Human spermatid-specific thioredoxin-1 (Sptrx-1) is a two-domain protein with oxidizing activity

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Received 30 July 2002; accepted 9 September 2002

First published online 26 September 2002

Edited by Gunnar von Heijne

Abstract **Spermatid-specific thioredoxin-1 (Sptrx-1) is the first member of the thioredoxin family of proteins with a tissue-specific expression pattern, found exclusively in the tail of elongating spermatids and spermatozoa. We describe here further biochemical characterization of human Sptrx-1 protein structure and enzymatic activity. In gel filtration chromatography human Sptrx-1 eluates as a 400 kDa protein consistent with either an oligomeric form, not maintained by intermolecular disulfide bonding, and/or a highly asymmetrical structure. Analysis of circular dichroism spectra of fragments 1–360 and 361–469 and comparison to spectra of full-length Sptrx-1 supports a two-domain organization with a largely unstructured N-terminal domain and a folded thioredoxin-like C-terminal domain. Functionally, Sptrx-1 behaves as an oxidant in vitro when using selenite, but not oxidized glutathione, as electron acceptor. This oxidizing enzymatic activity suggests that Sptrx-1 might govern the stabilization (by disulfide cross-linking) of the different structures in the developing tail of spermatids and spermatozoa.**

Key words: Thioredoxin; Spermatozoon; Fibrous sheath; Redox regulation

1. Introduction

In sexually reproducing species the fusion of the gametes is a prerequisite for the generation of the offspring whose genetic makeup is different from both parents. This mode of reproduction via fertilization emerged during evolution and has been maintained in most metazoans including mammals [1]. In higher vertebrates the complexity of the fertilization process is increased by the advent of internal fertilization and by the functional adaptations of the oocyte vestments. To circumvent associated difficulties, the spermatozoon has acquired a highly specialized morphology consisting of cytoskeletal components, which appear to have no structural counterparts in somatic cells [2]. The most evident among these cytoskeletal structures are the perinuclear theca (PT) of the sperm head and the outer dense fibers (ODF) and fibrous sheath (FS) of the sperm tail [3]. The mammalian ODF and FS are composed of numerous polypeptides of which only a few have been cloned and characterized to date (reviewed in [4]). ODF and FS are believed to regulate the beat of the sperm flagella by adding stiffness, elastic recoil and protection against shearing forces generated during sperm transit through epididymis [5]. However, there is increasing evidence for a more active role of ODF and FS in sperm function than merely structural, also supported by the fact that among their constituent proteins there are a few displaying either enzymatic or regulatory functions.

Thioredoxins are a class of multifunctional protein disulfide reductants that participate in different cellular processes by the reversible oxidation of cysteine residues in their conserved active site Cys-Gly-Pro-Cys. Thioredoxins have both intracellular and extracellular roles such as electron donor for ribonucleotide reductase, regulator of cell growth and apoptosis, embryonic implantation, modulator of the DNA binding activity of transcription factors, co-cytokine and as protective mechanism against oxidative stress (reviewed in [6,7]). In the human genome, three ubiquitous forms of thioredoxins have been identified so far: Trx-1, a cytosolic enzyme that can translocate into the nucleus upon certain stimuli [7], Trx-2, a mitochondrial enzyme [8] and Txl-1/Trp32, a protein with no function described so far [9,10]. In addition, we have recently identified two novel members of the thioredoxin family, named spermatid-specific thioredoxin (Sptrx)-1 and Sptrx-2, whose expression is restricted to the haploid phase of spermatogenesis [11,12]. Sptrx-1 transiently associates to the longitudinal columns of the FS during sperm tail assembly but does not remain as a permanent component of the FS in the mature sperm [4]. In turn, Sptrx-2 is a structural component of the mature FS (R. Oko and A. Miranda-Vizuete, unpublished results). Sptrx-1 and Sptrx-2 represent the first two members of the family with tissue-specific distribution and interestingly, both located in close association to the sperm FS. We present here evidence that Sptrx-1 is organized into two independent structural domains: a 38.7 kDa unstructured polypeptide (residues 1–357) and a 12.7 kDa thioredoxin-like domain (resi-

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Abbreviations: FS, fibrous sheath; ODF, outer dense fibers; PDI, protein disulfide isomerase; Trx-1, -2, thioredoxin-1, -2

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03417-8

dues 358–469). Characterization of Sptrx-1 in vitro suggests that the protein behaves as a non-covalent oligomer in solution and that this organization might modulate its redox properties. The implications of an oxidizing character of Sptrx-1 are discussed in the light of its unique expression pattern during sperm tail formation

2. Materials and methods

2.1. Expression and purification of human Sptrx-1

The open reading frame encoding human Sptrx-1 was cloned into the *Bam*HI–*Eco*RI sites of the pGEX-4T-1 expression vector and transformed in *Escherichia coli* BL21(DE3). The induction of the fusion protein was performed as previously described [11]. Briefly, overexpressing cells were disrupted by 10 min sonication and the supernatant was cleared by centrifugation at 15 000×*g* for 30 min and loaded onto a glutathione Sepharose 4B column (Amersham Pharmacia Biotech). Binding to the matrix was allowed to occur during 2 h at room temperature. Thrombin (5 U per mg fusion protein) was used to remove glutathione-*S*-transferase by incubation overnight at 4°C. The resulting protein preparation was then subjected to ion exchange chromatography using a HiTrap Q column (Amersham Pharmacia Biotech) and human Sptrx-1 was eluted using a gradient of NaCl. Protein concentration was determined from the absorbance at 280 nm using a theoretical molar extinction coefficient of 7690 M^{-1} cm^{-1}, calculated using the Protean Program included in the DNASTAR Software Package (DNASTAR Inc.).

2.2. Gel filtration chromatography

Gel filtration experiments were carried out on a Superdex 200 HR10/30 column (Amersham Pharmacia Biotech). The column was pre-equilibrated with 100 mM Tris–HCl pH 8.0, 100 mM NaCl. Human Sptrx-1 was applied to the column in a volume of 5 ml at a final concentration of 2 mg/ml and the flow rate was 5 ml/cm^2/h.

2.3. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis

The spectra were acquired in the linear mode using a Reflex III mass spectrometer from Bruker Daltonik GmbH (Germany). Data processing and evaluation were carried out with the XMASS software (Bruker). On the target plate, a microcrystalline layer was prepared with a saturated solution of sinapinic acid (Sigma) in ethanol. The samples were mixed with an equal volume of a saturated solution of sinapinic acid in 33% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid, and 0.5 μl of this mixture was crystallized on the microcrystalline sinapinic acid layer [13]. Calibration of the spectra was carried out with the high mass protein standard from Aglient Technologies that contained bovine serum albumin (66 430.2 Da), equine cardiac myoglobin (16 951.5 Da), and equine cardiac cytochrome *c* (12 359.2 Da).

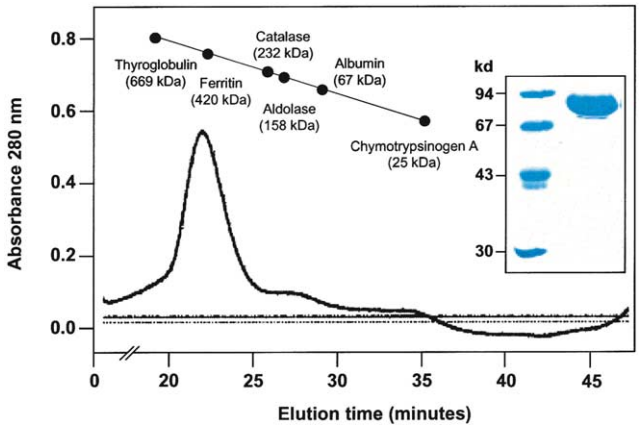

Fig. 1. Gel filtration chromatography of human Sptrx-1. Elution profile of human Sptrx-1 applied to a Superdex 200 column in a volume of 5 ml, at a final concentration of 2 mg/ml and a flow rate of 5 ml/cm^2/h. Inset shows the calibration of the column using high and low molecular weight markers and a sodium dodecyl sulfate (SDS)–PAGE analysis of an aliquot of Sptrx-1 preparation applied to the column.

2.4. Human Sptrx-1 crystallization

Crystallization conditions were screened with the sitting drop vapor diffusion method. Promising conditions were further optimized to obtain crystals suitable for X-ray analysis. The crystallization droplet was prepared by mixing equal volumes of protein solution (protein concentration 30 mg/ml in 10 mM Tris–HCl pH 7.4) and reservoir solution (25% polyethylene glycol 5000 monomethyl ether in 0.1 M Tris buffer pH 8.0 with the addition of 0.3 M sodium acetate). The droplet was equilibrated against 0.5 ml reservoir solution at 293 K. X-ray diffraction data were collected at 100 K with Cu-Kα radiation using a MAR research image plate. 20% glycerol was added to the reservoir solution as a cryo-protectant. The data were processed with the HKL package [14]

2.5. Circular dichroism spectroscopy

Circular dichroism spectroscopy was performed using an Aviv 202DS spectropolarimeter (Lakewood, NJ, USA). Spectra were obtained using protein solutions in 100 mM potassium phosphate buffer pH 7.0 containing 10 mM NaCl and 1 mM ethylenediamine tetraacetic acid (EDTA) at protein concentrations ranging from 0.5 to 0.9 mg/ml at 25°C in 0.01 cm cuvettes. Spectra were recorded for 8 s per

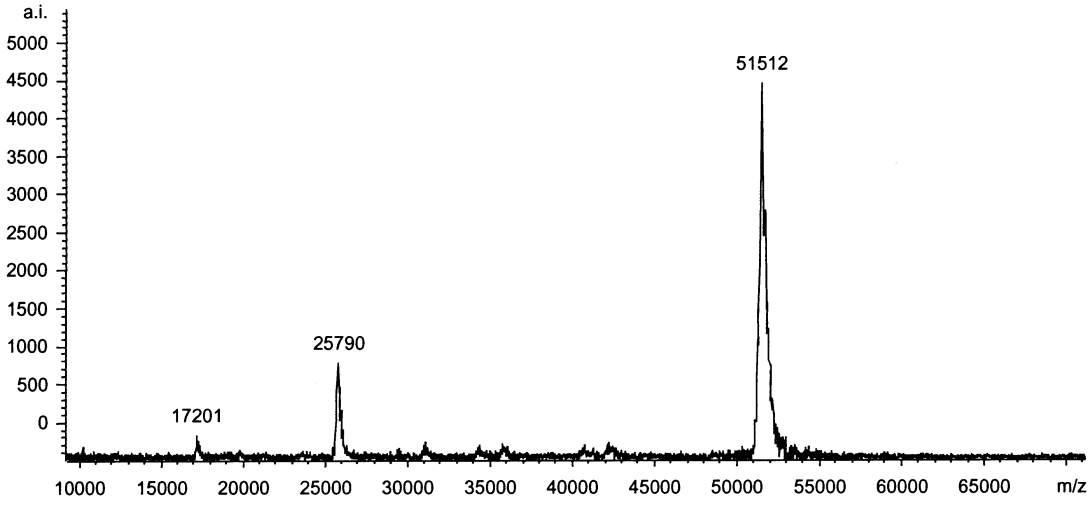

Fig. 2. MALDI-TOF analysis of human Sptrx-1. The spectrum shows the single, double and triple charged ions of Sptrx-1 at *m/z* 51512, 25790 and 17201, respectively. The mass determination of Sptrx-1 was carried out using the single charged ion of the protein.

nm between 260 and 184 nm. Protein concentrations were determined by quantitative amino acid analysis [15]. Contributions from cuvette and solvent were removed by subtracting an identically recorded spectrum of buffer without protein. No signal processing (e.g. smoothing) was used.

2.6. Enzymatic activity assays

Disulfide bond formation was measured in a reduced model peptide, NRCSQGSCWN (Genemed Synthesis) which was acetylated and amidated at the N- and C-terminus, respectively by the manufacturer essentially as described [16,17]. The peptide concentration was calculated using the molar extinction coefficient at 280 nm of 5690 (M^{-1} cm^{-1}). The oxidation reaction was performed in 100 ml of buffer consisting of 100 mM Tris–HCl pH 7.4, 200 mM KCl, 1 mM EDTA and either 1 mM selenite (Na_2SeO_3) or 2 mM reduced glutathione (GSH) and 0.5 mM oxidized glutathione (GSSG) which corresponds to a redox potential of −202 mV at pH 7.4. Protein disulfide isomerase (PDI) or Sptrx-1 was added at a final concentration of 1–2 μM. The reaction was initiated by adding 20 μM of the reduced peptide and quenched after different time points by addition of HCl to a final concentration of 0.2 M. No attempt was made to control the oxygen concentration in the samples. After quenching, the samples were immediately analyzed on reverse phase high-performance liquid chromatography (HPLC) (Pharmacia SMART System) on a C18 column (2.1 mm × 10 cm) using a linear gradient of 5–25% acetonitrile, 0.1% trifluoroacetic acid in 25 min at a flow rate of 100 μl/min. Elution of the peptide was determined by absorbance at 215 nm. The degree of peptide oxidation was calculated from the relative peak area of the oxidized peptide and total peak area. Control experiments were performed using fully oxidized and reduced peptide.

3. Results

3.1. Oligomeric structure of human Sptrx-1

In addition to the two cysteine residues in the active site of the thioredoxin domain, human Sptrx-1 has four additional cysteine residues located in the C-terminal thioredoxin domain [11]. Interestingly, when determining the thioredoxin activity of recombinant Sptrx-1 we observed that prior reduction of the protein by dithiothreitol (DTT) diminished its activity, in contrast to what happens to Trx-1. Previously, we showed that Sptrx-1 migrated anomalously (~180 kDa) on non-denaturing polyacrylamide gel electrophoresis (PAGE) thus suggesting a possible oligomeric form [11]. In an attempt to further define the oligomeric state of Sptrx-1 in vitro, we first performed gel filtration chromatography. As shown in Fig. 1, recombinant human Sptrx-1 eluted at a position consistent with that of a 440 kDa globular protein. With a monomeric molecular mass of 53 kDa, this result indicates that native Sptrx-1 is either oligomeric or is highly asymmetrical. We used MALDI-TOF mass spectrometry to elucidate whether potential Sptrx-1 oligomers are maintained by intermolecular disulfide bonds. Sptrx-1 behaves as a single peak of 51 512 Da (Fig. 2) demonstrating that intermolecular disulfide bonding is not responsible for the anomalous behavior of the native protein. The average value of the mass of Sptrx-1 determined from 10 independent spectra was 51 485 Da ± 0.2%, which was significantly lower than the reported theoretical mass of human Sptrx-1 monomer (53 274 Da) [11] and indicated a difference in the primary structure. To see whether partial N-terminal digestion could account for this difference, we performed N-terminal sequencing of the protein, which was in agreement with that reported previously (data not shown). The clone overexpressing human recombinant Sptrx-1 used in these experiments was sequenced and found to lack one of the repetitive motifs in the N-terminal domain of the protein. The expected size of this shorter variant is 51 582 Da, which is in good agreement with the MALDI-TOF results. We have recently reported the mouse Sptrx-1 gene and protein [18] and found that it also lacks one of the repetitive motifs compared to the orthologous human sequence. Interestingly, we have identified the two human variants among several patients diagnosed of sperm tail defects and normal controls (unpublished results).

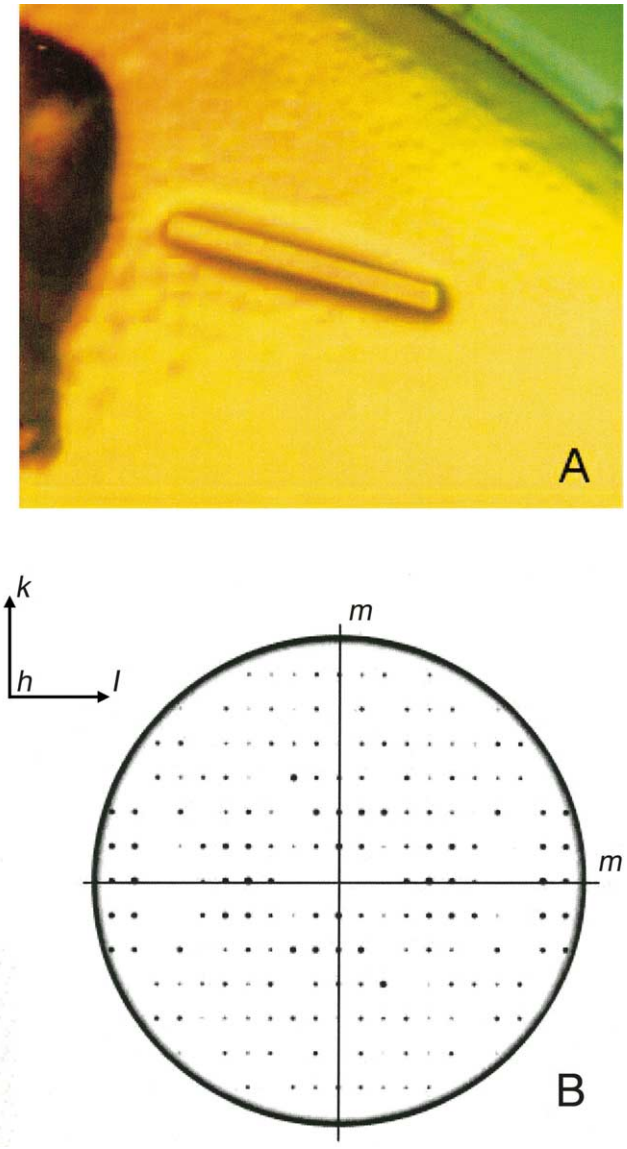

Fig. 3. Crystallographic analysis of the thioredoxin domain of human Sptrx-1. A: Monoclinic crystal of the C-terminal thioredoxin domain of Sptrx-1. The maximal dimension of the crystal is about 0.4 mm. B: A pseudo-precession photograph (0kl layer) showing the Laue symmetry 2/m. Systematic extinctions of $k = 2n+1$ can be clearly observed. The diffraction data were evaluated in space group P1. The outer edge of the photograph corresponds to 4.0 Å resolution. The figure was produced with the program PATTERN [30].

3.2. Crystallization and preliminary X-ray analysis of human Sptrx-1 protein

To gain more insight into Sptrx-1 three-dimensional structure we attempted the crystallization of the full-length protein. Needle-like crystals, with a maximum dimension of about 0.4 mm, were obtained after nearly one year (Fig. 3A). The crys-

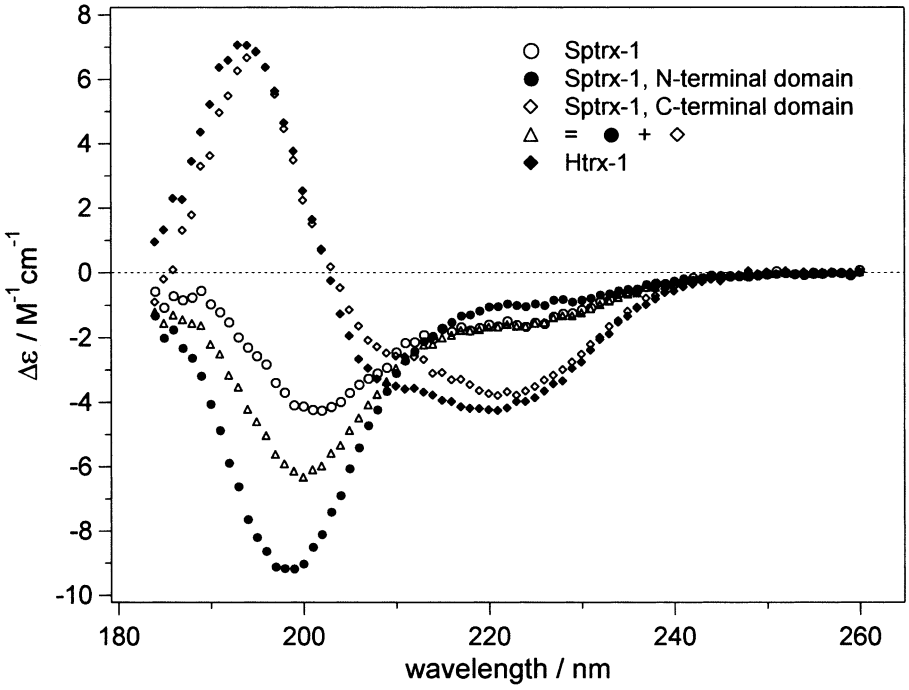

Fig. 4. Circular dichroism spectra of Sptrx-1. Plot of decadic molar (mean residue) CD, $\Delta\varepsilon$, as a function of wavelength for native Sptrx-1 (1–463), open circles; N-terminal domain (1–360), filled circles; C-terminal domain (361–463), open diamonds; combined N- and C-terminal spectra, open triangles; and human Trx-1, filled diamonds. Spectra were recorded for 8 s/point at 1 nm intervals between 260 and 184 nm using 0.01 cm cuvettes. No signal processing (smoothing) was used.

tals diffracted beyond 3.0 Å resolution and autoindexing of the diffraction data [19] suggested that the crystals belong to space group C2, which was further confirmed by inspecting the systematic extinctions and the Laue symmetry of the diffraction pattern (Fig. 3B). A preliminary data set was collected to 2.9 Å resolution. A total of 4105 observations were merged to give 1823 unique reflections with a merging R factor $(\Sigma(I-\langle I\rangle)/\Sigma(I))$ of 0.135. The data set is 83% complete to 2.9 Å resolution with 52% in the last shell of 3.0–2.9 Å. The cell parameters are $a=45.4$, $b=55.2$, $c=42.1$, $\alpha=\gamma=90°$ and $\beta=93.7°$.

With the knowledge of unit cell and the molecular weight of the protein molecule, the crystal volume per unit of protein molecular weight (V_m), can be calculated. For protein crystals, this value ranges from 1.6 to 3.5 Å^3/Da, with the most commonly observed values of V_m around 2.1 Å^3/Da [20]. Assuming one monomer of Sptrx-1 with a molecular weight of 51.5 kDa per unit cell, an unlikely V_m value of 0.49 Å^3/Da is obtained, which suggests the existence of a much smaller protein molecule in the unit cell. Protein sequence analysis using protein from dissolved crystals verified that a specific cleavage between residues 359 and 360 had occurred during the crystallization. The molecular weight of the remaining residues 360–463 is 12 kDa, a value typical for thioredoxins, that would lead to a more realistic V_m value of 2.2 Å^3/Da. This result indicates that Sptrx-1 underwent cleavage during crystallization, with apparent degradation of the N-terminal domain. Thus, crystals consisted of the remaining C-terminal thioredoxin domain.

3.3. Sptrx-1 structural characterization using circular dichroism

Circular dichroism spectra of the intact human Sptrx-1 (Fig. 4) are consistent with the full-length protein (1–469) containing a majority of disordered structure as evidenced by the intense minimum near 200 nm. To ascertain whether the polypeptide responsible for this disorder is confined to a continuous segment of the protein, we recorded spectra of the purified N- and C-terminal domains separately. The circular dichroism (CD) spectra of the N-terminal domain (1–357) contained an intense negative band between 195 and 200 nm consistent with a significant content of disordered structure. In contrast, the spectra of the C-terminal domain (358–469) resembled that recorded of native human Trx-1. When the CD spectra of the two domains are combined and compared to that of full-length Sptrx-1 (Fig. 4), the close coincidence of the two spectra is strong evidence that the two domains do not influence each others structure significantly, supporting the picture of a largely disordered N-terminal domain and a folded C-terminal thioredoxin-like domain.

3.4. Redox activity of human Sptrx-1 protein

We have previously reported a weak reducing activity of human recombinant Sptrx-1 using both DTT or NADPH and thioredoxin reductase as electron donors [11]. We sought to determine whether human Sptrx-1 might in fact be capable of catalyzing disulfide bond formation, in vitro. For this purpose, a model peptide whose oxidized and reduced forms can be separated by HPLC after reaction with Sptrx-1 or PDI was used. For a functional assay, an electron acceptor is also needed. GSSG is a common oxidant of protein thiols. However, in the presence of a glutathione redox buffer with a redox potential corresponding to −202 mV, no activity could be detected by Sptrx-1 (Fig. 5A). In the presence of selenite (which is known to oxidize the active site of Trx-1 in a non-stoichiometric and oxygen-dependent manner [21]) Sptrx-1

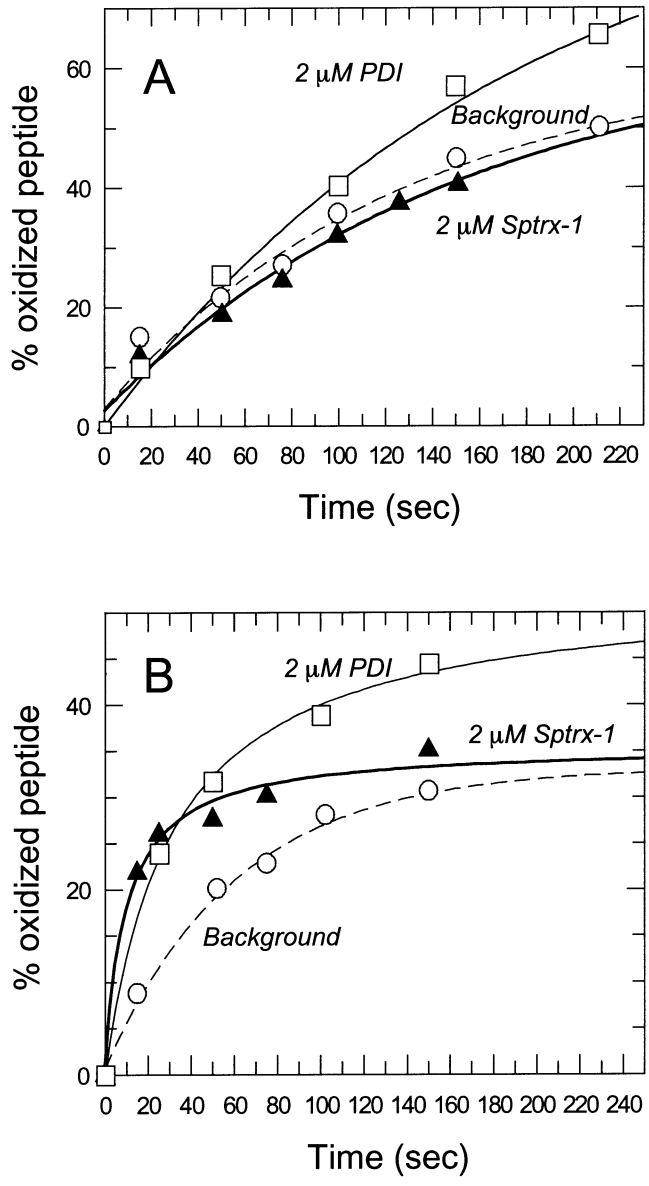

Fig. 5. Disulfide formation in a reduced model peptide in the presence of Sptrx1 or PDI. A: Using 2 mM GSH and 0.5 mM GSSG and B: using 1 μM selenite as electron acceptor. The reaction was performed in 100 μl buffer consisting of 100 mM Tris–Cl pH 7.4, 200 mM KCl and 1 mM EDTA. The reaction was initiated by addition of peptide to a final concentration of 20 μM. Aliquots were removed and quenched at different time points by addition of HCl to a final concentration of 0.2 M. The samples were analyzed immediately on reverse phase HPLC (Pharmacia SMART system) on a C18 column (2.1 mm×10 cm) using a linear gradient of 5–25% acetonitrile, 0.1% trifluoroacetic acid (TFA) for 25 min at a flow rate of 100 μl/min. The fraction of oxidized peptide was calculated from the total peak area in the chromatogram. Open squares, PDI; black triangles, Sptrx-1; open circles, background.

oxidized the peptide at a similar initial rate compared to PDI but with a lower final yield (Fig. 5B). Similar results were obtained using fluorometric and colorimetric assays with GSSG and selenite (data not shown).

4. Discussion

The transformation of spermatids to spermatozoa (spermiogenesis) involves a complex sequence of events by which a conventional cell is converted into a highly organized motile structure. During spermiogenesis the spermatid undergoes striking and unique morphological and biochemical changes that include nuclear condensation, acrosome formation and sperm tail organization [22]. A common link for most of these processes to occur is a progressive increase in disulfide bonding, which starts at the spermatid stage and continues through epididymal transit [23–26]. However, despite the prominent role of disulfide bonding in spermiogenesis, the molecular mechanisms that regulate this process are still poorly understood, mostly due to the lack of in vitro models of spermiogenesis.

Thioredoxins are a class of proteins typically considered as key players in redox-regulated cellular mechanisms [7]. Sptrx-1 is the first member of this family with a tissue-specific distribution, located in the sperm tail [11]. A developmental expression analysis through the spermatogenic cycle in rodents has shown that Sptrx-1 transiently associates to the longitudinal columns of the sperm FS during its elongation and

assembly but does not remain as a structural component of the FS in the mature sperm [4]. When analyzing Sptrx-1 thioredoxin activity in the presence of NADPH and thioredoxin reductase we found that preincubation of the protein with DTT (to fully reduce the protein) resulted in a decrease of the activity compared to that of oxidized Sptrx-1, in contrast to what occurs to Trx-1 used as control [11]. These data suggested an important role of the cysteine residues for the maintenance of Sptrx-1 in an active conformation. We decided to further characterize Sptrx-1 in vitro by gel filtration chromatography which indicated a very anomalous behavior comparable to a 400 kDa globular protein. Considering 51.6 kDa as the size of the Sptrx-1 monomer and assuming ideal globular behavior, the results of the gel filtration experiment are consistent with that of an octamer. However, MALDI-TOF analysis of Sptrx-1 showed that the solution conformation in vitro is not maintained by intermolecular disulfide bonding. The fact that the enzymatic activity of Sptrx-1 is affected by reduction of the protein suggests that intramolecular disulfide bonding may be important.

Despite extensive efforts to crystallize the native Sptrx-1 protein, the crystals contained only the C-terminal thioredoxin domain indicating that the N-terminal domain is prone to spontaneous degradation. Indeed, during the Sptrx-1 purification process, we could detect some degradation of this domain resulting in a 'laddering' pattern of bands, most probably suggesting that the spontaneous degradation occurs at specific residues within the N-terminal domain. This spontaneous degradation might be facilitated by the disordered structure of the N-terminal domain as shown by CD spectroscopy.

FS is a spermatozoa cytoskeletal structure, with no counterpart in somatic cells, characterized by its elastic rigidity to provide support to the sperm tail during its beating [27]. This function is dependent on the tight association of the proteins that integrate the FS, achieved through extensive disulfide bonding and resulting on its high insolubility. As we have demonstrated, Sptrx-1 is able to function as a reductant as well as an oxidant in vitro. Together with its spatial and temporal expression in the tail of elongating spermatids, simultaneous to the assembly of the FS, our data suggest that Sptrx-1 could indeed participate in the regulation of this process by favoring the formation of disulfide bonds during sperm tail morphogenesis. In addition, its dual reducing/oxidizing activity might be required to rectify non-correct disulfide pairing and generate the suitable ones between the different sperm FS constituents, similarly to what occurs to the disulfide bond reshuffling activity of PDI [28]. The relatively mild reducing and oxidizing activities of recombinant Sptrx-1 should be viewed in a wider perspective. First, the oxidizing activity found in vitro with the model peptide as a substrate could be limited by the reoxidation of Sptrx-1. In the case of PDI and the protein DsbA, this has been shown to be the rate-limiting step in similar experiments [16,29]. Second, it is plausible that additional factors are required in the assay, most probably testis-specific, or post-translational modifications might be necessary to achieve a fully active protein. Third, higher activity could be obtained upon interaction with substrates. In this context, the N-terminal domain, which is unstructured in solution, could acquire a more structured conformation after binding to an interacting protein.

As noted previously, the morphogenesis and assembly of FS (as well as other components of sperm tail) require stabilization by disulfide bonding between its different constituent proteins. Therefore, failure of proper functioning of Sptrx-1 could lead to spermatozoa tail defects, thus justifying further work on Sptrx-1 as a potential target for male factor infertility studies.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council (Projects 03P-14096-01A, 03X-14041-01A), the Åke Wibergs Stiftelse and the Karolinska Institutet to A.M.-V. and the Fundación Margit y Folke Pehrzon to A.J.

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