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ORIGINAL ARTICLE

Evidences of a natively unfolded state for the human topoisomerase IB N-terminal domain

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Abstract The N-terminal domain of human topoisomerase IB has been expressed, purified and characterized by spectroscopic techniques. CD spectra as a function of concentration and pH indicate that the domain does not possess any defined secondary structure. The protein is probably in a natively unfolded state since its denaturation curve is indicative of a non-cooperative transition. Evidence of a partially folded structure comes from the fluorescence spectrum of ANS, whose intensity increases in presence of the domain. Indication of a partial structural arrangement of the domain comes also from the endogenous fluorescence of tryptophans that is centred at 350 nm in the native and shifts to 354 nm in the fully denaturated protein. Interestingly despite the poor structural degree, as also confirmed by a predictive approach, the domain efficiently binds DNA, suggesting that the absence of a defined 3D structure has a functional meaning that permits the domain to be available for the interaction with different molecular partners.

Keywords Human topoisomerase IB · Natively unfolded · Fluorescence measurements · DNA binding · Hydrophobic site · Chemical denaturation

Abbreviations

Topo I	Human topoisomerase IB
WRN	Werner protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
CD	Circular dichroism
ANS	8-Anilino-1-naphthalene sulfonate
GST	Glutathione transferase

Introduction

Human topoisomerase IB (topo I) is a monomeric enzyme that controls the topological states of DNA during essential nuclear processes such as replication, transcription, DNA repair, chromatin assembly and chromosomal segregation (Champoux 2001). Topo I acts by cutting a single strand of the DNA double helix and generating a covalent 3′-phosphotyrosyl linkage (Redinbo et al. 1998). During this state, named cleavable-complex, the 5′-hydroxyl-end can rotate around the intact strand, allowing relaxation of positive and negative supercoils (Stewart et al. 1998; Frøhlich et al. 2007). Both cleavage and religation events are transesterification reactions (Redinbo et al. 1998; Redinbo et al. 2000). Besides the DNA relaxation activity, topo I is also involved in the control of transcription of genes which contain a TATA box in their promoter region and in the

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phosphorylation of proteins controlling RNA splicing (Merino et al. 1993; Shykind et al. 1997; Rossi et al. 1996; Andersen et al. 2002). It is also involved in DNA repair and the apoptosis process (Pommier et al. 2003; Søe et al. 2004).

The enzyme is composed of four domains: N-terminal (Met1-Gly214), core (Ile215-Ala635), linker (Pro636-Lys712) and C-terminal domain (Gln713-Phe765) (Staker et al. 2002) as illustrated in Fig. 1. The N-terminal domain has been considered to be dispensable for the relaxation activity of topo I (D'Arpa et al. 1988; Bronstein et al. 1999; Stewart et al. 1996a, b), although the domain binds DNA, participates in the control of the strand rotation and has been reported to be anchored to the nose-cone and the C-terminal domain of topo I (Lisby et al. 2001; Frøhlich et al. 2004; Laco and Pommier 2008). Within the N-terminal region there are nuclear localization sequences (NLSs) controlling the enzyme's distribution (Alsner et al. 1992; Mo et al. 2000) as well as phosphorylation sites that modulate the enzyme activity (Staron et al. 1995; Hackbarth et al. 2008; Pommier et al. 1990; Cardellini et al. 1994). The domain has been found to interact with numerous nuclear proteins, such as p53, poly(ADP-ribose) polymerase and WRN, which have a role in the regulation of topo I activity (Bauer et al. 2001; Czubaty et al. 2005; Mao et al. 2000; Redinbo et al. 1999a, b). Despite its central role little is known about its structure and no study of the N-terminal domain in solution has appeared (Chrencik et al. 2004; Johnson 1988). The domain is supposed to be highly disordered, as suggested by experiments performed on truncated forms of topo I (Stewart et al. 1996a, b) and by the fact that crystals of the protein have been obtained only upon its deletion (Redinbo et al. 1999a, b; Lesher et al. 2002). However, a definite demonstration of its order/disorder has never been provided.

In this work we have expressed the N-terminal region in *E. coli* and characterized it by several spectroscopic techniques. The data indicate that the domain is highly flexible: it does not have a defined 3D structure and can be described as a naturally unfolded state, that likely assumes different conformations according to the specific interaction with each of its numerous molecular partners.

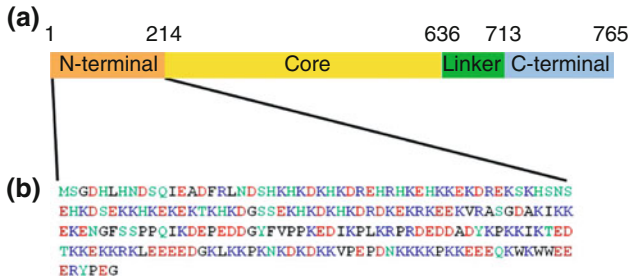

Fig. 1 **a** Schematic representation of the human topoisomerase IB domains. **b** Amino acidic sequence of the N-terminal domain with hydrophobic, hydrophilic, positive and negative residues represented in *black*, *green*, *blue* and *red* colour, respectively. The net charge and the isoelectric point of the domain, calculated with the ProtParam tool of the expasy database (http://www.expasy.org/tools) are +13 and 9.36, respectively (colour figure online)

Results and discussion

Prediction of the degree of disorder of the N-terminal domain

The degree of order/disorder of the N-terminal domain of the protein (1–218) has been evaluated using the PONDR program. Figure 2 shows that there are five regions with a PONDR score larger than 0.5, indicative of disorder (Romero et al. 2001), the two longer ones being between residues 17–104 and 129–215. Actually, about 90% of the domain is predicted to be in a disordered conformation, and a great number of residues have a score value higher than 0.7 (Fig. 2), diagnostic of a high degree of disorder. This result was in part expected since it has been reported that low hydrophobicity and a relatively high net charge promote disorder in proteins (Uversky et al. 2000a, b; Romero et al. 2001; Obradovic et al. 2005; Tompa 2005). The domain reflects many of the sequence characteristics typical of intrinsically disordered proteins: in fact only 11% of the amino acids belong to the so-called order-promoting residues (including bulky hydrophobic and aromatic amino acids residues), while 66% of the amino acids represents disorder-promoting residues. In addition, about 60% of total residues consists of charged amino acids, which may be responsible for charge–charge repulsion, driving unfolding not balanced by the hydrophobic interactions

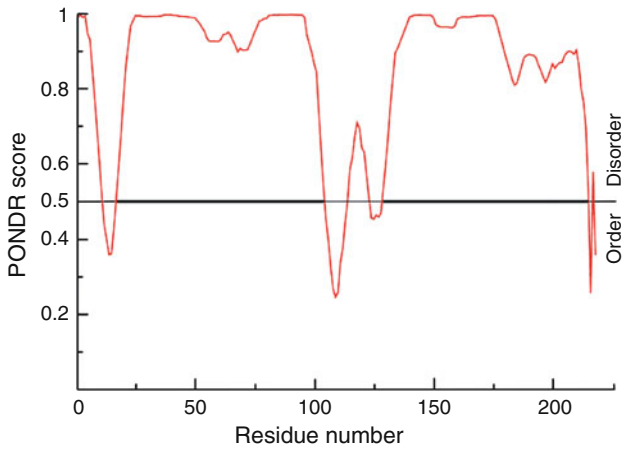

Fig. 2 Scale of disorder obtained with the program PONDR (Redinbo et al. 1999a, b). Values range from 0, complete order, to 1, complete disorder; 0.5 being the discriminating value between the ordered and disordered regions

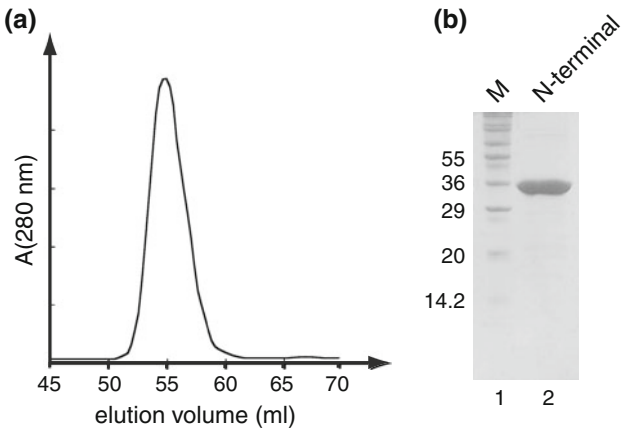

Fig. 3 **a** Elution profile of the N-terminal domain of the human topoisomerase IB injected onto a HiLoad16/60 Superdex 75 gel filtration FPLC column, equilibrated with 20 mM Tris–HCl, 0.15 M NaCl, pH 7.1. The *bars* indicate the elution volumes. The molecular weight standards (Sigma), blue dextran (2,000 kDa), albumin (67 kDa), ovalbumin (43 kDa), bovine superoxide dismutase (32 kDa), ribonuclease (13.7 kDa) eluted at the 41st, 49th, 54th, 59th and 74th millilitre, respectively. **b** Coomassie blue-stained SDS-polyacrylamide gel showing 10 μg of N-terminal domain (*lane 2*). The size of the protein markers (*lane 1*) in kDa are shown on the *left side*

that instead drive the folding (Uversky et al. 2000a, b; Uversky 2009).

Protein purification

The N-terminal domain of human topoisomerase IB was overproduced in *E. coli* BL21 as a fusion protein with glutathione transferase (GST). Before the final purification column, GST was removed, exploiting the presence of a cleavage site for thrombin between the GST and the N-terminal domain. The gel filtration chromatography elution profile of the N-terminal-domain (Fig. 3a) shows that the protein elutes with an apparent molecular weight of 43 kDa, much higher than that calculated from the amino acid sequence. This can be expected given that the hydrodynamic volume in the premolten globule in comparison with that of the native state increases up to three times (Uversky 2002). The product is fairly pure, as shown by the gel electrophoresis reported in Fig. 3b, but it migrates anomalously in SDS gels with an apparent molecular weight of 35 kDa, higher than the expected 27 kDa. A similar result is found also in the full-length topoisomerase IB, where the SDS protein gel displays a band with an apparent molecular weight of ~100 kDa instead of the expected 91 kDa (Stewart et al. 1996a, b). This may be attributed to the negatively charged residues in the domain, since it has previously been observed that negatively charged residues retard the migration of proteins in SDS gels (Stewart and Vogt 1991; Stewart et al. 1996a, b). Analysis of the amino acid sequence confirms that the product is constituted of the first 218 residues of topoisomerase I plus nine residues (Gly, Ser, Arg, Arg, Ala, Ser, Val, Gly, Ser), coming from the linker used to join the GST and the topoisomerase N-terminal domain.

CD spectra and chemical denaturation

The CD spectrum of the N-terminal domain in the far UV region at pH 7.1 is reported in Fig. 4a. The spectrum is characterized by two minima at 205 and 222 nm, the peak at 222 being about four times less intense than that at 205 nm. An all-helical protein is expected to have two minima at similar wavelengths but with comparable intensity (Woody 1995; Li and Jing 2000), whilst a reduced ellipticity at 222 nm is observed for pre-molten globule or native coils (Uversky et al. 2000a, b; Uversky 2002; Petzold et al. 2008). No major change is observed after dissolving the domain at pH 9.0, corresponding to its isoelectric point (Fig. 4a) indicating that a low degree of secondary structure is present in the domain independently of its protonation state, as described for other natively unfolded proteins (Uversky 2009). A fourfold decrease in protein concentration does not affect the shape and intensity of the spectrum, indicating the absence of any aggregation processes in this range of concentration (data not shown). Different programs for the deconvolution of CD spectra predict a fraction of disordered structure between 50 and 60% both at pH 7 and at pH 9, corresponding to the percentage of residues with a PONDR score >0.9.

The chemical denaturation curve of the domain obtained measuring the ellipticity at 222 nm as a function of urea concentration is reported in Fig. 4b. The transition curve is characteristic of a non-cooperative transition, at variance with what is observed in the denaturation curve of any natively folded domain (Kriwacki et al. 1996; Semisotnov et al. 1991). This observation indicates that the N-terminal domain must be in a conformation similar to a pre-molten globule.

Interaction with ANS

The fluorescence spectrum of ANS in the absence or presence of the N-terminal domain is shown in Fig. 5a. Upon addition of the domain the ANS fluorescence intensity increases and this enhancement is accompanied by a blue shift of the emission maximum. ANS is a hydrophobic fluorescent probe used to verify the presence of accessible hydrophobic sites, and it is widely used to monitor the partially folded states of proteins (Uversky et al. 2000a, b; Uversky 2009; Jones and Rumsby 1975). The increase in intensity and the blue shift observed in Fig. 5a are diagnostic of binding of ANS to a hydrophobic region of the

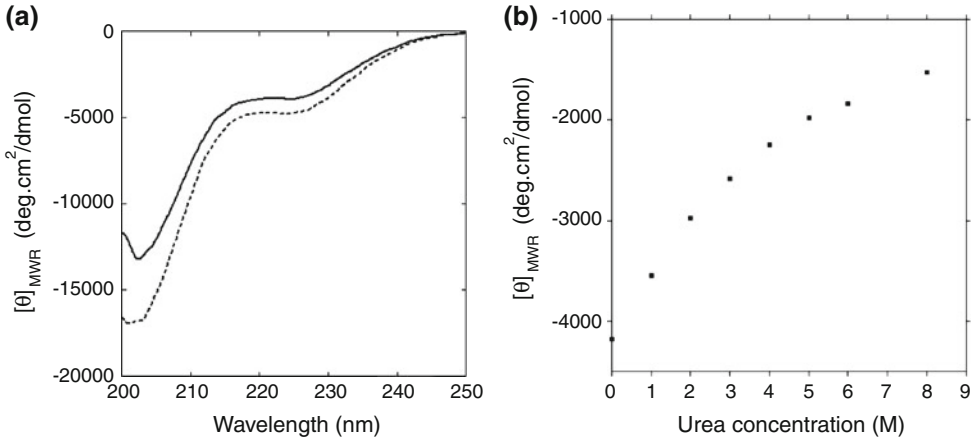

Fig. 4 **a** CD spectra in the 200–250 nm region of 20 μM N-terminal topo I domain in 20 mM Tris–HCl, 0.15 M NaCl buffer pH 7.1 (*full line*) or 9.0 (*dashed line*), at 20°C. **b** Chemical denaturation curve of N-terminal topo I domain measured through the ellipticity value at 222 nm as a function of urea concentration. A 20-μM domain solution in 20 mM Tris–HCl, 0.15 M NaCl buffer pH 7.1 was used

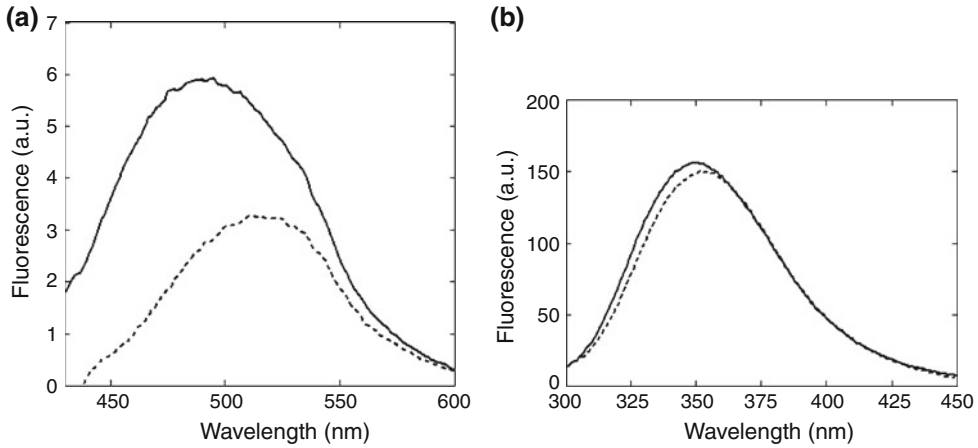

Fig. 5 **a** Fluorescence spectrum of 150 μM ANS in the absence (*dashed line*) or in the presence (*full line*) of the N-terminal domain. A 20-μM protein solution in 20 mM Tris–HCl, 0.15 M NaCl buffer pH 7.1 was used for the experiment. **b** Fluorescence spectrum of 20 μM N-terminal-domain, in the presence (*dashed line*) or absence (*full line*) of 8 M urea. The protein was dissolved in 20 mM Tris–HCl, 0.15 M NaCl buffer pH 7.1 The spectrum was recorded between 300 and 500 nm with an excitation wavelength set at 295 nm. The fluorescence intensity is expressed in arbitrary units (a.u.)

N-terminal domain, despite the large number of charged amino acids in its sequence. It is interesting to notice that no ANS binding is observed for the domain fully denatured in 8 M urea (data not shown), demonstrating that an ANS-accessible hydrophobic site is present in the native but not in the fully unfolded protein state.

Tryptophan fluorescence

The N-terminal domain contains four tryptophan residues located in position 203, 205, 206 and 217, respectively. The intrinsic fluorescence spectra can be used to monitor the environment surrounding these residues. The emission spectrum of the domain when excited at 295 nm is characterized by a band centred at 350 nm (Fig. 6), typical of indole groups relatively well exposed to the solvent. Irradiating the sample at 275 nm does not change the emission wavelength that remains centred at 350 nm with an apparent increase in the signal intensity. However, after normalization the spectra irradiated at 295 or at 275 nm are superimposable (insert Fig. 6). These results show that it is impossible to detect the emission spectrum of the tyrosine residues, either because an efficient tyrosine–tryptophan energy transfer occurs or because the fluorescence emission of tyrosines is quenched, possibly due to their proximity to positively charged residues (Wiczk et al. 2001; Liu et al. 2006). In fact, two of the three tyrosines in the domain are located adjacent to lysine or arginine (Fig. 1).

The fluorescence spectrum has been also recorded after protein denaturation. In this case the spectrum shifts slightly toward longer wavelengths as expected for a domain having the tryptophans fully accessible to the water solvent (Fig. 5b); the spectrum has in fact an emission intensity at 354 nm. This means that the band centred at 350 nm in the native N-terminal domain, although characteristic of tryptophan residues at the protein surface, is also indicative of a partially folded domain with the indole groups partially screened from the solvent.

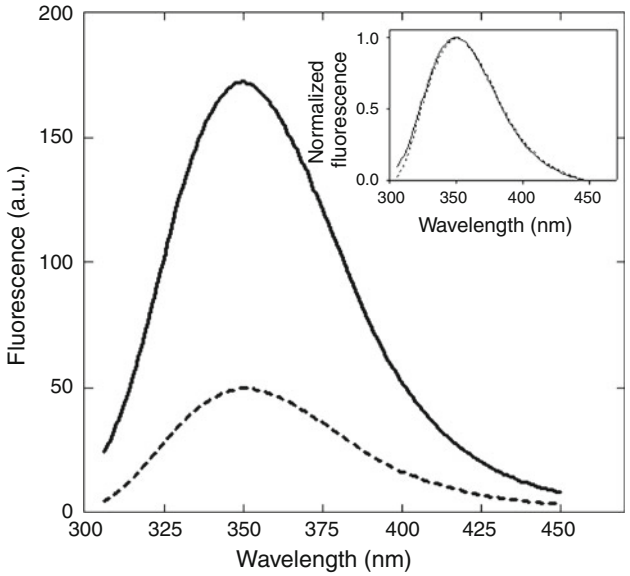

Fig. 6 Fluorescence spectrum of the N-terminal-domain (20 μM) in 20 mM Tris–HCl, 0.15 M NaCl buffer pH 7.1 The spectrum was recorded between 300 and 450 nm with an excitation wavelength set at 295 nm (*dashed line*) or 275 nm (*full line*). In the insert the same spectra were normalized in intensity to show substantial overlap. The fluorescence intensity is expressed in arbitrary units (a.u.)

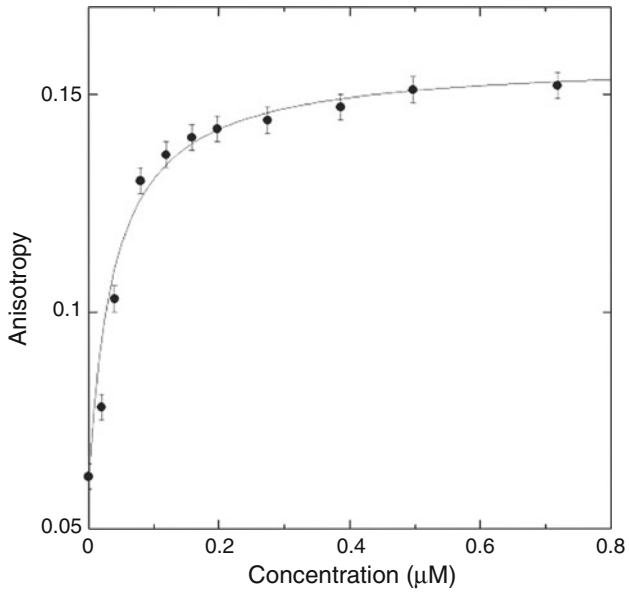

Fig. 7 Fluorescence anisotropy of a fluorescein-labelled 24-base double-stranded DNA (10 nM), as a function of topoisomerase IB N-terminal domain concentration

DNA binding

Binding of the N-terminal domain to DNA has been monitored through the anisotropy fluorescence signal of a fluorescein probe covalently attached at the 5′ end of a 24 base double-stranded DNA. The variation of the anisotropy signal of the fluorescein labelled DNA as a function of the N-terminal domain concentration is reported in Fig. 7. The anisotropy increases upon increasing the domain concentration, due to its binding to DNA that slows down the tumbling of the DNA substrate. Fitting of the experimental data permits the calculation of the dissociation constant K_d that was found to be $(2.9 \pm 0.4) \times 10^{-8}$ M, consistent with an efficient DNA–N-terminal domain interaction.

Conclusion

Experimental and computational analysis of the N-terminal domain depicts it as a largely unstructured and disordered region. In fact, a CD spectrum with a reduced ellipticity at 222 nm, when compared with the one at 205 nm (as seen in Fig. 3) has been found for many pre-molten globule or natively unfolded proteins, characterized by a low degree of secondary structure (Petzold et al. 2008). The chemical denaturation curve (Fig. 4) leads towards the same conclusion since its shape is typical of a non-cooperative process as expected for a natively unfolded domain (Semisotnov et al. 1991; Uversky 2009). The N-terminal domain has, however, some structure as indicated by the presence of a hydrophobic site where the ANS can bind which is lost after denaturation (Fig. 5). After denaturation we also observed a shift of tryptophan fluorescence from 350 to 354 nm (Fig. 7). The disordered structure of the domain is also predicted by the program PONDR, that indicates the presence of two large regions (17–104 and 129–215) characterized by an high intrinsic disorder (Fig. 1), likely due to the presence of a low and a large number of hydrophobic and charged residues, respectively (Uversky et al. 2000a, b; Obradovic et al. 2005; Dyson and Wright 2002). The domain, despite the absence of a defined structure binds DNA with high efficiency (Fig. 7), suggesting that the disorder is coupled to an efficient recognition mechanism. In fact, although one of the protein science dogma is that structure determines function, there are now many known proteins where disorder is required to confer recognition capabilities (Tompa 2005; Dunker et al. 2001, 2005; Uversky et al. 2005; Radivojac et al. 2007; Uversky et al. 2008; Wright and Dyson 2009). The common characteristic of these proteins is the presence of unstructured regions that can be useful to bind different targets with long surface of interaction and with different conformations. As a matter of fact, the N-terminal domain is known to be able to interact with several molecular partners (Mao et al. 2000) and its intrinsic disorder confer to the domain the necessary plasticity to efficiently bind to many them.

Materials and methods

Expression and purification of N-terminal domain of human topoisomerase IB

The plasmid pGEX-2TK-N-terminal domain was used to produce the protein (Lisby et al. 2001). It contains the sequence for glutathione transferase (GST), a linker sequence coding for a thrombin cleavage site, and the coding sequence for the first 1–218 amino acids of topo I, corresponding to the entire N-terminal domain plus four amino acids of the core domain of the enzyme.

Protein production was carried out as described by Lisby et al. (2001) with some modifications. Briefly, *E. coli* strain BL21 (Invitrogen) was transformed with the pGEX-2TK-N-terminal domain and grown at 37°C in Luria–Bertani broth, containing 2% of glucose and ampicillin (100 μg/ml) until absorbance value at 600 nm reached 0.5. At this point 0.15 mM IPTG was added and the cells were grown for 4 h at 27°C. Cells was harvested by centrifugation (4,000g × 10 min) and resuspended in PBS buffer. Crude cell extract was obtained by sonication and cell debris was removed via centrifugation (17,000g × 20 min). The supernatant was loaded onto a Sepharose 6B column activated with GSH (as described in technical bulletin) and equilibrated with 10 mM phosphate buffer pH 7.0. The column was washed with 100 mM phosphate buffer pH 7.0 and eluted with five column volumes of 20 mM phosphate buffer pH 7.8, 500 mM NaCl. The sample was diluted with the same buffer without NaCl to reduce the ionic strength, and the fusion protein was cut with thrombin at the cleavage site between the amino acidic sequences of GST and N-terminal domain. The digested sample was loaded onto a cationic exchange HiLoad™ 16/10 SP column, equilibrated with 20 mM phosphate buffer pH 7.8, 50 mM NaCl and eluted using a 50 mM–1 M NaCl linear gradient. The peak fractions corresponding to the N-terminal domain were loaded onto HiLoad™ 16/60 Superdex 75 equilibrated with 20 mM phosphate buffer pH 7.1, 0.15 M NaCl. To denature the protein, the gel filtration chromatography was carried out with 20 mM phosphate buffer pH 7.1, 0.15 M NaCl, 8 M urea buffer. Where indicated, the purified protein in 20 mM phosphate buffer pH 7.1, 0.15 M NaCl was denaturated by heating at 95°C for 20 min. The protein concentration was determined by the Lowry method (Lowry et al. 1951).

To determine the apparent molecular weight of the N-terminal domain, based on gel filtration, the proteins were chromatographed over a HiLoad™ 16/60 Superdex 75 equilibrated with 20 mM phosphate buffer pH 7.1, 0.15 M NaCl, and the elution profiles were monitored by UV absorbance at 280 nm. Molecular standard proteins (Sigma) were Blue dextran (2,000 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Bovine Superoxide Dismutase (32 kDa) and Ribonuclease (13.7 kDa). The data were plotted as log (molecular weight) versus the ratio of observed elution volume (V_e) to excluded volume (V_0). The calculated apparent molecular weight for the N-terminal domain of human topoisomerase I is 43 kDa.

Circular dichroism measurements

The protein samples used for CD measurements were at 20 μM concentration, in a 20-mM Tris–HCl, pH 7.1 or pH 9.0, 0.15 M NaCl, buffer. CD spectra, in the peptidic region 200–250 nm, were recorded on a Jasco J-710 spectropolarimeter using 0.1-cm quartz cuvettes. All experiments were carried out at 25°C using an external circulating bath. CD spectral deconvolution was performed according to the K2D (Andrade et al. 1993), ContinLL (Provencher and Glockner 1981), Selcon (Sreerema and Woody 1993) and CDsstr (Johnson 1999) algorithms by using the CDPro (Sreerama and Woody 2004) and K2D (http://www.embl.de/~andrade/k2d.html) web servers.

In the unfolding experiments the protein was incubated in the presence of increasing amounts of urea and after equilibration the dichroism at 222 nm was monitored. The final value was obtained after blank subtraction. Each measurement is the average of three replicates.

Fluorescence measurements

The native and denatured proteins were diluted, at a concentration of 20 μM, in a 20-mM Tris–HCl, pH 7.1, 0.15 M NaCl buffer or 20 mM Tris–HCl, pH 7.1, 0.15 M NaCl, 8 M urea buffer, respectively. The intrinsic fluorescence spectra of the tryptophan residues were recorded using a Perkin Elmer LS50B spectrophotometer, between 300 and 450 nm with an excitation wavelength set at 295 or 275 nm.

1-Anilinonaphthalene-8-sulfonic acid (ANS) dye was used to determine exposed hydrophobic patches in each protein sample. 150 μM ANS was added to samples, and its intrinsic fluorescence spectra was recorded from 450 to 600 nm with an excitation wavelength set at 350 nm. All the spectra were blank subtracted. Each measurement is the average of three replicates.

DNA binding

Oligonucleotide substrate CP25 (5′-TAAAAATTTTTC-TAAGTCTTTTTTC-3′) was incubated with the complementary strands CL14 (5′-GAAAAAAGACTTAG-3′) labelled with fluorescein at its 5′ end, and R11 oligonucleotide (5′-AGAAAAATTTT-3′). The strands were annealed to equimolar amounts in 10 mM Tris–HCl, pH 7.8,

100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol by heating to 95°C for 5 min, followed by slow cooling to room temperature.

Protein–DNA association was monitored by following the fluorescein steady-state anisotropy. Anisotropy experiments were performed on a SPEX Fluoromax fluorimeter (Edison, NJ, USA), with 488 nm excitation wavelength, collecting the emission at 530 nm (7 nm bandpass), through a 495-nm cutoff filter. A fixed DNA concentration (10 nM) was titrated with increasing amounts of protein, under continuous agitation. Each measurement is the average of nine replicates, with an integration time of 4 s. Temperature was controlled to 20°C, within 0.1°C, with a thermostatted cuvette holder.

Prediction of the protein disorder

The program PONDR (Romero et al. 2001; Obradovic et al. 2005), freely available at the website http://www.pondr.com, has been used to predict the degree of disorder of the N-terminal. This program is made of a set of neural networks that predict the degree of disorder of proteins and peptides considering amino acid composition, flexibility, hydropathy and coordination factors. For this purpose, we used the VL-XT method (Obradovic et al. 2005), where the VL1 (*V*ariously characterized *L*ong disordered regions) algorithm (Romero et al. 1997) is applied to long segments of more than 30 residues, and the XT (*X*-ray characterized *T*erminal disordered regions) algorithm (Li et al. 1999) to the N-terminal and C-terminal portions. The analysis has been done using the sequence having SwissProt code P11387, from residues 1 to 218. The results have been graphed with the Grace program (http://www.plasma-gate.weizmann.ac.il/Grace/).

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Conflict of interest The authors declare that they have no conflict of interest.

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