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Does azurin bind to the transactivation domain of p53? A Trp phosphorescence study

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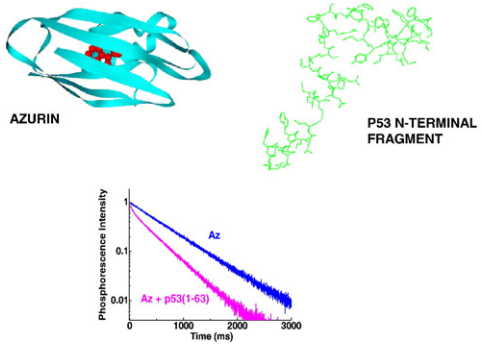
HIGHLIGHTS

- N-transactivation domain of p53 interacts with azurin by inducing structural changes.
- The estimated dissociation constant of the complex ranges from 5 to 10 μM.
- The binding site of azurin on p53 seems to differ from that of MDM2.

GRAPHICAL ABSTRACT



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ABSTRACT

The bacterial redox protein azurin has been shown to be able to enter into cancer cells and induce apoptosis by stabilizing p53. Although the formation of a complex between the two proteins has been demonstrated, little is known about their binding features. We investigated the interaction between the transcription activation domain of p53 (p53(1-63)) and *Pseudomonas aeruginosa* azurin using fluorescence and phosphorescence spectroscopic techniques. Trp phosphorescence lifetime measurements revealed conformational changes in azurin induced by the interaction with p53(1-63). Acrylamide quenching of Trp phosphorescence also indicated a significant increase in the overall flexibility of azurin upon binding to p53 (1-63). We show that azurin binds to the N-terminal region of p53 with a dissociation constant in the 5–10 μM range. No change in the fluorescence and phosphorescence emission of p53(1-63) was detected in the presence of azurin. This result indicated that no Trp residue of p53(1-63) is located in the interaction site with azurin and therefore suggested that the azurin binding site does not overlap that of MDM2, the protein that plays a crucial role in the p53 regulation. The present results may assist in the design of novel cancer treatments based on p53 stabilization by azurin.

1. Introduction

p53 is the major tumor suppressor protein in the human body. It has been at the center of intense investigation since its mutated or inactive forms were detected in over 50% of human cancers [1–3]. The protein is a homo-tetramer where each monomer is made up of four distinct domains: the well structured DNA binding core and tetramerization domains, and the naturally unstructured N-terminal and C-terminal regulatory domains. The N-terminal domain (residues 1-93) consists of two distinct regions: the transactivation domain (residues 1-58) and the proline-rich regulatory domain (residues 64-93) [4–6].

p53 responds to a variety of stress conditions including oncogene activation, DNA damage, and hypoxia [1]. In such stress conditions, p53 builds up and triggers cell growth arrest or apoptosis through

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transcriptional regulation of specific target genes. This contributes to eliminating cells carrying oncogenic lesions or damaged DNA, thus preventing tumor development. p53 is at the center of a network of signals leading to its induction, control and degradation. Regulation of p53 takes place through interactions with several proteins [7], among which the repressor oncoprotein MDM2 plays a relevant role. MDM2 binds to the 18-23 residues of the p53 transactivation domain, which changes its conformation from unstructured to α-helical [8]. In fact, MDM2 inhibits p53 transcriptional activity, favors its nuclear export and stimulates its degradation [9]. The inhibition of the p53-MDM2 interaction stabilizes p53 and rescues its function. Finding compounds able to prevent the interaction between the two proteins is therefore an attractive strategy for activating p53 tumor suppressor protein [10–13].

Azurin is a copper-oxidoreductase normally involved in denitrification reactions. It has been demonstrated that *Pseudomonas aeruginosa* azurin triggers cell death in a well-established J774 macrophage cell line [14,15]. Azurin is able to induce apoptotic cell death also in human cancer cells, such as human melanoma UISO-Mel-2 cells [16] and breast tumor MCF7 cells [17]. In vivo, the injection of azurin in immunodeficient mice harboring human breast cancer [17] or melanoma cells [15] results in a significant tumor regression. Azurin enters into the cytosol of cancer cells and moves to the nucleus. The resulting increase in the intracellular levels of p53 thereby triggers the apoptotic process [14,15,17–23].

Although there is evidence of the formation of a complex between azurin and p53, little is known about the molecular and structural mechanism of this interaction [24–26]. The decrease in p53 fluorescence after its binding to azurin suggests that azurin binds in the N-terminal domain at a site that probably overlaps with the MDM2 binding site [26]. This hypothesis has been validated by a modeling study of the interaction between the N-terminal domain and azurin [27]. On the other hand, the same authors, by means of AFM experiments, support the formation of a complex between azurin and the DNA binding domain of p53, as proposed also by a docking and free energy calculation study [24]. More recently, it has also been suggested that azurin binds to the C-terminal domain of p53 and that the interaction is mediated by nucleic acid [28]. It is therefore evident that there are still many open issues regarding the binding site of azurin on p53. The identification of the association site is of paramount importance for a possible therapeutic use of azurin. Likewise, knowledge of the determinants of the binding specificity, would allow the design of high affinity peptides with low molecular weight and therefore higher bioavailability than the entire protein.

In this study we directly investigated the involvement of the p53 transactivation domain (residues 1-63) in the p53–azurin interaction. We characterized the binding features and the conformational change induced by the interaction by using phosphorescence and fluorescence spectroscopic techniques. The phosphorescence emission of tryptophan residue is extremely sensitive to the chemical nature and dynamics of the surrounding protein matrix [29–32]. In particular, the room temperature phosphorescence lifetime (τ) provides information on the local flexibility of the protein matrix around the chromophore, whereas the bimolecular rate constant (k_q) for the phosphorescence quenching by acrylamide is related to the flexibility of the whole protein structure. Together, these parameters are useful in detecting even subtle protein structural perturbations resulting from the interaction with other macromolecules [33,34]. The single tryptophan (W48) of azurin is deeply embedded in a highly hydrophobic core and surrounded by a closely packed β barrel structure [35,36]. Many different criteria, crystallographic B-factors [37], T1 NMR relaxation times [38], H/D exchange rates [39], and molecular dynamic simulations [40,41], indicate that the aromatic ring of W48 is highly immobilized in an unusually rigid domain. According to a highly hydrophobic and rigid environment, W48 exhibits very peculiar fluorescence properties [42,43], a long phosphorescence lifetime in buffer at ambient temperature [44] and the migration rate of quenchers, such as acrylamide or oxygen, to the interior is one of the smallest ever found in proteins [45].

In this work we found an increase in the structural flexibility of azurin in the presence of p53(1-63), thus demonstrating the formation of a complex between the two proteins. The increase in azurin flexibility was p53(1-63) concentration-dependent and allowed us to estimate the K_D value of the complex. However, unlike the effect of MDM2 binding on the p53 fluorescence, azurin did not induce any change in p53(1-63) fluorescence. Taken together, our phosphorescence and fluorescence data indicate that azurin does bind to the transactivation domain of p53, although the binding site apparently spans residues that are different from those responsible for MDM2 binding.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of the highest purity grade available from commercial sources and used without further purification. Tris (hydroxymethyl)aminomethane, EDTA (ethylenediaminotetracetic acid disodium salt), spectroscopic grade glycerol, KI (Suprapure) and NaCl (Suprapure) were purchased from Merck (Darmstadt, Germany). Acrylamide (>99%) was purchased from Bio-Rad Laboratories (Richmond, CA) and GdnHCl (guanidinium hydrochloride) was from Sigma-Aldrich (Deisenhofen, Germany). GdnHCl concentration was determined by the refractive index [46] and confirmed by density measurements.

2.2. Protein expression and purification

The plasmid carrying the wild type sequence of *P. aeruginosa* azurin, was kindly provided by Prof. A. Desideri (Tor Vergata University, Rome, Italy). The W48Y mutant of the azurin gene was synthesized using the QuickChange kit (Stratagene, La Jolla, CA). The protein was expressed in *E. coli* strain BL21 and successively isolated and purified as described by van de Kamp et al. [47].

The pUC18p53 plasmid containing the gene coding for whole length human p53 was kindly provided by Prof. Nobua Tsuchida, Dept. of Molecular Cellular Oncology and Microbiology, Tokyo, Medical and Dental University. Cloning and purification of the whole-length p53 and p53(1-63) N-terminal fragment were performed as previously described [48,49]. Briefly, the portion of the human p53 gene encoding residues 1-63 was amplified by PCR from the pUC18p53 plasmid by using the 5′-AAAGGGGGATCCATGGAGGAGCCGCATCAGATCCT-3′ and 5′-AAAAGG-GAATTCTCAAGCTTCATCTGGACCTGGGTCT-3′ oligonucleotides. The fragment resulting from PCR amplification was digested with BamHI and EcoRI restriction enzymes and legated into pGEX-2T vector downstream and in frame with the glutathione S-transferase gene sequence. The fusion protein was expressed in *E. coli* BL21 cells transformed with the pGEX-2T(1-63) plasmid and purified as previously described [49].

2.3. Protein expression and purification

Protein concentration was measured spectrophotometrically by using the extinction coefficients ($\varepsilon_{280} = 8800\ cm^{-1}\ M^{-1}$ for apoazurin [50], $\varepsilon_{280} = 17{,}130\ cm^{-1}\ M^{-1}$ for p53 [48], $\varepsilon_{280} = 4595\ cm^{-1}\ M^{-1}$ for W48Y-azurin, and $\varepsilon_{280} = 11{,}000\ cm^{-1}\ M^{-1}$ for p53(1-63)). The latter values were calculated using Gill and von Hippel's method [51].

2.4. Fluorescence and phosphorescence measurements

Steady-state fluorescence measurements were performed on a Perkin-Elmer LS50B spectrofluorimeter (Perkin-Elmer Corporation, Norwalk, CT) at 20 °C. All measurements were made in 2.0 mM Tris–HCl pH 7.5/1.0 mM EDTA, at a p53(1-63) concentration of 2.0 μM. The excitation wavelength was at 295 nm with an excitation and emission slit width of 5.0 nm. All spectra were corrected for background fluorescence

and instrument response. Relative fluorescence quantum yields were calculated by integrating the corrected emission spectra taking into account the differences in the absorbance of the samples. Iodide quenching experiments were carried out in 25 mM Tris–HCl buffer, pH 8.0, containing increasing amounts of KI in the 0.0–0.2 M range. Compensating amounts of NaCl were added to the samples to keep the ionic strength constant. A small amount of $S_2O_3^{2-}$ (about 10^{-4} M) was added to the iodide solution to prevent I_3^- formation.

Phosphorescence spectra and decays were both measured with pulsed excitation ($\lambda_{ex}=290$ nm) on a homemade apparatus [52]. Pulsed excitation was provided by a frequency-doubled Nd/Yag-pumped dye laser (Quanta Systems, Milan, Italy) with pulse duration of 5.0 ns, pulse frequency up to 10 Hz and energy per pulse varying from 0.1 to 1.0 mJ. In this work, one pulse was sufficient to obtain satisfactory signal-to-noise ratios. For spectra measurements the emission was collected at 90° from the excitation and dispersed by a 0.3 m focal length triplet grating imaging spectrograph (SpectraPro-2300i, Acton Research Corporation, Acton, MA) with a band pass ranging from 1.0 to 0.2 nm. The emission intensity in the 400–535 nm range was monitored by a back-illuminated 1340×400 pixel CCD camera (Princeton Instruments Spec-10:400B(XTE), Roper Scientific Inc., Trenton, NJ) cooled at −60 °C. Phosphorescence decays were monitored by collecting the emission at 90° from vertical excitation through a filter combination, given by WG405, (Lot-Oriel, Milan, Italy) plus an interference filter DT-Blau, (Balzer, Milan, Italy), with a transmission window of 405–445 nm. The photomultiplier (EMI 9235QA, Middlesex, UK) was protected against fatigue from the intense fluorescence pulse by a gating circuit that inverts the polarity of dynodes 1 and 3, for up to 1.5 ms after the laser pulse. When the phosphorescence lifetime was shorter than 5 ms, laser excitation was synchronized to a fast mechanical chopper opening the emission slit 40 μs after the laser pulse. The photocurrent signal was amplified by a current-to-voltage converter (SR570, Stanford research Systems, Stanford, CA) and digitized by a 16 bit high speed (1.25 MHz) multifunction data acquisition board (NI 6250 PCI, National Instrument Italy, Milan, Italy) supported by LabVIEW software.

Phosphorescence measurements at low temperature (T = 77 K) were performed in 60/40 (v/v) glycerol/buffer (2.0 mM Tris–HCl buffer pH 7.5/1.0 mM EDTA), at a protein concentration of 10 μM. Measurements at ambient temperature were done in 2.0 mM Tris–HCl buffer pH 8/25 mM NaCl/1.0 mM EDTA. For phosphorescence measurements in fluid solution, deoxygenation was obtained by adding an enzymatic system including 3.2 μM glucose oxidase, 1.1 μM catalase and 0.3% glucose [53]. No emission from these proteins was detected at the amplification levels of the phosphorescence measurements. Satisfactory deoxygenation was achieved in 30 min. The thoroughness of O_2 removal was tested by the dependence of the triplet lifetime on the amount of excitation [54].

3. Results and discussion

3.1. Trp fluorescence emission in the transactivation domain of p53

The p53(1-63) peptide contains two tryptophan residues (W23 and W53) and no tyrosine. The fluorescence spectrum was broad and unstructured, with a maximum at 355 nm, which are typical characteristics of solvent-exposed Trp residues (see Fig. 1A, where the fluorescence of the free chromophore in solution, N-acetyltryptophanamide (NATA), is also shown for comparison). The average quantum yield of the two Trp residues was 70% that of NATA, indicating that the fluorescence emission of one or both Trp residues is somewhat quenched. Under denaturing conditions (6.0 M GuHCl) the quantum yield rose from 70% to 95% with respect to that of NATA. This finding suggests that, although the p53(1-63) domain is naturally unstructured, there are intra-molecular interactions with one or more residues responsible for fluorescence quenching.

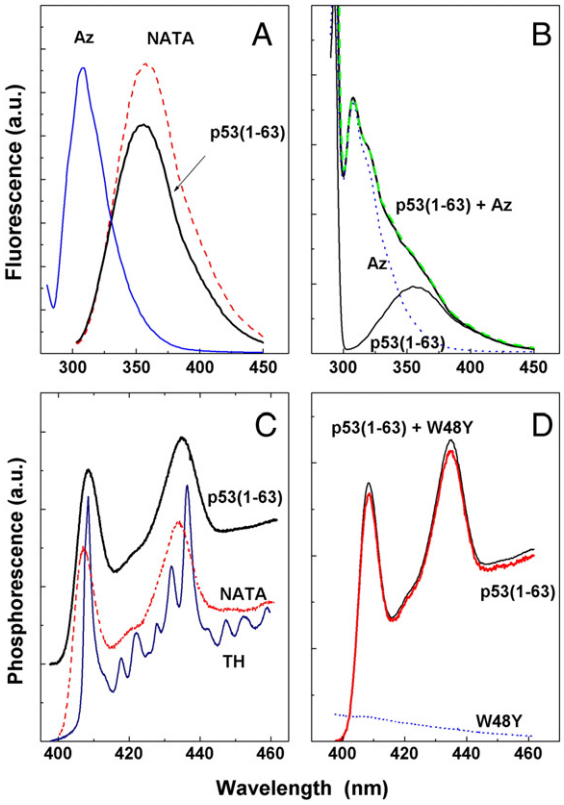

Fig. 1. Fluorescence and phosphorescence emission of p53(1-63). A) Fluorescence spectrum of p53(1-63) (thick line), apoazurin (Az) (thin line) and NATA (dash line) in 2.0 mM Tris–HCl buffer, pH 7.5/1.0 mM EDTA, at 20 °C. In all samples the chromophore concentration was 4.0 μM. λ_{ex} was 295 nm for p53(1-63) and NATA and 280 nm for azurin. B) Fluorescence spectrum of 1 μM p53(1-63)/5 μM Az mixture (thick line), 1 μM p53(1-63) (thin line) and 5 μM Az (dot line) under the same experimental conditions. λ_{ex} was 295 nm. The sum (dash line) of p53(1-63) and Az spectrum exactly overlaps the fluorescence spectrum of the mixture. The azurin concentration in the sample should give a p53(1-63) saturation ranging from 15% (assuming $K_D=30$ μM) to full saturation (for $K_D=1$ μM). C) Trp phosphorescence spectrum of p53(1-63) (thick line), NATA (dash line) and *Rhodospirillum rubrum* transhydrogenase (thin line) in glycerol/buffer (60/40, v/v) glass, at 77 K. Protein concentration was 10 μM. Background emission was subtracted. λ_{ex} was 290 nm. D) Comparison between high resolution phosphorescence spectrum of p53(1-63) alone (thick line) and in the presence of W48Y azurin (thin line). W48Y phosphorescence emission (dash line) was subtracted from the emission of the p53(1-63)/W48Y complex. Experimental conditions were as in panel C.

The hydrated, negatively charged iodide quenches solvent-exposed Trp residues effectively by a collisional mechanism [55,56]. Consequently, to measure the accessibility of W23 and W53 to the solvent, we carried out fluorescence quenching by iodide. The linear fit of F_0/F vs. $[I^-]$ led us to estimate a Stern–Volmer quenching constant, K_{SV}, of $2.8\pm0.9\ M^{-1}$. Under the same experimental conditions, the iodide quenching effectiveness of NATA fluorescence yielded $K_{SV}=8.8\pm0.5\ M^{-1}$. Considering that the exposure to quenchers of a macromolecule-bound chromophore is about 50% of that of the chromophore free in solution [57] and that the average quantum yield of p53(1-63) Trp residues is 70% of that of NATA, we concluded that the second order quenching rate constant, k_q, ($K_{SV}=k_q\tau$) of these residues is similar to that of NATA. The accessibility of W23 and W53 to the aqueous solvent was thus comparable to that of the freely accessible chromophore.

The fluorescence spectrum of Az is about 30 nm blue-shifted with respect to that of p53(1-63) (Fig. 1A and B). The difference in the emission wavelengths along with the use of red-edge excitation ($\lambda_{ex}=300$ nm), which selectively excites p53(1-63) and not blue absorbing azurin, allowed the fluorescence properties of p53(1-63) to be monitored in the presence of Az. Furthermore, we repeated the experiments with a Trp-less mutant of Az, W48F, which allowed to

significantly increase the azurin concentration. Based on the crystallographic structure of W48M [58], whose fold is practically identical to that displayed by the wild type protein, we assumed that substituting W48 with Tyr would not modify the structural determinants responsible for the interaction with p53. The shape of the fluorescence spectrum, the quantum yield and the accessibility of iodide to W23 and W53 were unaffected by the addition of azurin up to 60 μM. This result implies that neither of the two Trps is involved in the interaction with Az. This is a significant difference with respect to the binding of MDM2 to p53, where W23 is directly involved [8] and the binding leads to a blue-shift of the spectrum and changes in the fluorescence quantum yield [59]. The fluorescence emission of p53(1-63) unchanged in the presence of Az strongly suggests that the binding sites of azurin and MDM2 on p53 do not overlap.

3.2. Trp phosphorescence emission in the transactivation domain of p53

Phosphorescence spectra measured in glass matrices provide information on the polarity of surrounding groups and on the conformational homogeneity of the Trp site. The wavelength of the 0,0 vibronic band ($\lambda_{0,0}$) and its bandwidth (BW), which is the width at half height, are normally taken as indicators of spectral energy and spectral broadness, respectively. The 0–0 vibronic band is related to the polarity of the medium around the indole ring, and the bandwidth to the homogeneity of its micro-environment [60]. Fig. 1C shows the high resolution phosphorescence spectrum of p53(1-63) that we measured in glycerol/buffer (60/40, v/v) glass at 77 K. The phosphorescence emission of the free chromophore in solution (NATA), and that of W72 of *Rhodospirillum rubrum* transhydrogenase, a very buried chromophore [61], are shown for comparison. The p53(1-63) spectrum was broad and lacked the vibrational fine structure characteristic of well-structured protein sites (see the transhydrogenase spectrum in Fig. 1C). The 0–0 band was centered at 408.4 nm, which is consistent with polar, solvent-exposed sites for both Trp residues, and is in agreement with the red-shifted fluorescence spectrum. The single, broad 0,0-vibrational band indicates that the excited triplet-state energy of the two Trp residues is very similar. In other words, the two chromophores do not make distinct dipolar interactions with their surroundings even though the bandwidth (11 nm), which was larger than that of the free chromophore (9.6 nm), suggested that the spectra of W23 and W53 did not exactly overlap. Addition of W48Y azurin induced no significant changes in the spectrum features, confirming that the azurin interaction does not affect the polarity and heterogeneity of the Trp environment (Fig. 1D).

At room temperature, the Trp phosphorescence decay gives information on the local flexibility of the protein matrix around the chromophore [62–64]. We found that the phosphorescence lifetime (τ) of p53(1-63) at 20 °C was less than 40 μs, the instrument detection limit, and shorter than that generally exhibited by highly flexible, unstructured peptides ($\tau = 200$–250 μs) [63]. The short triplet lifetime implies effective intra-molecular quenching of the excited-triplet state. Even in the presence of azurin, τ remained below the detection limit as no phosphorescence emission could be measured upon the addition of 50 μM W48Y. Unlike Az, which has a strong phosphorescence signal at room temperature, W48Y is silent and thus does not interfere with p53 emission. All phosphorescence measurements performed in the presence of W48Y indicate that the polarity, the heterogeneity and the flexibility of the Trp environment of p53(1-63) are unaltered by the interaction with azurin.

In conclusion, all the characteristics of fluorescence and phosphorescence emission of p53(1-63) are unaltered by the interaction with azurin. Both Trp residues are largely exposed to the solvent and presumably located in an unstructured region of the polypeptide. Our results agree with those obtained with SAXS and NMR studies by Wells et al. [65]. They also agree with tryptophan fluorescence, CD and NMR spectroscopy studies by Dawson et al., who used a longer (1-93) p53 N-terminal domain [59]. Dawson ruled out the existence of a well-defined tertiary structure for this fragment and pointed out that it is intrinsically unstructured also under physiological conditions [59]. The unstructured dynamic fold of the p53 N-terminal may be important for the interaction with multiple receptors. Indeed, to achieve the tight regulation of p53, several inactivating proteins, such as MDM2, bind to the N-terminal domain by masking the transcription activation segment.

3.3. Effect of p53(1-63) on the phosphorescence lifetime of azurin

Spectroscopic studies were performed with the metal-free protein apoazurin (Az) because the native copper in both reduced and oxidized states extensively quenches the azurin fluorescence and phosphorescence emission [42,44]. In the presence of high concentrations (100 μM) of p53(1-63), we did not observe any significant alteration in the Az fluorescence and phosphorescence spectra and the fluorescence quantum yield. In contrast, the phosphorescence lifetime at room temperature (T = 20 °C) was significantly perturbed by the presence of p53(1-63). Fig. 2 compares the decay kinetics of Az both free and in the presence of p53(1-63). W48 of azurin is deeply buried in a rigid core and exhibits a mono-exponential phosphorescence decay at room temperature with a lifetime $\tau = 630 \pm 20$ ms. However, in the presence of p53(1-63) the Az decay became non-exponential and τ decreased by about 50%. According to the χ^2 criterion and the distribution of residuals (Fig. 2), the decay was adequately fitted in terms of two discrete components with $\tau_S = 48$ ms ($\alpha_S = 0.3$) and $\tau_L = 420$ ms ($\alpha_L = 0.7$), where α_S and α_L are the amplitudes of the short (τ_S) and long (τ_L) components, respectively. Addition of micromolar quantities of the whole p53 to Az induced a similar change in τ, i.e. the azurin decay became shorter and non exponential (Fig. 2). On the other hand, no significant alteration in the phosphorescence decay was measured in the presence of equal concentration of non related proteins, such as HSA or RNase A. These results suggest that the isolated N-terminal fragment and the whole p53 induce in azurin similar conformational changes and indicate that these changes, as monitored by the triplet lifetime, are specifically induced by p53. Since any p53(1-63) contribution could be ruled out

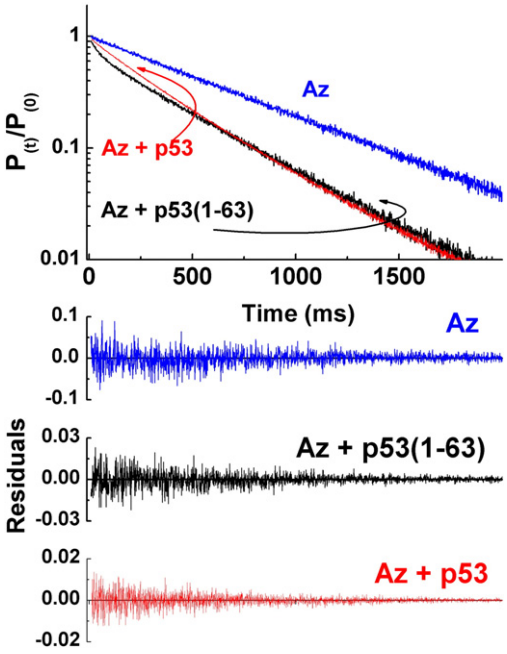

Fig. 2. Trp phosphorescence decay of apoazurin (4 μM) in 2.0 mM Tris–HCl buffer, pH 8.0/25 mM NaCl/1.0 mM EDTA, before and after the addition of 100 μM p53(1-63) or 10 μM full length p53 at 20 °C. λ_{ex} was 290 nm. The plot of residuals obtained by fitting the decay to one exponential component (Az) and two exponential components (Az + p53(1-63) and Az + p53) is shown.

because its lifetime is under the instrumental detection limit, multiple components of the decay were ascribed to conformational heterogeneity of Az induced by p53(1-63) binding.

Changes in the intrinsic lifetime of W48 induced by p53(1-63) provided direct evidence that the peptide interacts with sufficient strength to alter the internal region of Az, where W48 is located. An estimate of the flexibility around the indole ring of W48 may be obtained from the empirical relationship between τ and solvent viscosity derived in model systems [62]. Based on this criterion, we found that the local "viscosity" (η_τ) around W48 estimated from $\tau = 630$ ms was 5×10^4 cPoise. On the basis of the average lifetime ($\tau_{AV} = \alpha_S\tau_S + \alpha_L\tau_L$) of 308 ms, the local viscosity in the presence of p53(1-63) appeared to be reduced by about 3 fold ($\eta_\tau = 1.5\times10^4$ cPoise). The decrease in internal viscosity along with the appearance of a multiple component decay were indicative of conformational changes in the internal region of azurin induced by its interaction with p53(1-63).

3.4. Effect of p53(1-63) on quenching of azurin phosphorescence by acrylamide

Acrylamide is a neutral solute, which quenches Trp phosphorescence by a relatively short-range, near-contact quenching reaction [66]. Quenching phosphorescence of internal Trp residues needs therefore the penetration of the quencher through the protein matrix. Given the relatively large size (Mw = 71 D), acrylamide does not quickly diffuse inside proteins, and its diffusion rate varies by several orders of magnitude among different protein sites depending on the amplitude and frequency of the protein structural fluctuations [67]. In azurin, W48 is located at least 8 Å inside the protein, and quenching by through-space interactions with free acrylamide in solution or bounded on the protein surface can be ruled out [68]. Phosphorescence quenching therefore depends on the diffusion rate of acrylamide and is directly related to the flexibility of the protein structure. To establish whether the flexibility enhancement of azurin revealed by the decrease of phosphorescence lifetime is local or involves large regions of the macromolecule, we monitored the ease with which acrylamide diffuses inside the protein in the presence of saturating amounts (100 μM) of p53(1-63). Measurements were made at 20 °C in 50 mM Tris–HCl buffer pH 8.0/25 mM NaCl/1.0 mM EDTA. The acrylamide concentration was increased until the lifetime decreased by at least 10 fold. We found that $1/\tau$ increased linearly with acrylamide concentration, as expected for a dynamic quenching reaction (Fig. 3). The bimolecular acrylamide quenching rate constant (k_q) was determined from the slope of the phosphorescence lifetime Stern–Volmer plot ($1/\tau = 1/\tau_0 + k_q \cdot$ [acrylamide], where τ_0 is the unperturbed lifetime). In the presence of p53(1-63) the k_q value increased to 52 $M^{-1}\,s^{-1}$ from 32 $M^{-1}\,s^{-1}$ measured for the free azurin. A k_q value of 32 $M^{-1}\,s^{-1}$ with respect to 10^9 $M^{-1}\,s^{-1}$ expected for a solvent-exposed Trp residue [69] strongly indicates that acrylamide migration to the W48 site is dramatically hindered. Fig. 3 shows that the presence of p53(1-63) enhanced the accessibility of the acrylamide to the Az core, as shown by 1.6 fold increase in k_q. The relationship among changes in protein structure flexibility induced by specific ligand binding, different phosphorescence lifetimes, and different quencher k_q values is well discussed for the protein liver alcohol dehydrogenase [68]. The reduced viscous drag to the acrylamide migration to the W48 site is consistent with the loosening of the globular structure following binding to p53(1-63), as measured by the decrease in lifetime. This result also suggests that the increased structural flexibility provided by p53(1-63) extends over the proximity of the phosphorescence probe.

3.5. Dependence of the azurin lifetime on p53(1-63) concentration

To investigate the Az/p53(1-63) interaction more in detail, we studied the effect of increasing the p53(1-63) concentration on the Az phosphorescence lifetime. Addition of micro-molar quantities of p53 (1-63) to 4.0 μM Az was sufficient to alter the phosphorescence decay of Az and to make it non-exponential. All the decay curves were adequately fitted by a bi-exponential law. At increasing concentrations of p53(1-63) the long lifetime component, τ_L, decreased progressively from 630 ms down to a limit value of about 400 ms, and its relative amplitude decreased from 1.0 to 0.7. The short lifetime component, τ_S, was initially 90 ± 10 ms (α_S increasing from 0 to 0.1). Above a p53(1-63)/Az ratio of about 2.0 ([p53(1-63)] ≥ 10 μM), τ_S decreased at about 40 ms. As p53(1-63) concentration was further increased, this value remained roughly constant while α_S varied between 0.1 and 0.3, the average lifetime decreasing up to 300 ms. Fig. 4 shows that the trend of both $1/\tau_L$ and $1/\tau_{av}$ vs p53(1-63) concentration followed a roughly hyperbolic curve, reaching a plateau at relatively low p53(1-63) concentration. The non-linear dependence of $1/\tau$ on the p53(1-63) concentration is a strong indication that quenching by impurities was not effective under our experimental conditions. In addition, the hyperbolic behavior confirms a binding process and indicates that the observed change in τ Az can be ascribed to conformational changes induced by interaction with p53.

The change in τ azurin upon the formation of a complex with the non-phosphorescent p53(1-63) is described as follows:

$$\begin{array}{ccc} & k_{on} & \\ Az^* + p53(1\text{–}63) & \longleftrightarrow & Az^*\ p53(1\text{–}63) \\ & k_{off} & \\ \downarrow 1/\tau_0 & & \downarrow 1/\tau_c \end{array}$$

where $1/\tau_0$ and $1/\tau_c$ are the phosphorescent decay rates of Az in the free and complexed form, respectively. The scheme shows that the

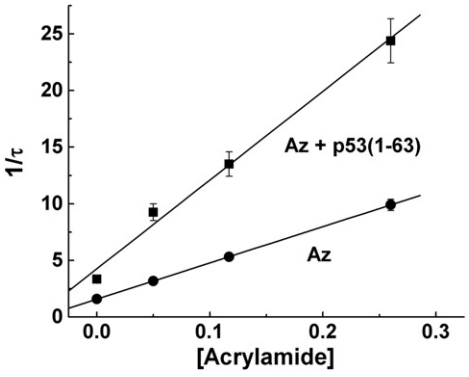

Fig. 3. Lifetime Stern–Volmer plots for the quenching of W48 Az phosphorescence by acrylamide in 50 mM Tris–HCl buffer, pH 8.0/25 mM NaCl/1.0 mM EDTA at 20 °C, before (●) and after (■) the addition of 100 μM p53(1-63). Each point was the average of at least three independent experiments and the error bars indicated the range of τ variations. λ_{ex} was 290 nm.

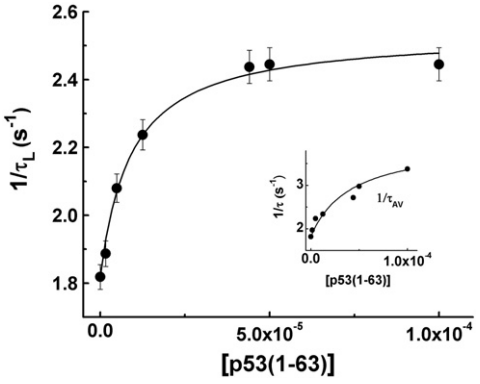

Fig. 4. Effect of p53(1-63) concentration on the long phosphorescence lifetime of Az. Az concentration was 4.0 μM in 2.0 mM Tris–HCl buffer, pH 8.0/25 mM NaCl/1.0 mM EDTA. T = 20 °C. The inset shows the change of the average phosphorescence lifetime at increasing p53(1-63) concentrations. Continuous lines through $1/\tau$ data represent the theoretical binding curve assuming $K_D = 7$ μM for $1/\tau_L$ or $K_D = 15$ μM for $1/\tau_{AV}$.

measured phosphorescent decay depends not only on the decay rates of the free and bound azurin, $1/\tau_0$ and $1/\tau_c$, but also on the interconversion between the two forms, that is k_{on} and k_{off}. Under rapid equilibration, i.e. k_{on}, $k_{off} > 1/\tau_0$, $1/\tau_c$ the phosphorescence intensity, $P_{(t)}$, decays with a single lifetime given by:

$$1/\tau = f_0/\tau_0 + (1-f_0)/\tau_c \quad (1)$$

where f_0 is the fraction of Az in the free form, which is related to the dissociation constant of the complex $K_D = k_{off}/k_{on} = f_0 \cdot [p53(1\text{-}63)]/(1-f_0)$. On the other hand, when the interconversion is slow with respect to the decay rate, k_{on}, $k_{off} < 1/\tau_0$, $1/\tau_c$, the phosphorescence decay is nonexponential with lifetimes equal to the lifetime of the free and complexed protein, and amplitudes corresponding to the free and complexed protein fraction, i.e.

$$P_{(t)}/P_{(0)} = f_0 \exp(-t/\tau_0) + (1-f_0)\exp(-t/\tau_c). \quad (2)$$

Under intermediate kinetic conditions, i.e. the interconversion takes place during the phosphorescence lifetime a non exponential decay is expected with lifetime components different from τ_0 and τ_c. Although the mathematical formulation is more complex and involves additional parameters [33], also in this case the average decay rate, $1/\tau_{av}$, is governed by the dissociation constant of the complex.

Clearly, the decay behavior observed during the binding experiments is not simply described by Eq. 1 or 2 hence the complex change in τ may in part be explained by intermediate kinetic regimes. However, the progressive increase in the short-lived fraction (α_S) up to the highest concentration of p53(1-63) used suggests that additional, weaker, 1:2 or 1:3 complexes may form. For the sake of simplicity, if we assume that the decrease in τ_L can be ascribed to the formation of a 1:1 complex in accordance with Eq. 1, we can estimate a dissociation constant $K_D = 7 \pm 3$ μM. The higher asymptotic value in the $1/\tau_{AV}$ profile, in comparison to $1/\tau_L$, raised the estimated value of K_D to 15 ± 4 μM (see Fig. 4, inset). The estimated affinity in the p53 peptide-Az complex is roughly 10 times weaker than that measured by isothermal titration calorimetry in the interaction between apo-azurin and the whole p53 ($K_D = 1.1 \pm 0.5$ μM, pH 7.5, T = 25 °C) [26]. The lack of contribution to the binding by other parts of p53 may explain the reduced affinity value suggested by our study. Analogously a similar difference between the K_D of p53 and p53(1-93) binding to MDM2 has been reported [59] and ascribed to the presence of synergy among different domains of p53.

4. Conclusions

P. aeruginosa azurin exhibits anti-cancer activity through formation of a complex with the tumor suppressor protein p53. The sites of the two proteins involved in the binding are not clearly identified. We believe that our results support the notion that azurin interacts with p53 at the level of the transactivation domain. The interaction with the p53(1-63) N-terminal fragment alters the internal dynamics of azurin, as revealed by the change in phosphorescence lifetime and the increase in the acrylamide diffusion rate inside the protein. Phosphorescence lifetime measurements reveal that comparable changes in the internal flexibility of azurin are also induced by the interaction with the whole p53. This result supports the assumption that p53(1-63) represents a suitable model to characterize the interaction between p53 and azurin. The dissociation constant of the p53(1-63)/Az complex is about 10 times larger than that measured for the interaction between azurin and the whole-length p53, suggesting that other domains of p53 may concur to the binding to azurin. Our measurements give no indications on the involvement of the tryptophan residues of p53(1-63) in the binding to azurin. This finding suggests that the site of p53 responsible for binding to azurin is different from the one involved in the interaction with MDM2, where the contribution of W23 is clearly shown. The p53 protein is subject to tight regulation at multiple levels. This is achieved by a variety of positive and negative regulators. Several inactivating proteins, such as MDM2, bind to the N terminal domain of p53, thus masking the transcription activation segment. Bound azurin, even if it does not overlap to the MDM2 binding site, may sterically shield p53 from interacting with inactivation and degradation proteins. The ability of inactivating proteins to associate with p53 highly depends also on the phosphorylation status of p53. In response to stress and damage when p53 phosphorylation takes place on multiple residues, MDM2 no longer associates with p53. Several phosphorylations are known to occur in the N-terminal transactivation domain of human p53 [70]. We could speculate about the possibility that binding of azurin to the NT domain of p53 could mimic the effect of phosphorylation on the protein structure.

In conclusion, our results contribute to the molecular understanding of the interaction of p53 with azurin, and help to understand how azurin can be employed to increase the half-life of such an important anti-tumoral protein.

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