (37 °C for 18 h). Reactions were stopped by adding 1 μL of 5% formic acid

Table 1
The fractions of the main secondary structures were calculated by using the software CONTIN/LL [21]. H (helix) and S (strand) fractions are the sum of regular (r) and distorted (d) α-helix and β-strand, respectively. The data are displayed as the average ± SD of the results obtained by using SP29, SP37 and SP43 as protein reference sets.

	Solvent	H(r + d)	S(r + d)	Turn	Unordered
$p53R^{72}$	Water	0.044 ± 0.006	0.446 ± 0.04	0.210 ± 0.007	0.300 ± 0.031
	TFE	0.593 ± 0.010	0.044 ± 0.008	0.138 ± 0.007	0.224 ± 0.007
	HFIP	0.390 ± 0.014	0.120 ± 0.017	0.210 ± 0.002	0.280 ± 0.008
	SDS	0.229 ± 0.016	0.252 ± 0.017	0.219 ± 0.004	0.300 ± 0.006
$p53P^{72}$	Water	0.044 ± 0.006	0.449 ± 0.049	0.208 ± 0.009	0.300 ± 0.035
	TFE	0.565 ± 0.007	0.058 ± 0.011	0.141 ± 0.008	0.235 ± 0.015
	HFIP	0.431 ± 0.027	0.093 ± 0.024	0.193 ± 0.003	0.283 ± 0.007
	SDS	0.243 ± 0.011	0.250 ± 0.014	0.218 ± 0.003	0.289 ± 0.012

2.4.2. MALDI-TOF analysis of $p53R^{72}$ and $p53P^{72}$ tryptic digest

Trypsin digest from PAGE was analyzed in reflector, positive detection mode over an *m/z* range from 500 to 5000 using a 20,000-V accelerating voltage, a 15,200-V grid voltage, and an extraction delay time of 40 ns. The spectrum of each spot was obtained by averaging the results of 100 laser shots. P53 digest spectra were internally calibrated on 343–363 (2212.1203), 320–333 (1707.9428), 102–110 (1078.4952) and 338–342 (729.3380) theoretical P53 digest peptide masses that were sufficiently abundant in the spectra. 5 mg/mL R-cyano-4-hydroxycinnamic acid in 1:1 (v/v) ACN/H2O containing 0.1% (v/v) TFA was used as matrix. The analysis was performed by spotting 1 μL of the sample mixed with an equal volume of the matrix solution onto the target plate.

2.4.3. LC–ESI-MS/MS analysis of $p53R^{72}$ and $p53P^{72}$ tryptic peptides

Analysis was performed using a CapLC capillary cromatography system (Waters, Milford, MA) coupled online with a quadrupole-TOF hybrid mass analyser (Q-TOF Micro, Micromass, Manchester, UK) employed with a Z-spray ion source. Samples (10 μL) were loaded onto a RP trap column (Waters, Milford, MA) for desalting and concentrating at a flow rate of 10 μL/min using mobile phase A (100% water containing 0.1% formic acid). Peptides were then eluted from the precolumn and separated on a Zorbax C18 (150 mm × 0.3 mm; 3 μm particles) column at a flow rate of 5 μL/min, under linear gradient elution from A [water:acetonitrile:FA (95:5:0.1)(v/v)]/B (acetonitrile:water:FA (95:5:0.1) (v/v)] 5/95 (v/v) to A/B 50/50 (v/v) in 40 min.

The mass spectrometer was operated in positive ion mode with a source temperature of 100 °C, desolvatation temperature of 220 °C and a cone gas flow of 30 L/h. A capillary voltage of 3.1 kV was applied. MS and MS/MS spectra were acquired in an automated data-dependent mode, and all data were processed using MassLynx, version 4.1 software. The protein identification was performed with the MASCOT MS/MS ion search software http://www.matrixscience.com and SwissProt protein database [19].

2.5. Circular dichroism characterization of $p53R^{72}$ and $p53P^{72}$

CD spectra were recorded by a J-810 (Jasco, Japan) spectropolarimeter equipped with a Peltier type temperature control system set at 20 °C. Solutions of the two isoforms $p53R^{72}$ and $p53P^{72}$ were prepared at 3.6 μM in water, hexafluoroisopropanol (HFIP), trifluoroethanol (TFE) and sodium dodecyl sulfate (SDS) 20 mM, dissolved in Native Elution Buffer (50 mM Na_2HPO_4, 0.5 M NaCl, pH 8). The spectra were recorded from 260 nm down to 185 nm by 1 mm path length quartz cell at a scan rate of 10 nm/min, time constant 2 s, data pitch 0.2 nm, sbw 1 nm. All the spectra were corrected for the respective blank and smoothed by the Savitzky–Golay algorithm [20]. The secondary structure was estimated by using the algorithm CONTIN/LL contained in the CDPro software package [21]. The reported fraction values of secondary structure content are the average of results obtained by using SP29, SP37 and SP43 as protein reference sets (Table 1).

The thermal stability of the two isoforms was studied in water monitoring the CD spectra upon changing the temperature from 20 °C up to 90 °C.

3. Results and discussion

3.1. Purification and structural identification of $p53R^{72}$ and $p53P^{72}$

$p53R^{72}$ and $p53P^{72}$ were individually prepared by the DNA recombinant technology in *E. coli*. This system allowed us to obtain native proteins without post-translational modifications such as phosphorylations. Two different methods have been used to purify the expressed proteins: an affinity purification system (Invitrogen), taking advantage of the 6xHis-tag present at the N-terminus of the proteins expressed by pET151 plasmids, and an LC–UV–ESI-MS analysis.

As far as the first method, briefly, we choose a hybrid protocol in order to preserve protein native conformation, thus lysate and columns were prepared under non-denaturing conditions, then native buffers were used during the wash and the elution steps to refold the protein. Specifically, the recombinant proteins were individually and selectively retained by affinity interaction between the protein histidine tags and the residual Ni^{2+}-charged chelating groups on the sepharose column. Then, protein elution was obtained by using a buffer containing 250 mM imidazole as a competing agent for the charged groups on the stationary phase. Most of the cell lysate content was eliminated, however, the affinity chromatography step alone was not sufficient for a complete protein purification and a further separation step was required. A reversed phase liquid chromatography interfaced with an iontrap mass analyser (LC–UV–ESI-IT) under potentially denaturing gradient elution was carried out for analytical and preparative purposes. Preparative purification of recombinant human p53 isoforms was performed on a C4 stationary phase, assembled with a C18 guard cartridge for protein concentration and salt elimination. In this chromatographic system both $p53R^{72}$ and $p53P^{72}$ showed the same retention time (Rt = 17 min), when individually analysed. In Fig. 1a and b, the chromatogram of the same impure $p53R^{72}$ sample with respectively MS and UV detection is reported. In Fig. 1c and d, the clean chromatographic profiles of the same purified sample are shown under respectively MS and UV detection. Identification of the two isoforms was feasible by the hyphenated ESI-IT analysis. Multicharged mass spectra acquired on peak apex eluting at 17 min, obtained by ion trap, and the relative deconvolution allowed their molecular weight, increased of the histidine tag, to be determined (Fig. 1a, inset). In the purified sample chromatograms (Fig. 1c) all along peak tailing, protein molecular weight was consistent, excluding degradation and/or impurities. The two purified p53 isoforms were further characterized by MALDI-TOF mass spectrometry, by following the procedure reported in Section 2.3. In Fig. 2, MALDI mass

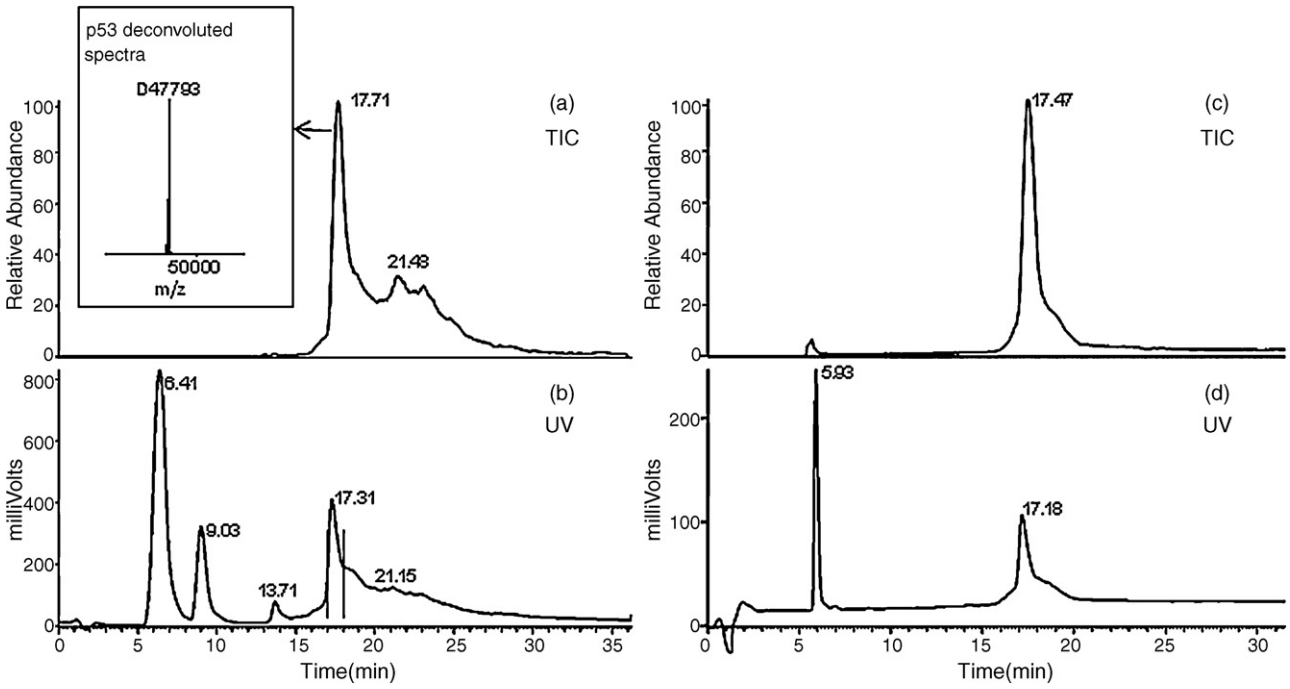

Fig. 1. LC–ESI-ion trap purification of p53. (a) LC–ESI-ion trap full scan chromatogram of p53R^{72} sample before purification (inset: p53R^{72} deconvoluted MS spectrum). (b) LC–UV chromatogram of same p53R^{72} sample before purification. (c) LC–ESI-ion trap chromatogram of p53R^{72} purified collected sample; LC–UV chromatogram of same p53R^{72} purified collected sample. Chromatographic conditions: C4 (5 μm, 300 Å, 4.6 mm × 150 mm I.D.) column, assembled with a C18 guard cartridge (4.0 mm × 3.0 mm I.D.) under gradient elution from A [water: TFA (100:0.04) (v/v)]/B [acetonitrile:TFA (100:0.04) (v/v)] 80/20 (v/v), to A/B 30/70 (v/v), in 30 min, at a flow rate of 0.4 mL/min; the injection volume was 20 μL.

spectra of purified intact p53 isoforms, p53P^{72} (a) and p53R^{72} (b), using sinapinic acid matrix material are reported, where the mono charged specie by one proton ionization are detected. Despite the low difference in molecular weight between the two isoforms, the instrumental resolution was found to be sufficient to distinguish between the two proteins molecular weight.

However, since the p53R^{72} (MW = 43,712) differs from the p53P^{72} (MW = 43,652) for just 60 mass units, it was necessary to verify the sequence information on the two p53 isoforms adopting a bottom-up approach, using both MALDI-TOF and HPLC–ESI-MS/MS analysis under tandem mass spectrometry. To this purpose, peptide maps were obtained by trypsin proteolysis on the two p53 isoforms.

MALDI-TOF was here used in conjunction with existing protein databases to perform protein mapping. Once mass spectral data on the peptides were obtained by MALDI-TOF MS, they were compared with a database of peptide mass values. These database values are calculated by using the enzyme cleavage rules to a collection of sequence data (SwissProt). The closest match to p53 was then identified. Not only the protein was identified, but it was possible to distinguish between p53R^{72} and p53P^{72}, since only the former underwent a trypsin enzymatic cleavage at R^{72} amide bond, whereas the latter showed a miscleavage at P^{72}. This gave rise to two shorter peptides in the case of p53R^{72} (65–72 and 73–101) and to a longer one (65–101) in the case of p53P^{72}. In Fig. 3, the MALDI-TOF spectra of the two isoforms digests are reported, zoomed on the identification peptides. In particular in Fig. 3a, p53R^{72} peptide digest MALDI-TOF spectrum is shown, where specific fragments (65–72 and 73–101) at 771 *m*/*z* and 2751 *m*/*z* are present. These fragments are missing in p53P^{72} peptide digest (Fig. 3b), in which on the other hand the longer peptide (65–101) was identified at 3444 *m*/*z* (Fig. 3d), not present in p53R^{72} (Fig. 3c).

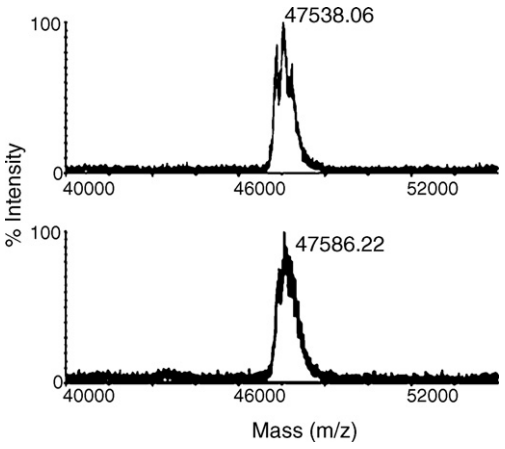

Fig. 2. MALDI-TOF analysis of purified p53 isoforms. (a) Intact p53P^{72} and (b) intact p53R^{72} samples.

Besides, the two isoforms proteolytic digests were separated by reversed phase LC and subsequent mass spectrometry analysis of both the whole and fragmented peptides was performed. In particular, precursor peptide ions obtained by ESI were subjected to collision by the triple quadrupole and TOF separation, allowing us to get information on the aminoacidic peptide sequence by peptide ladder sequencing. Fragmentation is in fact a vital step in sequencing peptides and hence proteins. The fragment ions produced in this process can be separated into two classes. One class retains the charge on the N-terminal, whereas cleavage is observed on the C-terminal.

In Fig. 4a and d, the HPLC–ESI-QTOF (total ion current) chromatograms of the two isoforms trypsin digests are reported, along with the extract ion chromatograms. In the case of p53P^{72}, extract ion chromatograms show in (b) a selective peak at *m*/*z* 682 and in (c) at 1149, respectively corresponding to the uncleaved long peptide [65–101+4H]$^{4+}$ and [65–101+3H]$^{3+}$.

On the other hand, p53R^{72} extract ion chromatograms show peaks related to the 771 *m*/*z* monocharged peptide [65–72+H]$^{+}$

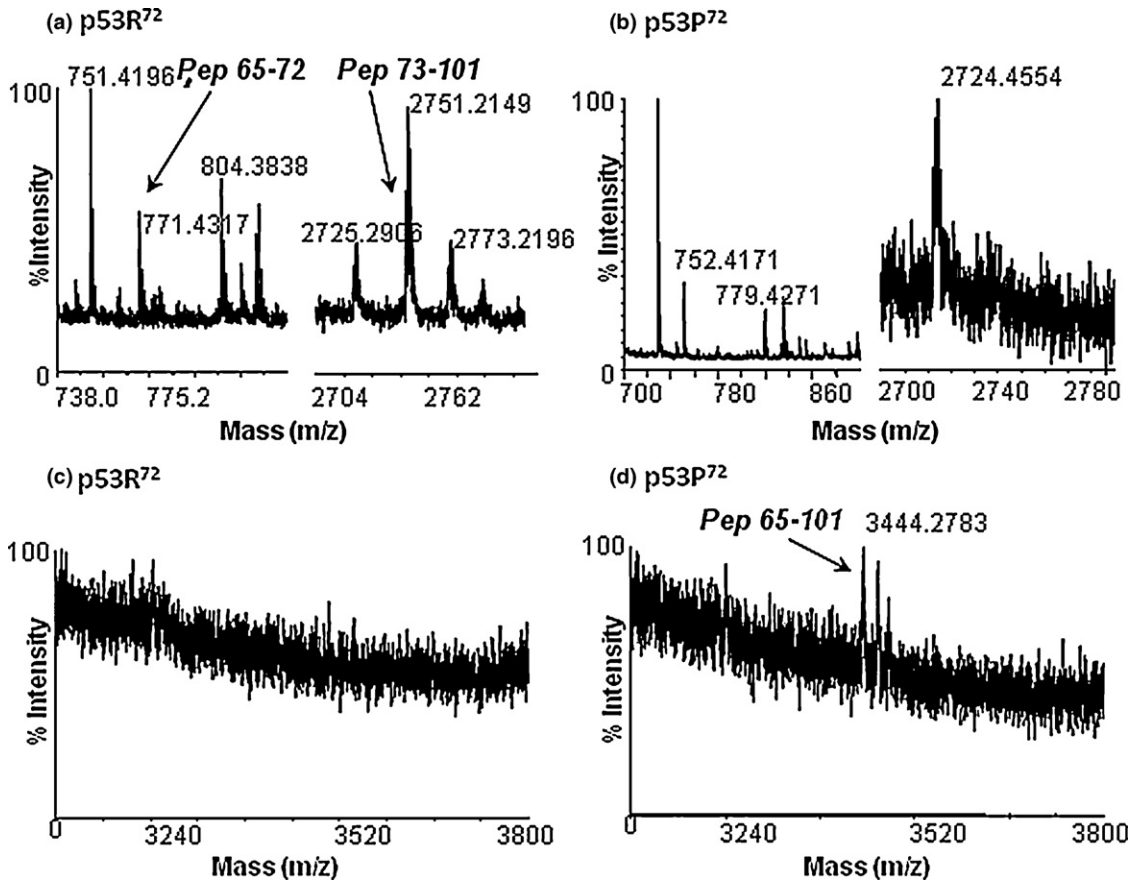

Fig. 3. MALDI-TOF analysis of p53 tryptic digests. MALDI-TOF spectra of p53R^{72} peptide digest (a and c), and p53P^{72} peptide digest (3b and d) are showed. Specific fragments (65–72 and 73–101) at 771 *m*/*z* and 2751 *m*/*z* are present in p53R^{72} digest (a), while a longer peptide (65–101) is absent at 3444 *m*/*z* (c). At variance, the short fragments are missing in p53P^{72} peptide digest (b), in which on the other hand the longer peptide (65–101) at 3444 *m*/*z* is present (d).

and to the *m*/*z* 1376 bi-charged peptide [72–101+2H]$^{2+}$, missing in p53P^{72} tryptic digest chromatogram.

Tryptic mass analysis was therefore found to be crucial in giving a further evidence of the different aminoacid sequence at position 72 in the two isoforms, confirming their primary sequence.

3.2. Secondary structure analysis of p53R^{72} and p53P^{72} by circular dichroism

After LC purification, the two p53 isoforms were analysed by CD spectroscopy to gain evidence of refolding and intrinsic structural differences between the isoforms that could explain their different behaviour *in vivo*. The far-UV CD spectra of p53R^{72} and p53P^{72} in water showed to be perfectly superimposable since the difference was of the same order of the background noise; both spectra were characterized by a negative maximum at around 201–202 nm (Fig. 5). This suggests that both isoforms assume mainly a non-ordered structure, as previously reported in the literature [22]. Anyway, both proteins showed a non-zero signal between 220 and 240 nm, suggesting a small but significant amount of ordered structure to be present. In fact denaturated p53 do not show any signal in that range of wavelengths [22]. Interestingly, the spectra recorded in water showed a good agreement with the spectrum previously reported for p53 containing a different histidine tag and recorded in a buffered solution [22]. This evidence demonstrated the small contribution of the histidine tag on the structural properties of p53 isoforms. The secondary structure analysis performed by the program CONTIN/LL on the spectra recorded in water, gives a surprisingly high value for the β-strand content. This reflects the limited capacity of the algorithm to solve structures of proteins characterized by a low grade of order. In fact the theoretical spectra elaborated by CONTIN/LL on the basis of the calculated values, overestimates the experimental signals in the range between 210 and 230 nm, where a β-strand signal stands. Anyway, this overestimation is maintained for the analysis of both p53 isoforms and the comparison of the results confirm that p53R^{72} and p53P^{72} assume the same secondary structure in water.

The propensity of the two p53 isoforms to assume an ordered structure was investigated, by analysing the spectroscopic behaviour upon changing the polarity of the solvent (Fig. 5). A reduction of solvent polarity led to significant differences in the CD spectra shape. In particular, the appearance of CD bands at 222 and 208 nm suggested an increase of the α-helix ordered conformation content of both p53 isoforms with respect to the water solution, where the CD was that of a mainly non-ordered structure (Fig. 5). This behaviour was more evident passing from water to SDS micelles, HFIP and finally to TFE, and it was observed for both the isoforms. Interestingly, a clean isosbestic point at 204 nm appeared by overlaying the CD spectra, suggesting a two-state transition from an unordered conformation to a high α-helix content for both the proteins. However, the secondary structure analysis performed by CONTIN/LL revealed a more complex conformational equilibrium depending on the nature of the solvent, because significant changes were observed also for the β-strand content (Table 1). The increasing of the ordered structure was particularly evident in the case of the TFE solution. This solvent is known to promote intramolecular hydrogen bonds in spite of intermolecular interactions with water molecules, thus enhancing α-helix forma-

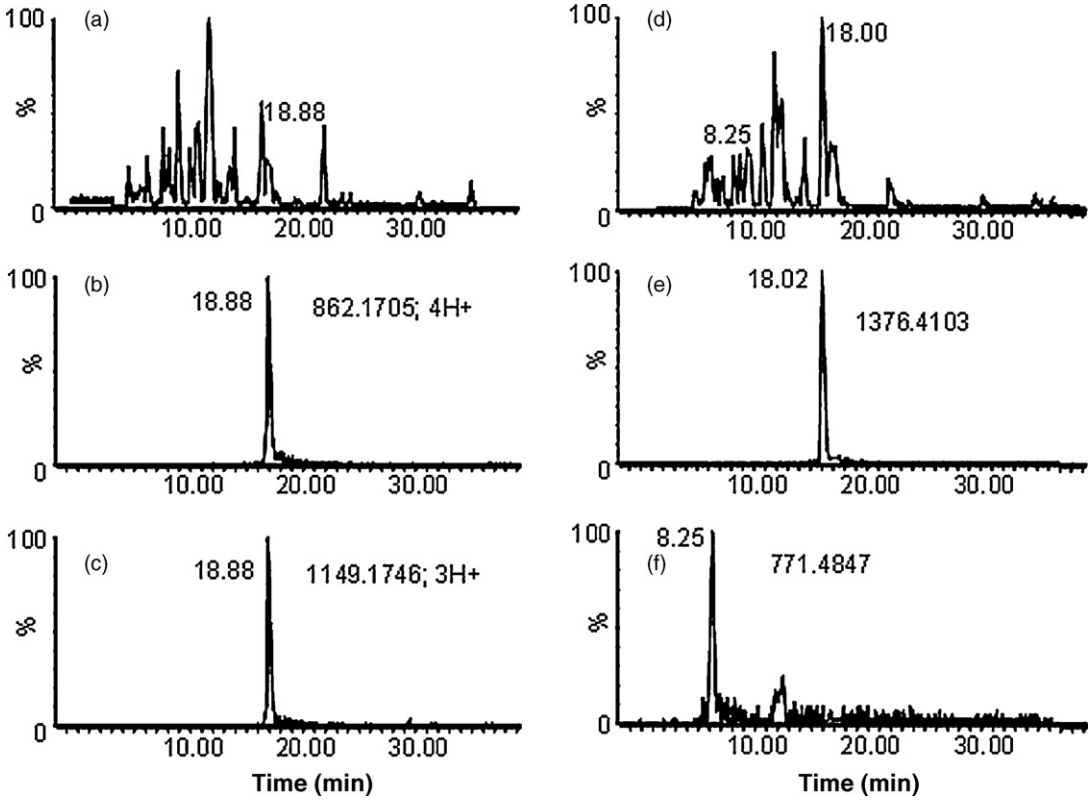

Fig. 4. p53P^{72} (a) and p53R^{72} (d) tryptic digests total ion current (TIC) LC–ESI-QTOF chromatograms. (b) $[65–101]^{4+}$ and (c) $[65–101]^{3+}$ p53P^{72} peptides extract ion chromatograms. (e) $[73–101]^{2+}$ and (f) $[65–72]^{+}$ p53R^{72} peptides extract ion chromatograms. Chromatographic conditions: C18 (3 μm, 150 mm × 0.3 mm I.D.) column under linear gradient elution from A [water:acetonitrile:FA (95:5:0.1)(v/v)]/B (acetonitrile:water:FA (95:5:0.1) (v/v)] 5/95 (v/v) to A/B 50/50 (v/v) in 40 min; at a flow rate of 5 μL/min.

tion [23]. The conformational analysis confirmed the very similar behaviour of the two p53 isoforms for their propensity to assume a higher ordered conformation by lowering the polarity of the solvent (Table 1).

The stereochemical stability of p53R^{72} and p53P^{72} isoforms was then checked by monitoring the changing of the secondary structure by increasing the temperature up to 90 °C. Both isoforms showed a similar changing upon increasing the temperature, suggesting a small shift of the conformational equilibrium. The process was not reversible by lowering the temperature back to 20 °C [data not shown].

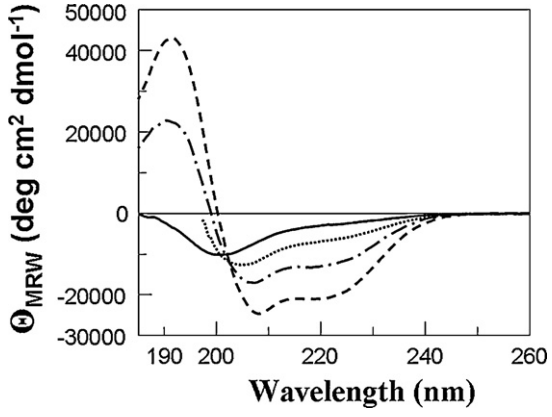

Fig. 5. Overlaid spectra of p53R^{72} in water (—), TFE (——), HFIP (– – –), and SDS micelles (·····).

4. Conclusions

The two p53 isoforms resulting from the polymorphism at codon 72 are known to be functionally different. In particular, it is reported that the two isoforms differ in the capability to localize at mitochondrial level [14], to induce the expression of specific genes [15–24] and to bind to specific proteins such as MDM2, Pin1 and iASPP [14,25,26]. There are convincing evidences that these differences have a deep impact *in vivo*, for example a number of studies reported an association with this polymorphism and risk of cancer [27], survival after cancer diagnosis [28], or longevity [29]. Recently some of us reported that this polymorphim modulates *in vivo* the levels of PAI-1, an important regulator of cell senescence [30]. In this work, we explored the possibility that these biological differences could be accounted for by a possible change in the structure of p53 introduced by this aminoacidic substitution in the PRD. In fact, the N-terminal domain (residues 1–94), and the C-terminal domain (residues 360–393) of p53 are intrinsically disordered [31,32]. Recent results of single-molecule FRET and ensemble FRET showed that the isolated N-terminal domain is extended in solution with a strong preference for residues 66–86 forming a polyproline II conformation, and that full length p53 can assume different conformations that are likely to result from the interactions of the N-terminal domain with the DNA-binding domain of p53 [33]. Thus, it was reasonable to hypothesise that the substitution of a proline residue with an arginine one, and in particolar at position 72, disrupting the second PXXP motif of the PRD, could introduce an alteration in p53 conformation. We addressed this issue with a number of different technical approaches. The two isoforms of p53 (p53P^{72} and p53R^{72}) were obtained by recombinant DNA technology. Their purification and primary structure characterization were carried out by means of liquid chromatography coupled with

mass spectrometry, MALDI-TOF, whereas secondary structure was studied by CD.

In particular, besides affinity chromatography, LC–ESI–UV-IT was employed for protein purification and for purity analysis. MALDI-TOF was used in all the steps of the purification as a quick and simple method for its ability to measure whole p53 isoforms mass also in complex mixture, as well as for tryptic mass analysis. As a further evidence, LC–ESI-QTOF analysis was carried out for tryptic digests of $p53P^{72}$ and $p53R^{72}$. p53 isoforms tryptic mass analysis was found essential to prove the primary structure and to confirm the substitution at position 72 of the two isoforms. In fact, due to the presence of arginine, a selective trypsin proteolytic cleavage occurred at R^{72}, giving rise to two selective shorter peptides in $p53R^{72}$, but was missing in the case of $p53P^{72}$ trypsin digest, in which the uncleaved long peptide was instead identified.

After primary structure elucidation, the two isoforms were studied by CD in order to characterize their secondary structure. CD studies allowed us to demonstrate a high propensity of the two p53 isoforms to assume ordered structures by lowering the polarity of the solvent. Nevertheless, no significant differences in the conformational equilibrium were observed amongst the two isoforms. It can be therefore excluded that the biological differences observed can be due to differences in the molecular structure of the two isoforms impinging upon their stereochemical properties. All these studies give an essential contribution for a basic knowledge of the two p53 isoforms, nevertheless further studies are needed to clarify the mechanism underlying the biological differences that are observed. In particular it could be interesting to elucidate the possible differential interaction of the two isoforms with the whole p53 interactome and in particular with proteins able to bind p53 at the level of PRD.

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