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An Arsenic Fluorescent Compound as a Novel Probe to Study Arsenic-Binding Proteins

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Abstract Arsenic-binding proteins are under continuous research. Their identification and the elucidation of arsenic/protein interaction mechanisms are important because the biological effects of these complexes may be related not only to arsenic but also to the arsenic/protein structure. Although many proteins bearing a CXXC motif have been found to bind arsenic in vivo, new tools are necessary to identify new arsenic targets and allow research on protein/arsenic complexes. In this work, we analyzed the performance of the fluorescent compound APAO-FITC (synthesized from p-aminophenylarsenoxide, APAO, and fluorescein isothiocyanate, FITC) in arsenic/protein binding assays using thioredoxin 1 (Trx) as an arsenic-binding protein model. The Trx-APAO-FITC complex was studied through different spectroscopic techniques involving UV–Vis, fluorescence, atomic absorption, infrared and circular dichroism. Our results show that APAO-FITC binds efficiently and specifically to the Trx binding site, labeling the protein fluorescently, without altering its structure and activity. In summary, we were able to study a protein/arsenic complex model, using APAO-FITC as a labeling probe. The use of APAO-FITC in the identification of different protein and cell targets, as well as in in vivo biodistribution studies, conformational studies of arsenic-binding proteins, and studies for the design of drug delivery systems for arsenic anti-cancer therapies, is highly promising.

Keywords Arsenic carcinogenesis · Circular dichroism · Conformational studies · Drug delivery systems · Thioredoxin

Abbreviations

APAO	p-Aminophenylarsenoxide
APAO-FITC	Compound between p-aminophenylarsen oxide and fluorescein isothiocyanate
ATR-FTIR	Attenuated total reflectance-fourier transform infrared
CD	Circular dichroism
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
O.N.	Overnight
PBS	Phosphate saline buffer
RFU	Relative fluorescence units
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Trx	Thioredoxin

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1 Introduction

Arsenic-binding proteins are under continuous research. Their identification and the elucidation of arsenic/protein interaction mechanisms are important because the biological effects of these complexes may be related not only to arsenic but also to the arsenic/protein structure [51].

Three of the most plausible biological theories of arsenic carcinogenesis are based on arsenic-protein binding, generation of oxidative stress and alteration of DNA methylation [30]. Interestingly, these theories are also the most acceptable to explain the efficacy of arsenic in cancer therapies such as that for acute promyelocytic leukemia [35], but the exact mechanism of action of arsenic remains somewhat obscure [20].

According to Kitchin and Wallace [30], some of the protein targets to which arsenite may bind in vivo include tubulin, poly(ADP-ribose)polymerase, thioredoxin reductase, thioredoxin 1, estrogen receptor-alpha, arsenic (III) methyltransferase and Keap-1. However, these proteins might not be the only ones interacting with arsenic, and further studies are needed to determine principal arsenic targets in vitro and in vivo [54].

Arsenic causes alterations in multiple cellular pathways, including alterations in the expression of growth factors, suppression of cell cycle checkpoint proteins, alterations in the promotion of resistance to apoptosis, inhibition of DNA repair, alterations in DNA methylation, a decrease in immunosurveillance, and an increase in oxidative stress, by disturbing the prooxidant/antioxidant balance. Oxidative stress may be induced by cycling between the oxidation states of elements such as arsenic and iron or by interacting with antioxidants and increasing inflammation, which results in the accumulation of free radicals in the cells [19, 35]. This, in turns, alters the regulation of the intracellular redox state, critical for cell viability [4, 15, 46]. Among the different proteins involved in the response to oxidative stress, thioredoxin 1 (Trx) is a redox protein which is found in nearly all cells, which presents an active site whose amino acid sequence is highly conserved between bacterial and mammalian species [39].

Thioredoxins are proteins of about 100 amino acid residues, with a distinctive α/β topology, whose three-dimensional structure is quite well characterized. They participate in diverse redox reactions via the reversible oxidation of the conserved active center WCGPC [11, 45]. Also, thioredoxins function in vitro as co-factors for reduction of proteins [31] as a protective system against oxidizing species [36] [18] and can catalyze the in vitro folding of proteins [33].

As mentioned above, Trx is an arsenic-binding protein, which binds arsenic through the interaction of one As (III) atom with the active site of two vicinal cysteines (thiols), with a constant dissociation value in the submicromolar order [10]. This interaction has been widely studied before and is particularly interesting since it is useful to model and understand the arsenic molecular basis of action at the cellular level [14].

Thiol active–reactive dyes have been used to label biopolymers such as peptides, proteins, and oligonucleotides, and to demonstrate protein–nucleic acid interactions [2, 3, 13, 23]. In addition, several authors agree on the great value of fluorescent dyes on different research topics like protein conformational studies or cellular markers design, since fluorescent techniques are widely used because of their low detection limits and broad work range [12, 16, 40, 47].

In a previous work, we published the synthesis of a new fluorescent compound between p-aminophenylarsenoxide (APAO) and fluorescein isothiocyanate (FITC), denominated APAO-FITC, able to induce apoptosis in a leukemia cell model, where most of the apoptotic population showed the highest intensity of FITC fluorescence [17].

In the absence of arsenic-target markers that can be measured easily and avoiding typical interference of amino acids signals, APAO-FITC was designed wherein the arsanilic acid is suitably joined to a fluorescent marker (FITC). Then, binding to Trx was tested, not only because it is a common arsenic target but also due to its biological function (oxidoreductase).

The aim of the present work was to evaluate APAO-FITC as a probe to characterize arsenic-binding proteins, using Trx as a model protein. It is important to highlight the relevance of this report as the fluorescent arsenical probe showed prospective usefulness as a potential biomarker to identify arsenic-binding proteins, to study arsenic targets in vitro and in vivo experiments, and to evaluate the dependence on the structure of protein binding sites as well as on the folding process.

2 Materials and Methods

2.1 Reagents

Fluorescein isothiocyanate (FITC) was from Merck and p-aminophenylarsenic from Carlo Erba. Analytical grade solvents (dimethyl sulfoxide, β-mercaptoethanol, chloroform, dichloromethane, methanol) were all from Bioanalitica. All other reagents were of analytical grade.

2.2 APAO-FITC Synthesis

APAO-FITC (Fig. 1) was obtained as previously detailed in Femia et al. [17]. Briefly, small aliquots (c.a. 5 µl) of a

0.5 M FITC solution, prepared in anhydrous dimethyl sulfoxide, were added every 5 min to 5.0 ml of a 23 μM p-aminophenylarsonic acid solution, prepared in carbonate buffer, pH 9, at 4 °C, with constant stirring, to obtain a 1:1 p-aminophenylarsonic acid: FITC final ratio. The reaction progress was followed by thin layer chromatography, using a chloroform:dichloromethane:methanol (2:1:2, volume ratio) developing solvent mixture. After 8 h, the reaction was stopped by adding an ammonium chloride solution up to a final concentration of 50 mM. Then, As^{+5} was reduced to As^{+3} with a β-mercaptoethanol (2.0 M final concentration) overnight (O.N.) incubation at 4 °C.

The reaction mixture was lyophilized O.N. in a Freezone 4.5, LABCONCO lyophilizer; pre-cooled at −50 °C, maintaining the lyophilization process pressure within the range of 33×10^{-3} to 65×10^{-3} mbar.

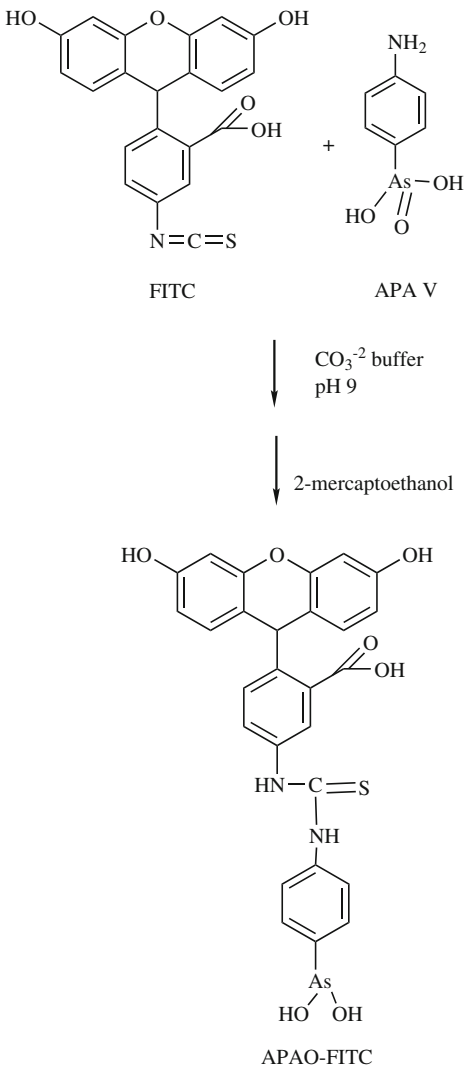

Fig. 1 Reaction scheme and compound chemical structures. Reaction has been performed at pH 9 in carbonate buffer

The reaction compounds were resolved in a silica column. To this end, 5 mg of the lyophilized reaction mixture was run in a silica chromatographic 10 cm × 3.5 cm column, collecting 15-ml eluate fractions. The elution was performed with the same thin layer chromatography solvent mixture described above. At the beginning, only low polarity solvents, like chloroform and dichloromethane, were used. The polarity was gradually increased by increasing the methanol proportion in the solvent mixture and the process was finished with 100 % methanol. A total of 25 fractions were collected and fractions 12–17 were rotoevaporated until dryness, and resuspended with 5.0 ml of phosphate buffer saline (PBS) for Trx association experiments.

The Trx-binding assay implied the use of two controls to ensure that APAO-FITC was bound to Trx through the As (III) atom. The positive control, p-aminophenylarsenoxide (APAO) was synthesized as described by Stevenson [49]. Briefly, 10.9 g of p-aminophenylarsonic acid was added to a solution containing 30 ml of methanol, 24 ml HCl and 100 mg of potassium iodide. Sulphur dioxide was bubbled through the stirred solution for 30 min. After this period, the solution changed its color from orange to pale yellow, and p-aminophenyldichloroarsine precipitated. The solution was cooled in an ice bath and the precipitate collected and washed with diethyl ether. Dichloroarsine was dissolved in 200 ml of 10 % ammonium hydroxide. After stirring for 15 min, p-aminophenylarsenoxide began to precipitate. The solution was cooled again in an ice bath. The precipitate was collected by filtration, washed with diethyl ether and dried under vacuum with sodium hydroxide. A 25 μM solution was prepared in a 0.1 M NaOH, and then neutralized with 0.1 M HCl.

A FITC negative control solution was prepared following APAO-FITC synthesis conditions but without p-aminophenylarsonic acid (see "APAO-FITC Synthesis"). It is important to remark that the addition of ammonium chloride deactivates reactive isothiocyanate groups in FITC. In this work, this control solution will be referred as FITC*.

2.3 Trx Purification

The thioredoxin 1 used in this work is a histidine-patch thioredoxin system that was purified as described previously by Carbajal et al. [7] from an *Escherichia coli* TOP10 strain transformed with the *pThioHis* plasmid (Invitrogen, Carlsbad, CA, USA). Briefly, this strain was grown in Luria–Bertani medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) containing 0.1 mg/ml ampicillin at 37 °C to an optical density of 0.5 at 550 nm. Isopropyl-β-D-thiogalactopyranoside was then added to a 0.5 mM final concentration. Then, *E. coli* cells were incubated for 3 h and harvested by centrifugation. Trx was released from

cells by an osmotic shock procedure and purified by column chromatography as described by the manufacturer.

The purification process was monitored following the presence of protein by measuring absorbance at 280 nm in a Nanodrop 1100 (Thermo Scientific). We identified the presence of Trx by enzymatic activity measurements and SDS-PAGE. The sample was lyophilized O.N. in sterile distilled water in a Freezone 4.5, LABCONCO lyophilizer, pre-cooled at −50 °C, maintaining the lyophilization process pressure within the range of 33×10^{-3} to 65×10^{-3} mbar. Finally, for binding assays, Trx was resuspended with a β-mercaptoethanol 1 mM solution prepared in sterile PBS and then, β-mercaptoethanol was removed by a molecular exclusion column PD10 (G25, Pharmacia, Sweden). This step was performed to reduce the cysteines on the active site of Trx and thus facilitate the binding of other molecules.

2.4 Trx Activity Assay

Trx activity determination is based on the precipitation of the insulin B chain that occurs after Trx-mediated reduction of the inter-chain disulfide bridges between insulin chains A and B [26]. Briefly, 70 µl of 300 mM phosphate, 3 mM ethylenediaminetetraacetic acid (EDTA), pH 6.6 buffer, 50 µl of sample, 40 µl of insulin and 40 µl of D-L-Dithiothreitol (DTT) (2 mM) were seeded in a 96-well plate. The reaction was monitored by measuring the turbidity of the medium at 595 nm in a microplate reader (MRXTC; Dynex Technologies) at room temperature. Turbidity at 595 nm versus time was plotted, determining the slope of the curve in the linear range. The slope allowed the calculation of the enzyme activity using Eq. 1:

$$y(\mathrm{AU/ml}) = m * \mathrm{x}\,(\mathrm{mg/ml}) + b \quad (1)$$

where y denotes the enzymatic units, m is the calculated slope and b is the intercept [26].

2.5 Trx/APAO-FITC Interaction

To prove the association of the synthesized compound to Trx, the protein was incubated for different times in the presence of APAO-FITC or controls (APAO, FITC* and PBS).

Labeling efficacy was tested in PBS, pH 7, and the compound (APAO-FITC or control compounds) was added maintaining a molar ratio of 5:1 mol of compound per mol of protein in all the experiments.

After 8, 24, 48 h and 7 days of incubation at 4 °C, 1 ml of each protein solution was seeded in a molecular exclusion column PD10 (Pharmacia, Sweden). Fractions of 0.5 ml were recovered and enzyme activity, SDS-PAGE, Bradford, A_{280} and fluorescence measurement were used to detect Trx.

2.6 Trx Unlabeling Assay Mediated by a Reducing Agent

To determine whether the interaction between Trx and the synthesized compound was effectively produced through the active site, we designed an "unlabeling test". In this test, the fluorescently labeled Trx fractions were incubated O.N. in the presence of the reducing agent DTT in 10 mM final concentration. DTT is a known strong reducing agent able to compete and displace arsenic from the Trx active site [8].

After DTT incubation, the protein was separated from the reaction mixture by size exclusion chromatography on a PD10 column (Pharmacia, Sweden). Then, fluorescence and UV–Vis spectra, concentration and enzymatic activity of the protein were measured. Note that the decrease in fluorescence intensity after DTT incubation is indicative of the displacement of the compound from the Trx active site.

2.7 UV–Vis Spectrophotometric Measurements

Protein concentration was estimated by determining absorbance at 280 nm ($\varepsilon_{\mathrm{Trx280}} = 14{,}070\ \mathrm{mM^{-1}\ cm^{-1}}$). In this sense, a UV–Vis spectrum was recorded in the range from 220 to 750 nm, with a 1 nm resolution with a UV–Visible 1600 Shimadzu Spectrophotometer. In addition, the peak observed at 490 nm was used to estimate FITC concentration, considering in this case an ε_{490} of 70,000 $\mathrm{cm^{-1}\ M^{-1}}$ [17, 22].

2.8 Fluorescence Measurements

The fluorescence spectra between 450 and 600 nm (3 nm resolution) were obtained at room temperature with an ISS K2 multifrequency phase fluorometer, using a 488 nm excitation wavelength. Relative fluorescence units (RFU) at 515 nm were measured with a Nanodrop 3300 (ThermoScientific), using a 488 nm excitation wavelength.

2.9 Arsenic Quantification

Arsenic was quantified by atomic absorption using an Analyst 800 Perkin Elmer spectrometer under electrothermal atomization stabilized temperature platform furnace conditions, including calibration curves with aqueous certified standards, performed at SECEGRIN-CONICET (Santa Fé, Argentina). The quantification limit of this method is 9 µg/l and the detection limit is 3 µg/l.

2.10 Attenuated Total Reflectance (ATR) FTIR Spectroscopy Measurements

The first approach to study the effects of APAO-FITC on Trx secondary structure was through ATR-FTIR since it is highly sensitive to detect small changes. FTIR measurements were performed for Trx, Trx treated with 2 mM of DTT, Trx-APAO and Trx-APAO-FITC, all in aqueous solutions, maintaining a protein of 15 μM concentration in all the experiments. Water spectrum was also determined. Measurements were performed by using a 0.6 ml aliquot per sample, in the measurement plate of the ATR accessory in an IRAffinity-1 Compact Fourier Transform Infrared Shimadzu spectrophotometer, and spectra were collected in transmittance mode after 350 scans in the range from 650 to 4,000 cm^{-1}, with a 4 cm^{-1} resolution. The spectra were processed and analyzed with the IR solution software (v. 1.50), provided by the manufacturer.

All spectra were subjected to an ATR correction [48]. To obtain a protein spectrum free of IR-water signals, the water spectrum was subtracted from every protein spectrum in an iterative way until a straight line was observed between 2,400 and 1,800 cm^{-1}. Finally, the spectrum thus obtained was smoothed using a 9-point Savitsky-Golay algorithm [48]. Peaks and relative intensities were calculated with the software, and settings for threshold, noise and minimal area parameters were adjusted to obtain the parameters of the main peaks.

2.11 Circular Dichroism (CD) Measurements

In order to evaluate the secondary and tertiary structure of Trx-APAO-FITC, Trx, Trx-APAO, and APAO-FITC, circular dichroism spectra were acquired. All compounds used were dissolved in 30 mM phosphate buffer, pH 7.

Spectra were obtained in a Jasco J-810 spectropolarimeter. For near-UV CD, five consecutive spectra were collected from 340 to 240 nm, with a 0.1 nm resolution and a 10 mm path length. In the case of far-UV CD, five consecutive spectra were collected for each sample from 320 to 200 nm, with a 0.1 nm resolution and a 1 mm path length. Data were acquired at a speed of 20 nm/min. Finally, the results obtained for the blank (buffer) were subtracted from the spectra obtained, and the ellipticity values (θ) were expressed as deg cm^2 $dmol^{-1}$.

In the case of near-UV spectra, protein and APAO-FITC concentrations were 11.0 and 7.0 μM, respectively, and in the case of far-UV spectra, 6 and 3.5 μM, respectively.

A thermal denaturation curve was also performed, measuring ellipticity at 222 nm, between 20 and 90 °C, and examining whether the process was reversible in any of the cases (Trx-APAO-FITC, Trx-APAO or Trx).

These experiments were carried out at a 4 μM protein concentration, in 10-mm light-path cuvettes. A ramp of 2 °C/min with a resolution of 0.5 °C was used.

3 Results

3.1 Trx/APAO-FITC Interaction

After purification, Trx purity was determined as previously described by Vizioli [52], and purity was over 90 %. To study the Trx/APAO-FITC interaction, different techniques involving UV–Vis, fluorescence, infrared and circular dichroism were used. Figure 2a shows the UV–Vis spectrum obtained for Trx/APAO-FITC, Trx and Trx incubated with FITC* (control spectra), after being solved in a molecular exclusion column PD10, to eliminate free APAO-FITC or FITC* molecules. The protein concentration in all cases was 32 μM. Note that in the visible region there is an absorbance peak whose maximum corresponds to that reported for the APAO-FITC compound [17] and that this peak is absent in both control spectra. Moreover, an emission maximum peak, corresponding to that found for APAO-FITC [17], is present only in the Trx/APAO-FITC fluorescence spectrum (see Fig. 2b), thus confirming that FITC* does not bind covalently to Trx.

After confirming that Trx interacts with APAO-FITC, we analyzed the kinetics of this interaction. As explained in "Materials and methods", "Trx/APAO-FITC interaction", aliquots of the different reaction mixtures (Trx/APAO-FITC, Trx, Trx/FITC*, and Trx/APAO-FITC/β-mercaptoethanol) were solved in a molecular exclusion column PD10, to obtain Trx or Trx complexes without free APAO-FITC or FITC* molecules. Different elapsed reaction times were analyzed (8, 24, 48 and 168 h) and the results obtained are shown in Fig. 3. As expected, Trx/APAO-FITC presented the highest fluorescence, for every time analyzed, when compared to Trx, Trx/FITC* and Trx/APAO-FITC/β-mercaptoethanol. Note that the maximum fluorescence in this sample is reached after 48 h. Trx alone did not present fluorescence at any of the times analyzed. This was not the case for Trx/FITC* and Trx/APAO-FITC/β-mercaptoethanol, which presented low fluorescence values with a slight tendency to increase over time (see Fig. 3). When β-mercaptoethanol, a known Trx active site reducing agent able to displace arsenic as DTT [26], was present in the reaction mixture, it caused a marked decrease in fluorescence intensity, when compared to Trx/APAO-FITC alone. The fluorescence obtained was found to be very similar to that obtained for Trx/FITC*, suggesting that FITC* and/or APAO-FITC interact poorly and unspecifically with Trx.

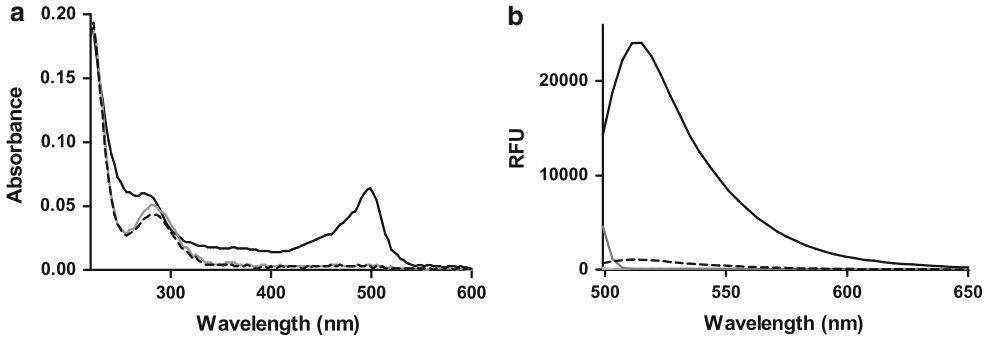

Fig. 2 UV-Vis (**a**) and fluorescence (**b**) spectra are depicted for Trx/APAO-FITC (*black solid line*), Trx (*gray solid line*), and Trx/FITC* (*dashed black line*). Spectra correspond to a 32 μM Trx concentration, measured after a 24-h incubation with APAO-FITC or FITC* and were performed on solutions at pH 7.4 in PBS buffer

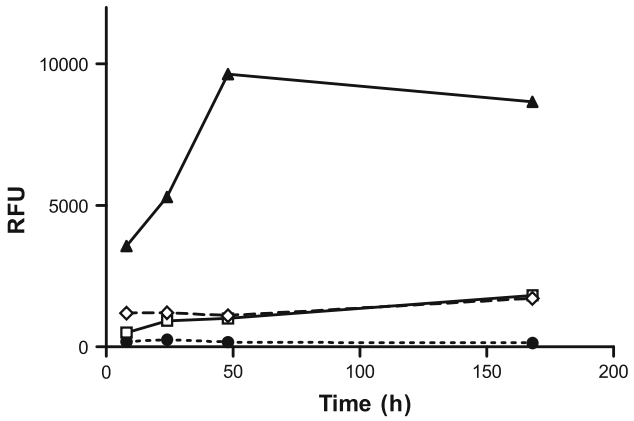

Fig. 3 Reaction kinetics depicted as RFU at 515 nm versus time (h) determined for Trx/APAO-FITC (*filled triangle*), Trx (*filled circle*), Trx/FITC* (*open square*), and Trx/APAO-FITC/*β*-mercaptoethanol (*open diamond*). Excitation was performed with a 488 nm wavelength radiation. In all cases Trx concentration was 32 μM and were prepared on solutions at pH 7.4 in PBS buffer

3.2 Trx Unlabeling Assay Mediated by a Reducing Agent

To confirm that APAO-FITC binds to Trx through the thiol groups present in its active site, a competition assay was designed. In this assay, the Trx/APAO-FITC complex was incubated with a high concentration of the active site competitor DTT and the effect was followed by UV–Vis spectroscopy, fluorescence and total arsenic determination.

Figure 4 shows the spectra corresponding to Trx, Trx/APAO-FITC and Trx/APAO-FITC treated with DTT. All the samples had the same protein concentration (32 μM) and activity at the moment of the assay. It is important to mention that after column exclusion chromatography, in this case, the samples were diluted in a 0.76 dilution factor. Figure 4 clearly shows a decrease in absorption at 490 nm in the UV–Vis spectrum (Fig. 4a) and a decrease in the emission maximum intensity at 516 nm in the fluorescence spectrum (Fig. 4b) of the Trx/APAO-FITC complex, after DTT treatment (compare the Trx/APAO-FITC spectrum with Trx/APAO-FITC/DTT presented in Fig. 4a, b).

The results obtained for Trx activity determination and arsenic quantification, as well as a summary of the data obtained from UV–Vis and fluorescence spectroscopy, are shown in Table 1. The percentage of Trx bound to As (III) (Trx/As %) was calculated based on arsenic concentration, and considering that one molecule of APAO or APAO-FITC is bound to one molecule of Trx. This percentage can be calculated using Eq. 2:

$$\text{Trx/As } \% = 100 \times [\text{As}]/[\text{Trx}] \quad (2)$$

where [As] is As concentration and [Trx] is Trx concentration, both in μM.

It is important to note that increasing As concentration is related to higher RFU values at 516 nm and that the Trx ratio is not significantly affected in the different conditions. It is also important to mention that, after an 8 h-DTT-incubation, no As was detected in the Trx/APAO-FITC

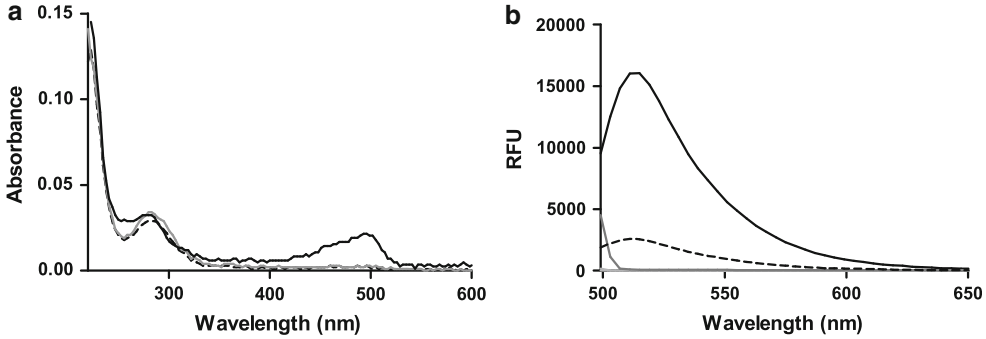

Fig. 4 UV-Vis (**a**) and Fluorescence (**b**) spectra for Trx-APAO-FITC (*black solid line*), Trx (*gray solid line*) and Trx-APAO-FITC/DTT (*dashed black line*), performed on solutions at pH 7.4 in PBS buffer

Table 1 Data obtained from the labeling and unlabeling events

Sample	Trx[‡] [uM]	FITC [uM]	As [uM]	RFU [516 nm]	Activity [UA/ml]	Trx/Activity ratio	Trx/As %[†]
Trx (24 h)	20	n/d	n/d	98	0.516	39	–
Trx-APAO (24 h)	19	n/d	7	134	0.47	40	37
Trx-APAO-FITC[a] (8 h)	32	8	7	3,850	ND	ND	22
Trx-APAO-FITC[b] (24 h)	32	13	18	6,870	ND	ND	56
Trx-APAO-FITC[c] (48 h)	32	24	26	12,250	0.62	52	80
Trx-APAO-FITC (24 h)/DTT[¥]	30	n/d	n/d	1,890	0.56	53	–
Trx/FITC* (24 h)	25	n/d	n/d	1,821	0.48	42	–

ND not determined

n/d non-detectable

[a, b, c] Aliquots of a unique protein sample analyzed at different times (see materials and methods)

[‡] The values informed correspond to the concentration determined after the molecular exclusion chromatography step

[†] Determined with Eq. 2

[¥] O.N. DTT incubation

sample (compare Trx/APAO-FITC (24 h) and Trx/APAO-FITC (24 h)/DTT sample, in Table 1, As column).

Finally, the maximum percentage of Trx bound to As was obtained after a 48 h-incubation (see column Trx/As percentage).

3.3 ATR-FTIR Measurements

ATR-FTIR experiments were carried out to study the secondary structure of Trx in the Trx/APAO-FITC complex. The comparison analysis was performed at amide I (1,690–1,630 cm^{-1}), II (1,500–1,550 cm^{-1}) and III (1,350–1,250 cm^{-1}) spectrum regions. A 12 μM protein concentration was maintained in all cases at the time assay measurement.

All proteins assayed (Trx, Trx-DTT treated, Trx-APAO, Trx-APAO-FITC) showed the same spectrum, with great coincidence on their maxima and relative intensity of peaks. Figure 5 shows this coincidence between spectra in the amide I, II and III regions for all cases, so it is possible to infer that no secondary structure modification or conformational changes are induced in Trx after APAO or APAO-FITC binding.

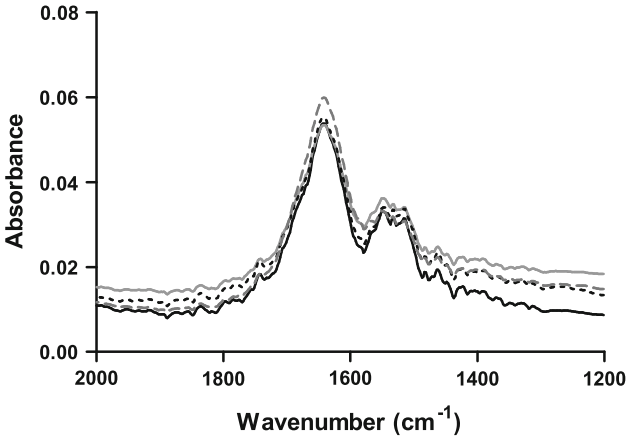

Fig. 5 IR spectra from Trx-APAO-FITC (*black solid line*), Trx-APAO (*gray solid line*), Trx/DTT (*black dotted line*) and Trx (*gray dashed line*), all performed in aqueous solution. The wavenumber range plotted corresponds to the amide I (1,690–1,630 cm^{-1}), amide II (1,500–1,550 cm^{-1}) and amide III (1,350–1,250 cm^{-1}) regions, which are typical for protein IR signals. Spectra were performed at room temperature

3.4 Circular Dichroism (CD) Measurements

To confirm the maintenance of the secondary and tertiary structures, CD experiments (in the far- and near-UV regions respectively) were performed for Trx, Trx-APAO, Trx/APAO-FITC and APAO-FITC (Fig. 6a, b, respectively). As seen in the FTIR spectra, no significant differences were detected for Trx/APAO-FITC respect to controls, neither in the far-UV nor in the near-UV CD experiments. Small differences seemed to appear when the Trx-APAO-FITC spectrum was compared to those of Trx and Trx-APAO. However, when the APAO-FITC spectrum was subtracted from the Trx-APAO-FITC one, the resulting spectrum was very similar to that found for Trx.

Also, thermal unfolding experiments were carried out to analyze the stability of the Trx/APAO-FITC complex respect to controls and the spectra obtained are shown in Fig. 7. The transition melting temperature (Tm) was calculated by first derivative spectra as reported by the groups of Yeh and Layton [32, 53]. The untreated Trx used in this work (Histidine-patch Trx) showed a Tm at 80 °C as reported by Brown et al. [5] for wild type Trx. However, reduced Trx (Trx-DTT), Trx-APAO and Trx-APAO-FITC

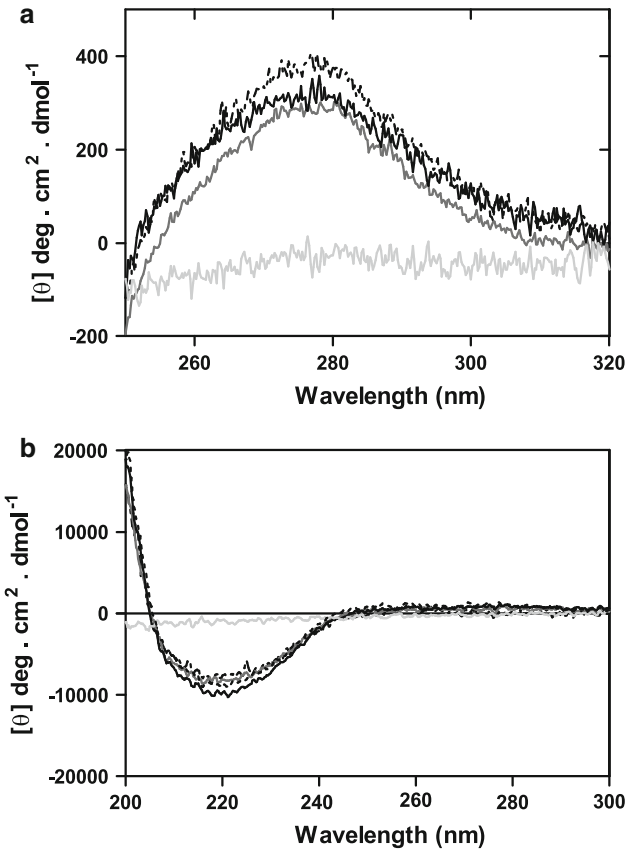

Fig. 6 Near-UV (**a**) and far-UV (**b**) CD experiments performed on Trx (*dark gray solid line*), Trx-APAO (*black dashed line*), Trx-APAO-FITC (*black solid line*) and APAO-FITC (*light gray solid line*). **b** shows the resulting spectrum obtained after the subtraction of the APAO-FITC spectrum from the Trx-APAO-FITC spectrum (*black dotted line*). The protein concentration used was 10 μM, whereas that of APAO-FITC was 5 μM in 30 mM phosphate buffer, pH 7. All spectra were performed at 20 °C

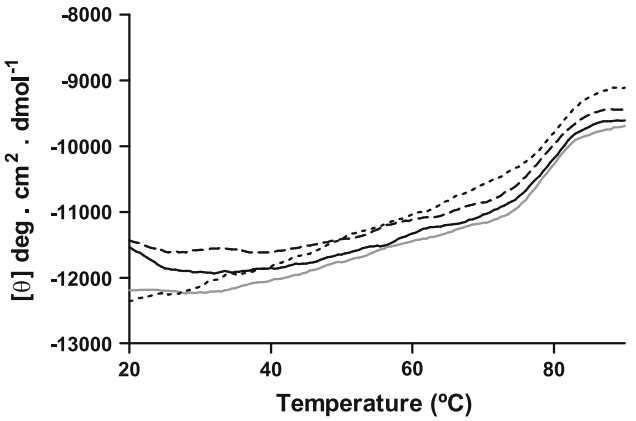

Fig. 7 Curves obtained for thermal unfolding experiments for Trx APAO-FITC (*black solid line*), Trx (*black dotted line*), Trx/DTT (*gray solid line*) and Trx-APAO (*black dashed line*) in 30 mM phosphate buffer, pH 7

showed a Tm at 78 °C for the fitted curve. It is known that in wild-type Trx variants, reduced Trx presents a lower Tm than oxidized Trx due to its lower stability at the reduced state [5]. The results obtained indicate that the process cooperativity is not quite affected in the Trx-APAO or Trx/APAO-FITC complexes and thus, that they may have a thermal stability similar to that obtained for reduced Trx. Interestingly, the process reversibility was fully confirmed in Trx-DTT, Trx-APAO and Trx-APAO-FITC, although slightly incomplete for untreated Trx.

Besides, after a detailed observation of the unfolding curves between 20 and 65 °C, small differences are observable. Untreated Trx shows a pre-transition slope that is not quite seen in the others and the difference is noticeable especially in the case of Trx-APAO-FITC. This pre-transition slope has been observed before [45], but this behavior has not been satisfactorily explained.

4 Discussion

Trx, as well as many other tumor-related proteins such as thioredoxin reductase and poly(ADP-ribose)polymerase, is known to bind arsenic [7]. In this work, we evaluated APAO-FITC, a previously synthesized fluorescent arsenic compound that was able to induce apoptosis in a leukemia cell model and to label the apoptotic cell population fluorescently [17], as a labeling probe for Trx.

The results indicate that APAO-FITC interacts with Trx efficiently and is able to label this protein fluorescently. It is widely known that As (III) binds to Trx through the vicinal cysteine groups in its active site. Our results, obtained with the APAO-FITC compound, are in agreement with this fact. We confirmed that the interaction between Trx and APAO-FITC takes place through the As atom present in the APAO-FITC molecule, since deactivated FITC (FITC*) slightly labeled Trx. Moreover, in the presence of DTT, a reducing agent used as a strong competitor for the Trx active site, APAO-FITC was displaced from Trx with the consequent loss of As and fluorescence. It is important to remark that unspecific binding of APAO-FITC or FITC* to Trx leads to small fluorescence values, 100 times lower than those obtained for Trx when APAO-FITC is bound to its active site. This is highly important because this means that the protein is efficiently fluorescently labeled only when the compound interacts specifically in this site.

The time necessary for the formation of APAO-FITC with high labeling efficiency was unusually longer than expected according to published data [29]. When experiments with short incubation times were performed, we found low labeling percentage (data not shown). Thus, we decided to extend the incubation period maintaining at 4 °C the mixture to reduce protease activity, and contamination. A significant

decrease in the protein activity (50 %) at 15 °C has been previously reported for a Trx family protein [21]. In this sense, incubation at 4 °C might reduce the binding kinetics of APAO-FITC to Trx′s active site. Although, at first, this might seem a disadvantage for APAO-FITC labeling, the incubation temperature can be optimized in function of a particular protein and condition. Besides the advantage of being a low-cost and simple process, the specific binding for vicinal di-cysteines and biological activity give rise to interesting applications of APAO-FITC.

Several authors [2, 3, 13, 23] have designed and synthesized probes for cysteine targeting, like porphyrinmaleimides [13]. However, this kind of probes result in a high percentage of cell protein labeling since many proteins have at least one cysteine residue [6], and thus, the resulting binding is too unspecific. On the other hand, a biarsenic fluorescein compound has been synthesized and is commercially available as FLAsH [24]. This compound has been proposed as a protein tag for chemical labeling of a particular protein in vivo, bearing the short tetracysteine peptide (CCXXCC), since this amino acid sequence is rarely seen in natural proteins.

Although it has been reported that FLAsH can bind non-specifically to cysteine-rich proteins [50], in the same work it was determined that, in the same experimental conditions, the fluorescence obtained for FLAsH interacting with a peptide containing the CXXC motif was only 38 % of that obtained for FLAsH interacting with a peptide containing the CCXXCC motif. In our case, we showed that APAO-FITC interacts with the WCGPC motif present in the Trx active site [45], and taking into account that reported by Ralph [42], we suggest that this compound can also label proteins containing the CXXC motif or structural vicinal cysteines. Thus, cell protein labeling with APAO-FITC would result in an intermediate situation between the very unspecific labeling due to porphyrinmaleimides and the high labeling with FLAsH.

Furthermore, it is important to note that although neither the cysteine-labeled compounds nor FLAsH are markers of naturally occurring targets of arsenic, APAO-FITC is. In line with this idea, APAO-FITC was able to induce apoptosis in a leukemia cell model [17]. This is not the case for FLAsH, which has been reported to present low or no toxicity due to its specific targeting [24].

On the other hand, it was of great relevance to determine whether APAO-FITC binding has an impact on protein native structure by inducing structural changes. To analyze this issue, we conducted ATR-FTIR studies because ATR-FITR is a very useful tool to observe changes in secondary structures of proteins, specifically those reflected in amide I (1,690–1,630 cm^{-1}), II (1,500–1,550 cm^{-1}) and III (1,350–1,250 cm^{-1}) regions [48], and because it has been previously used to analyse the effects of arsenic on proteins [41]. We observed and compared the ATR-FTIR spectra and found the same vibration peaks for Trx, Trx-APAO, Trx-APAO-FITC and Trx/DTT, in the regions mentioned above, with approximately the same relative intensity. Thus, the secondary structure of Trx was found to be maintained after APAO-FITC binding. It is important to mention that small conformational changes in the Trx active site might not be detected by this technique since it has been reported that oxidized or reduced Trx conformational differences can be observable only through Nuclear Magnetic Resonance spectroscopy [27, 28]. In this sense, Thorpe & Schneider have investigated the effect of As (III) binding on the structure of dicysteine-containing α helices and β-hairpin structure of small peptides. By using a methodology to obtain accurate Kd values sweeping UV–vis absorption, they proposed that As (III) compounds binding to dicysteine residues can be either detrimental of the helical structure in some cases or act as stabilizers in others, depending on the cysteine sequence distance. Also, when they studied the effects over the β-hairpin structure, they observed that As (III) binding to dicysteine motifs in proteins induced local changes in conformation without global rearrangements of the protein fold [10, 43, 44].

In line with this idea, it was important to confirm through circular dichroism experiments and FTIR that APAO-FITC binding to the active site of Trx does not affect its secondary structure. Moreover, when the tertiary structure was analyzed through this technique, we found that the Trx-APAO-FITC spectrum remained almost unchanged when compared to controls. This means that APAO-FITC binds to Trx without altering the tertiary structure of the protein, without affecting the package of aromatic residues that contributes to the near-UV CD spectrum. However, the existence of small changes near the tryptophan residues located relatively close to the active site cannot be overlooked. This is reflected in the spectrum region around 285–290 nm, as band broadening [1, 9, 34]. The fact that the secondary and tertiary structures are almost unchanged took even more importance when we found that Trx activity was not affected after APAO-FITC addition, implying the reversibility of Trx/APAO-FITC binding.

Taking into account the data obtained using Trx as an arsenic-binding protein model, we propose APAO-FITC as an arsenic compound able to fluorescently label proteins containing the CXXC motif, without altering their conformation, with the potentiality to be used in arsenic-binding protein structural/conformational fluorescence studies.

It is widely known that Trx has anti-apoptotic activity but it has also been proved that extracellular Trx administration does not lead to an anti-apoptotic effect in cells [37]. Also, high extracellular Trx levels have been related

to intracellular Trx down-regulation and cytoprotective effects [37]. In addition, it has been recently suggested that Trx can function as a therapeutic agent since it inhibits neovascularization and blocks neutrophil infiltration into inflammatory sites under several cellular stress-induced conditions or tissue injuries [38]. On the other hand, we have previously shown that APAO-FITC has an apoptotic effect on a leukemia cell model. In this work, we demonstrated the interaction between Trx and APAO-FITC (Trx/APAO-FITC), the labeling properties of the compound, and the reversibility of this link. Taking these facts into account, Trx might be useful as a delivery system of As (III) with the possible capacity to reduce its known systemic side effects [25], acting as a control releasing system.

Finally, we were able to study a protein/arsenic complex model, using APAO-FITC as a labeling probe. In this sense, the use of APAO-FITC to identify different naturally occurring As-binding proteins and/or in vivo cell targets, as well as to study the conformation of arsenic-binding proteins and design new drug delivery systems for arsenic anti-cancer therapies, is highly promising.

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