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Characterization of the zinc-metalloprotein nature of rat spermatidal protein TP2

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Abstract

Spermatidal transition protein, TP2, was purified from rat testes by Hg-affinity chromatography. The present study reports the details of the zinc-metalloprotein nature of TP2 by employing the ^{65}Zn-blotting technique. Chemical modification of cysteine by iodoacetic acid, and histidine by diethylpyrocarbonate, resulted in a near complete inhibition of ^{65}Zn-binding to TP2. The ^{65}Zinc-binding was localized to the V8 protease-derived N-terminal two-third polypeptide fragment. Circular dichroism spectroscopy studies of TP2 (zinc pre-incubated) and its V8 protease-derived polypeptide fragments revealed that the N-terminal fragment has a Type I-β-turn spectrum, while the C-terminal fragment has a small but significant α-helical structure. EDTA altered the circular dichroism spectrum of TP2 and the N-terminal fragment (zinc binding domain) but not that of the C-terminal fragment.

Key words: Spermatidal transition protein TP2; ^{65}Zinc blotting; Secondary structure

1. Introduction

The chromatin structure undergoes extensive modification during mammalian spermatogenesis resulting in the generation of highly compact and condensed spermatozoa [1–3]. In mammals, the spermiogenesis process is characterised by a transitory stage (stages 12–15) during which time the nucleosomal histones (both somatic and the testis-specific variants) are replaced by a set of small basic proteins TP1, TP2 [3,4] and the recently described TP4 [5]. Subsequently, these transition proteins are themselves replaced by protamine S1 (stages 16–19). Three important biochemical events occur coincident with the time of appearance of these proteins. They are (i) transformation of the nucleosomal type of chromatin into a smooth chromatin fibre, (ii) initiation of chromatin condensation, and (iii) cessation of transcription. TP2 has DNA-condensing properties [6] and was recently shown to contain two atoms of zinc bound per molecule by atomic absorption spectroscopy. Based on the cDNA-derived amino acid sequence, it was speculated that the protein could be folded to generate two zinc finger motifs [7]. Elucidation of the structure of the zinc coordinated motifs in the spermatidal protein TP2 and its interaction with DNA is very crucial to an understanding of the physiological significance of its transition. We report here our studies to demonstrate and localize the zinc binding sites within TP2 using a radioactive ^{65}Zn blotting technique. By using circular dichroism (CD) spectroscopy we have been able to show that there is a small but significant zinc-induced secondary structure in the TP2 molecule.

2. Materials and methods

Male albino Wistar rats (60 days) were used in all the experiments. All the chemicals used were of analytical reagent grade. $^{65}ZnCl_2$ (specific activity 618 mCi/g) was purchased from Bhabha Atomic Research Centre, Bombay, India.

2.1. Purification of TP2 and its V8 protease-digested fragments

TP2 was purified from the sonication-resistant spermatid (SRS) nuclei of rat testes. The SRS nuclei were prepared from rat testes as described by Platz et al. [8]. To generate larger quantities of TP2 for the present studies, we developed a Hg ligand-based affinity chromatographic technique. The SRS nuclei were extracted with 0.4 N HCl and was fractionated into 0–5% and 5–25% trichloroacetic acid-precepitable fractions. The 5–25% TCA fraction containing TP2 was washed with acidified acetone and ether after which the residue was dried under vacuum [8]. The dry powder was dissolved in 50 mM sodium acetate, pH 5, containing 1 mM EDTA, and incubated overnight at 4°C. The clear supernatant obtained after a low-speed centrifugation was loaded onto an organomercurial Sepharose column prepared according to the method of Sluyterman and Wizdenes [9]. The bound protein was eluted with 0.2 M cysteine hydrochloride, 1 mM EDTA, pH 7.5. The purity of the eluted protein was checked by electrophoresis on a 15% polyacrylamide acid-urea gel [10]. The authenticity of the purified TP2 was checked by determining the amino acid composition in a Shimadzu Amino Acid Analyser (post-column derivatization using OPA). TP2 was digested with V8 protease (*Staphylococcus aureus*; Sigma Chemical Co., USA) in 50 mM ammonium bicarbonate, pH 7.8, at 37°C for 12 h at an enzyme-to-protein ratio of 1:50. The N-terminal (88 aa) and C-terminal (28 aa) fragments were purified by preparative electrophoresis and subsequent electroelution followed by gel-filtration on Sephadex-G25 which was developed with 1% acetic acid. Histidine and cysteine residues in TP2 were chemically modified with diethylpyrocarbonate (DEPC) and iodoacetic acid (IAA) according to methods described by Miles [11] and Gurd [12], respectively.

2.2. Radioactive zinc blotting

^{65}Zn-Blotting was done according to the method of Mazen et al. [13]. For carrying out ^{65}Zn-blotting of the protein separated on an acid-urea gel, the gel was washed twice with 50 mM acetic acid containing 0.5%

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SDS for 30 min each before blotting [14]. In experiments wherein competition was carried out with different divalent cations, the nitrocellulose strips after the renaturation step were incubated in buffer B containing 10 mM competitor cations for 30 min. The control strip was incubated with only buffer B [15].

2.3. CD spectroscopy

CD spectra of TP2 and its V8 protease-derived polypeptide fragments (116–320 μg/ml based on amino acid analysis) were recorded in a JASCO (J-500 A) spectropolarimeter from 300 to 200 nm. Each spectrum is an average of 8 scans. Samples were in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 μM $ZnSO_4$, and with or without 0.2 mM Na_2EDTA. A mean residue weight of 115 was used to calculate molar ellipticity values (θ MRW).

3. Results and discussion

3.1. Localization of zinc binding sites in TP2.

To isolate TP2 we have combined the procedure of Platz et al. [8] with the Hg affinity chromatography technique to achieve maximum yield in a much shorter time. The protein pattern on an acid-urea gel of the fractions at different stages of purification is shown in Fig. 1. The 5–25% TCA precipitate contained predominantly 4 proteins, namely protein TP1, TP2 and two other polypeptides moving slower than TP2. After chromatography on an organomercury Sepharose column, only TP2 was retained on the column (lane 3), while TP1 and the upper two bands were in the flow-through fractions (lane 4). A small faint band above TP2 in lane 3 is the aggregated product of TP2, as demonstrated by Western blotting using polyclonal anti-TP2-antibodies (data not shown). The yield of TP2 obtained using this procedure was approximately 350 μg protein/50 g of testes.

To localize the zinc binding site within the TP2 molecule, we employed the ^{65}Zn-blotting technique [13]. The results presented in Fig. 2A demonstrate that TP2 indeed binds to ^{65}Zn, confirming earlier observations [7]. The cDNA-derived amino acid sequence of rat TP2 [16] shown in Fig. 2B has a glutamate residue at position 88. When TP2 was cleaved with protease V8, it yielded two polypeptide fragments (Fig. 2A, lane 2) corresponding to the N-terminal fragment (88 aa) and a C-terminal peptide (28 aa). Among these two fragment only the N-terminal fragment bound to ^{65}Zn (Fig. 2B, lane 2). In order to determine the specificity of metal ligand binding to TP2 we carried out competition studies with other divalent metal ions in a ^{65}Zn-blotting experiment. Preincubation with 0.1 mM unlabelled $ZnSO_4$ reduced the ^{65}Zn binding by 61%, while with 10 mM $ZnSO_4$ there was almost near complete inhibition of ^{65}Zn binding. On the other hand preincubation with 10 mM $CaCl_2$ and $MgCl_2$ did not affect ^{65}Zn binding to TP2. Cadmium and cobalt, which have been shown to replace Zinc in many of the Zinc-metalloproteins [17,18], also reduced ^{65}Zn binding of TP2 by 61 and 59%, respectively (Fig. 3 and Table 1).

There are 5 cysteine and 12 histidine residues in TP2 of which 5 cysteine and 11 histidines are located in the V8-derived N-terminal peptide. In order to demosntrate the involvement of cysteine and histidine residues of Tp2 in its binding to zinc, we carried out chemical modifications of these amino acids and checked the ability of modified protein to bind to zinc in the ^{65}Zn-blotting experiment. Iodoacetic acid treatment of TP2 greatly reduced its ^{65}Zn binding capacity (Fig. 4A, lane 4). From the densitometric scan of the autoradiogram, the per-

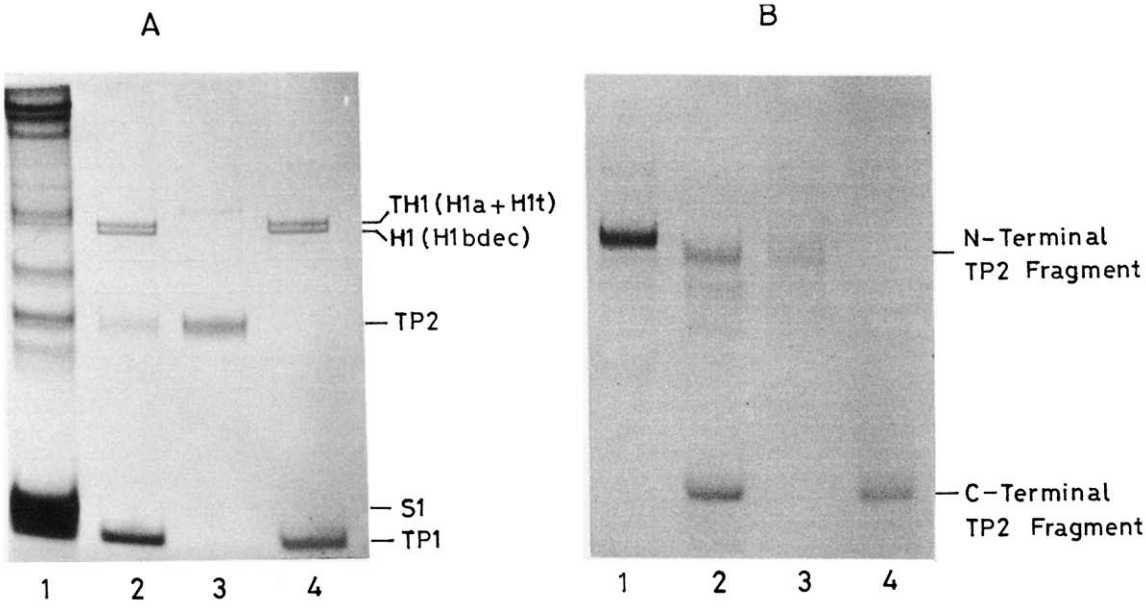

Fig. 1. Acid-urea polyacrylamide gel electrophoretic patterns of fractions at different stages of purification of TP2 (A) and its V8 protease-derived N-terminal and C-terminal fragments (B). (A) Lane 1, 0.4 N HCl-soluble proteins of SRS nuclei (60 μg); lane 2, 5–25% trichloroacetic acid precipitate (10 μg); lane 3, purified TP2 eluted from a Hg-affinity column with 0.2 M cysteine HCl (5 μg); lane 4, flow-through from a Hg-affinity column (10 μg). The proteins were visualized after staining with Amido black. (B) lane 1, intact TP2 (10 μg); lane 2, V8 protease-digested TP2 (10 μg); lane 3, purified N-terminal fragments (3 μg); lane 4, purified C-terminal fragment (3 μg).

centage decrease was calculated to be 70%. On the other hand DEPC treatment of TP2 resulted in a more than 95% decrease in the ability of TP2 to bind ^{65}Zn (Fig. 4B, lane 4). These results suggest that both histidine and cysteine residues are coordinated with each of the two zinc atoms since modifying either one of these amino acids resulted in a substantial decrease in ^{65}Zn binding to TP2.

3.2. *Zinc-induced alterations in the secondary structure of TP2*

The CF [19] and GOR algorithms [20] predict an α-helical structure between residues 85 and 100 (Fig. 4A). These algorithms predict extensive β-turns in the N-terminal region of TP2. It can be noted that within this region there are 14 proline residues between residues 11 and 81 (Fig. 2B), which favours β-turns in the polypeptide chain. Since the results presented above clearly demonstrated that TP2 binds to zinc and that the binding site(s) is located in the V8 protease-derived N-terminal polypeptide fragments, it was of interest to see whether zinc affects the folding of TP2 and its polypeptide fragments. For this purpose we have carried out a detailed analysis of the secondary structure by CD spectroscopy. Fig. 5B shows the spectrum of an intact TP2 molecule

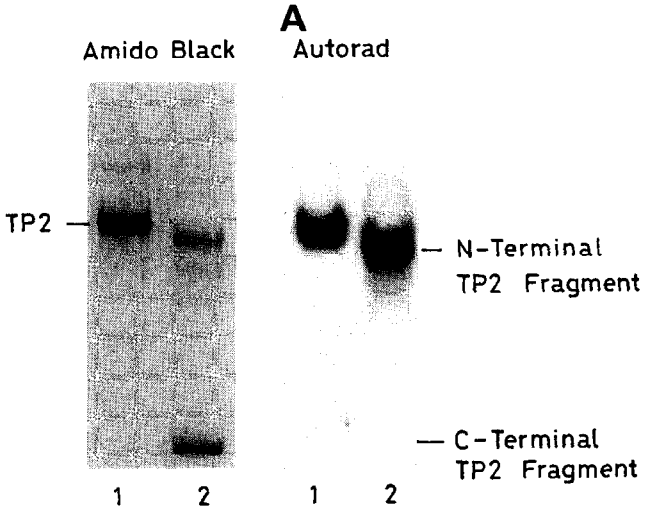

Fig. 2. (A) ^{65}Zn-blotting with TP2 and its V8 protease-derived fragments. Proteins were separated on a 15% acid urea polyacrylamide gel [10] and then transferred onto a nitrocellulose sheet. Lane 1, purified TP2; lane 2, V8 protease-digested TP2. (B) cDNA-derived amino acid sequence of rat TP2 [16]. The cysteine and histidine residues are encircled. The arrow indicates the site for V8 protease cleavage.

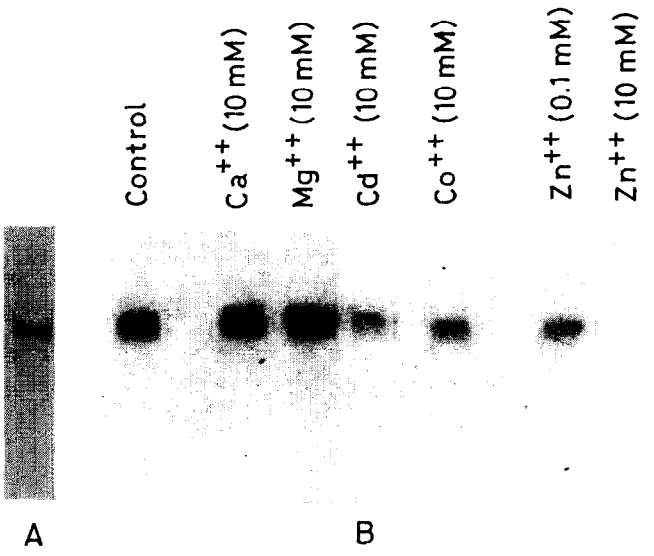

Fig. 3. Competition of ^{65}Zn-binding to TP2 with different divalent cations. For this experiment purified TP2 was electrophoresed on a 15% SDS-polyacrylamide gel [23]. (A) Coomassie blue-stained pattern of TP2. (B) Autoradiogram.

pre-incubated with 10 μM $ZnSO_4$. The spectrum reveals a large ellipticity minima at 200 nm ($\theta = -13{,}033$) and a shoulder at 222 nm ($\theta = -2682$). The shape of the curve is typical of a type I β-turn which has been observed for Cbz-Gly-Gly-Pro-Gly-*o*-stearate in acetonitrile [21].

When TP2 was pretreated with 0.2 mM EDTA, there was a decrease in the ellipticity at 200 nm from −13,033 to −10,733. Furthermore, the shoulder at 222 nm almost disappeared. Several conformers of β-turns are believed to be present in polypeptides [21]. Therefore, from these results one can conclude that zinc induces alterations in the secondary structure of the polypeptide fold, possibly stabilizing one of the conformers of the β-turn. Another possible interpretation for the generation of a shoulder at 222 nm is that zinc induces a small but significant α-helical structure in the polypeptide chain since the ellipticity minima at 220 nm is a characteristic feature of an α-helix. More refined structural studies are necessary

Table 1
Effect of different divalent cations on ^{65}Zn binding to TP2

Competitor divalent cations	% binding
None	100
+ $ZnSO_4$ (0.1 mM)	38.28
+ $ZnSO_4$ (10.1 mM)	0
+ Ca^{2+} (10.0 mM)	126.56
+ Mg^{2+} (10.0 mM)	100.56
+ Cd^{2+} (10.0 mM)	39.06
+ Co^{2+} (10.0 mM)	41.4

The autoradiogram was scanned in a LKB Laser densitometer and the area under the peak was calculated. The area obtained for the control was taken as 100%. The results presented are the average of 3 independent experiments.

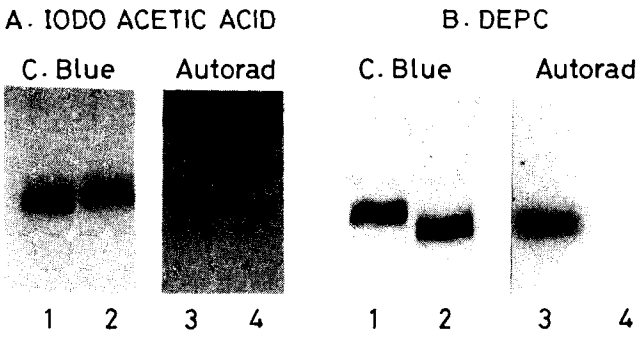

Fig. 4. Effect of modification of cysteine and histidine residues with iodoacetic acid (IAA) and diethylpyrocarbonate (DEPC), respectively, on the binding of ^{65}Zinc to TP2. TP2 (20 μg) was treated with either IAA (10 mM) or DEPC (20 mM) after which the excess reagents were quenched with 20 mM cysteine hydrochloride and 20 mM histidine, respectively. ^{65}Zinc blotting was subsequently carried out. Lane 1, control TP2; lane 2, chemically modified TP2; lane 3, ^{65}Zinc-blotted autoradiogram of TP2; lane 4, ^{65}Zinc-blotted autoradiogram of modified TP2.

for a finer analysis of zinc-induced alterations in the structure of TP2. Fig. 5C and D show the dichroic spectra of the V8 protease-derived N-terminal and the C-terminal polypeptide fragments of TP2, respectively. The shape of the spectrum of the N-terminal fragment (Fig. 5C) is very similar to that of intact TP2 in that a major minima at 200 nm (θ = −9030) and a shoulder at 222 nm (θ = −1548) were observed. It is also apparent from Fig. 5C that removal of zinc by treatment with EDTA resulted in a decrease in $\theta_{200\ nm}$ from −9030 to −7740, and also in the abolition of the shoulder at 222 nm, as was the case with intact TP2. The CD spectrum of the C-terminal fragment, on the other hand, shows a typical α-helical spectrum characterized by two minima at 208 nm (−790) and at 222 (−416), in agreement with the structure predicted by the two algorithms (Fig. 4A). The percentage α-helical content of the C-terminal fragment was calculated to be 2%. This can be localized to the polypeptide sequence between residues 85 and 100 in TP2 (Fig. 2B). It is interesting to note that pre-incubation of the C-terminal fragment with EDTA did not affect either the shape or the ellipticity value, which confirms our finding that zinc binding sites are located in the N-terminal fragment.

At this juncture, it may be pertinent to point out that human sperm protamine (which replaces TP1, TP2 and TP4 during the final stages of spermiogenesis) was also shown to be a zinc-metalloprotein by Bradbury and coworkers [22]. Using the CD spectroscopic technique,

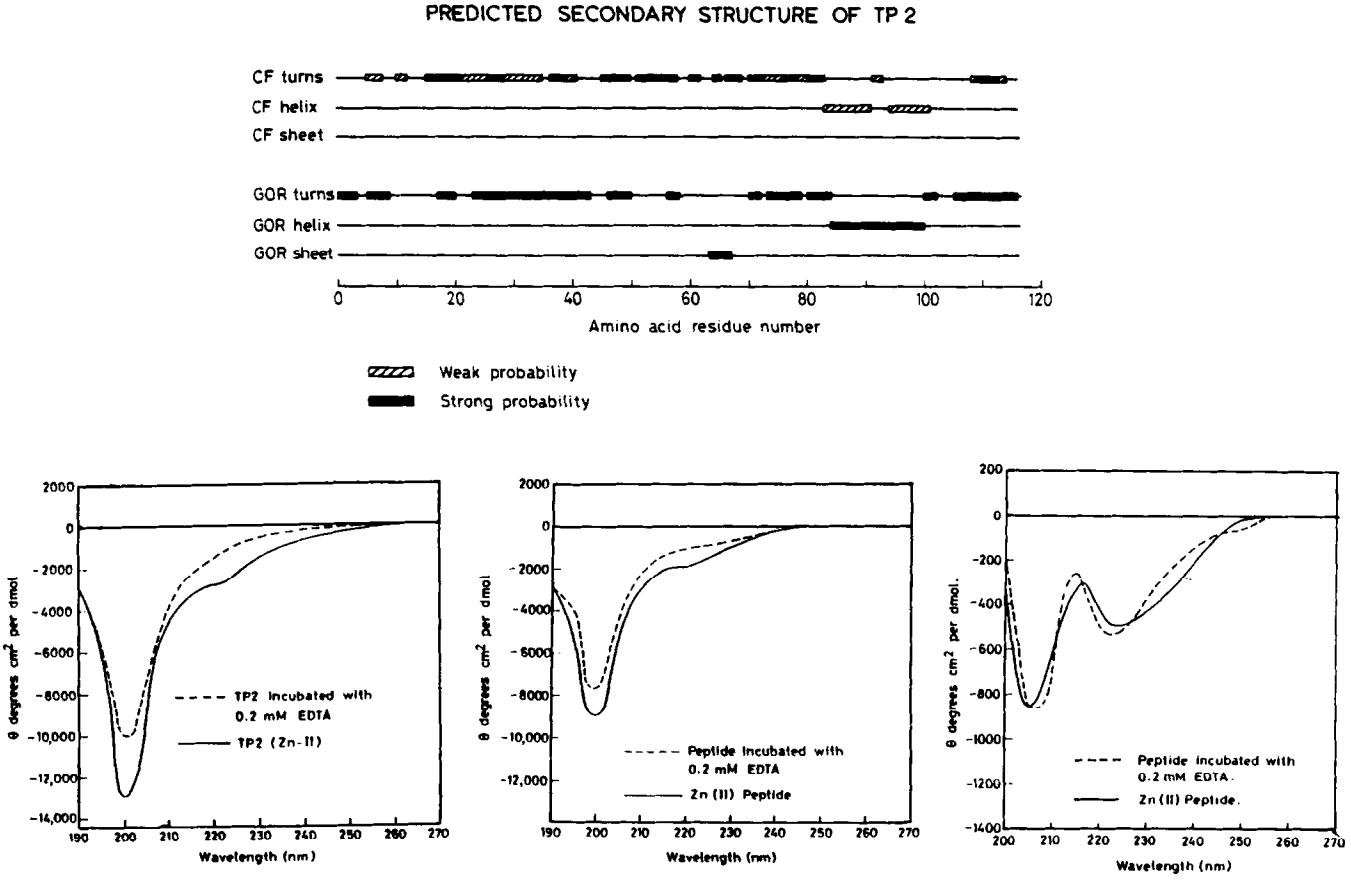

Fig. 5. Secondary structure prediction of TP2 and CD spectra of TP2 and its V8 protease-derived fragments. (A) Secondary structure prediction according to Chou and Fasman [19] and GOR algorithms [20]. (B–D) CD spectra of TP2, and its N-terminal and C-terminal fragments, respectively. The spectra were recorded both in the presence of zinc (10 μM) and after treatment with EDTA (0.2 mM).

they showed that zinc induces both β-turns and antiparallel β-sheets in protamine. The ellipticity minima of protamine in the absence of zinc is at 197 nm, which is characteristic of a random coil. There is a decrease in the ellipticity at 197 nm upon zinc binding and the ellipticity minima is then observed at 200 nm. On the other hand, in the case of TP2, zinc increases the negative ellipticity at 200 nm while at the same time generates a shoulder at 222 nm. However, both TP2 and protamine are rich in β-turns. It remains to be seen what this structural feature means in terms of their DNA binding properties.

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