Identification of Bull Protamine Disulfides†

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ABSTRACT: We have identified the disulfide cross-links in bull protamine by titrating intact bull sperm with dithiothreitol (DTT) and following the modification of each cysteine residue with tritiated iodoacetate. The derivatization of each cysteine was monitored by a combination of HPLC, peptide mapping, and protein sequencing. Analyses of total free sulfhydryls show that all seven of the bull protamine cysteines are cross-linked as disulfides in mature sperm. The first disulfide is reduced at a DTT:protamine cysteine (DTT:Cys) ratio of 0.3 and the last at a ratio of 2.0. Intra- and intermolecular disulfides were identified by correlating the reduction of specific disulfides with the dissociation of protamine from DNA in partially reduced sperm and sperm treated with *N,N'*-ethylenedimaleimide, a bifunctional disulfide cross-linking agent. Three intermolecular and two intramolecular disulfides were identified. The results of these experiments demonstrate that the amino- and carboxy-terminal ends of the bull protamine molecule are folded inward toward the center of the molecule and are locked in place, each by a single intramolecular disulfide bridge. Three intermolecular disulfides cross-link neighboring protamine molecules around the DNA helix in such a manner that the protamines cannot be dissociated from DNA without first reducing the interprotamine disulfides.

The protamines of eutherian mammals are unique in that they contain large amounts of cysteine. Numerous studies have demonstrated that these cysteines are not present as free sulfhydryls in mature sperm (Calvin & Bedford, 1971; Calvin et al., 1973; Marushige & Marushige, 1974, 1975; Saowaros & Panyim, 1979; Pellicciari et al., 1983; Balhorn, 1989) but that they are progressively oxidized to disulfides as the sperm descend through the epididymis (Calvin & Bedford, 1971; Calvin et al., 1973; Marushige & Marushige, 1975; Saowaros & Panyim, 1979; Pellicciari et al., 1983; Balhorn, 1989). The protamines of mature sperm cannot be dissociated from DNA with acids, salts, or denaturants unless the disulfide cross-links are first reduced to free sulfhydryls. These findings suggest that disulfide cross-links interlock neighboring protamine molecules around the DNA helix.

Two different classes of protamine molecules have been isolated from mammalian sperm. These two proteins, designated protamine 1 and protamine 2, differ in both sequence and mode of synthesis. The sperm of all species appear to contain protamine 1. This protein is smaller than protamine 2 and appears to be more highly conserved. Once protamine 1 is synthesized and deposited onto DNA, it remains there intact (Green et al., 1987; Yelick et al., 1987). Numerous rodents and primates have been shown to produced sperm containing a second protamine, protamine 2. Protamine 2, unlike protamine 1, is synthesized as a much larger precursor protein that is processed by removing the amino-terminal third of the molecule during the first 24–30 h after it binds to DNA (Green et al., 1987; Balhorn, 1989; R. Balhorn, M. Corzett, and J. A. Mazrimas, manuscript in preparation).

Little is known about the actual structure of the sperm chromatin complex in mammals or the intermolecular interactions that occur between neighboring protamine molecules. Several years ago, we proposed a hypothetical model that described the binding of bull protamine to DNA (a species that produces sperm containing only protamine 1), as well as specific intra- and intermolecular disulfides that form within and between neighboring protamine molecules (Balhorn, 1982). While the general features of bull sperm chromatin structure remain consistent with this model, subsequent experiments have shown that the proposed disulfide cross-links are incorrect. In this paper we describe these experiments and identify the intra- and intermolecular disulfides as they exist in vivo in ejaculated spermatozoa.

Experimental Procedures

Pooled bull semen was obtained from the American Breeders Service (DeForest, WI) and maintained at −20 °C until use. Tritiated sodium iodoacetate (5 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). The *N,N'*-ethylenedimaleimide (EDM) was synthesized according to the procedure developed by Searle (1948).

Titration of Protamine Disulfides and Carboxymethylation of Free Sulfhydryls. Pooled bull semen was centrifuged at 4100*g*, and the sperm were washed by sonication (Branson Sonifier, level 5 for 20 s) in a buffer of 0.01 M Tris, pH 6, and 0.9% sodium chloride (Tris–saline). The sperm were resuspended in a buffer of 0.5 M Tris, pH 8, and 2 mM EDTA (Tris–EDTA), aliquots were added to 1 mL of a solution of 5 M guanidine hydrochloride (GuCl), 0.01 M Tris, pH 8, and 10 mM dithiothreitol (DTT) to dissolve the sperm and release the DNA, and the absorbance of the released DNA was monitored at 260 nm. The sperm and protamine concentrations were determined by using standard absorbance curves that relate the A_{260} of the dissociated sperm in 5 M GuCl to actual sperm counts. The amount of protamine in the sample was calculated by using the value of 3 pg of protamine/pg of sperm DNA.

Aliquots of intact sperm were treated at various DTT:Cys ratios for 1 h at 20 °C. The sperm were centrifuged at 4100*g* for 3 min and resuspended in Tris–EDTA. Immediately following resuspension, the free sulfhydryls were carboxy-

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methylated with tritiated sodium iodoacetate (14 mg/mg of protamine) for 1 h at 20 °C in the dark under nitrogen. The reaction was stopped by the addition of cysteine (20 mg/mg of protamine), and the samples were dialyzed extensively to remove the unbound label. The basic nuclear proteins were extracted from the DNA by adding GuCl and mercaptoethanol to 5M and 0.5 M, respectively; the GuCl was then diluted to 0.5 M by addition of urea and sodium chloride, and the DNA was precipitated with HCl (Balhorn et al., 1977). After centrifugation to remove the DNA, the supernatant containing the protein was dialyzed against 0.01 M HCl, and the protein was precipitated with 20% trichloroacetic acid (TCA), washed with acidified acetone to remove residual TCA, and dried.

Protamine Purification. Protamine was isolated from each of the basic nuclear protein samples by HPLC on a Nucleosil RP-C18 column using a linear acetonitrile gradient (Mazrimas et al., 1986). The unmodified cysteine residues of the purified protamines were reduced with DTT (50-fold excess over total protamine cysteine) and recarboxymethylated with unlabeled sodium iodoacetate (2-fold excess over DTT concentration) at 20 °C in the dark under nitrogen. The protamine was subsequently desalted by chromatography on a 1-cm × 30-cm Sephadex G-25 column in 10 mM HCl and lyophilized.

Analysis of Protamine Solubility and Cross-Linking of Cysteines with EDM. The sequential reduction of intermolecular disulfides as a function of the DTT:cysteine ratio was determined by monitoring the solubility of the sperm (and the associated dissociation of protamine and release of DNA) in 5 M GuCl and 0.01 M Tris, pH 8. Upon reduction of these disulfides, the protamines can be dissociated from DNA by GuCl and the sperm dissolve. Sperm containing intact intermolecular disulfides are insoluble in 5 M GuCl and sediment upon centrifugation at 8000*g*.

Aliquots of sperm treated with DTT at various DTT:Cys ratios were diluted 20-fold in 5 M GuCl and 0.01 M Tris, pH 8, sonicated for 20 s, allowed to sit for 10 min at 20 °C, and centrifuged at 4100*g* for 10 min. The released DNA was determined by measuring the absorbance of the supernatant at 260 nm. Identical aliquots were added to a solution of 5 M GuCl, 0.01 M Tris, pH 8, and 10 mM DTT to determine the A_{260} of the totally reduced samples.

The sperm (treated with increasing concentrations of DTT) were washed once in 0.1 M Tris, pH 5, and the pellets were resuspended in 0.1 M Tris, pH 5. Ten aliquots of EDM in 0.1 M Tris, pH 5, were added every 5 min (final concentration of 96 mg of EDM/mg of protamine), and the reaction was allowed to proceed for 60 min. The sperm were centrifuged at 4100*g* and washed once in pH 5 Tris–saline. The extent of disulfide re-cross-linking by EDM and the attendant solubility/insolubility of the sperm were determined by adding aliquots to a solution of 5 M GuCl, 0.01 M Tris, pH 8, and 10 mM DTT as described above.

Analysis of Protamine Disulfide Re-Formation after Reduction. Aliquots of sperm were treated with different concentrations of DTT for 1 h at 20 °C. The sperm were subsequently centrifuged, washed in Tris–EDTA, resuspended in the same buffer, and allowed to sit at 20 °C for various lengths of time. The extent of intermolecular disulfide re-cross-linking was monitored by adding aliquots of the sperm to 5 M GuCl and 0.01 M Tris, pH 8, sonicating the suspension, centrifuging the insoluble sperm, and quantitating released DNA (and protamine dissociation) by using the absorbance at 260 nm.

Tryptic Digestion of Protamine and Isolation of Peptides by HPLC. The carboxymethylated protamines isolated from sperm exposed to increasing concentrations of DTT were digested with trypsin as previously described (Mazrimas et al., 1986). Following digestion and lyophilization, the peptide digest was fractionated by HPLC on a 5-μm Nucleosil RP-C18 column as described previously (Mazrimas et al., 1986). Peptides 4, 5, 8, and 10 were collected and lyophilized.

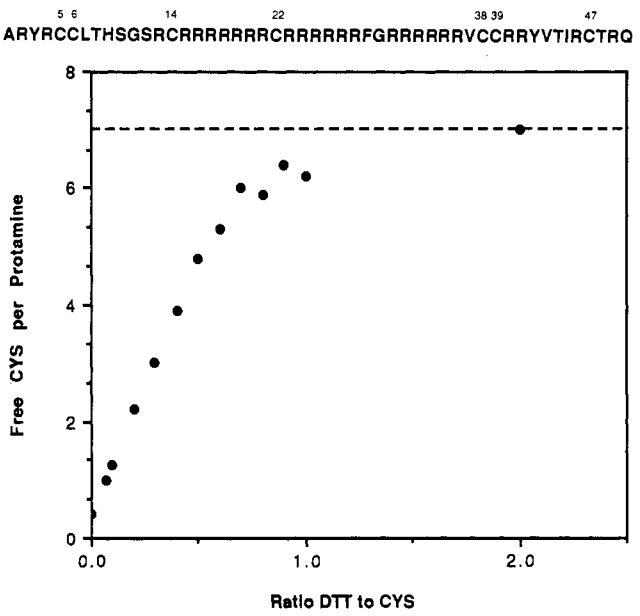

FIGURE 1: (Top) Amino acid sequence of bull protamine (Mazrimas et al., 1986). The single-letter nomenclature for amino acids follows the IUPAC–IUB rules: A, Ala; C, Cys; F, Phe; G, Gly; H, His; I, Ile; L, Leu; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr. (Bottom) Titration and carboxymethylation of bull protamine cysteine residues in intact bull sperm. Aliquots of sperm were treated with increasing concentrations of DTT, the reduced sulfhydryls were carboxymethylated with tritiated sodium iodoacetate, and the number of free cysteine residues was determined as described under Materials and Methods. The dashed line corresponds to complete carboxymethylation of all cysteines in protamine.

Amino Acid Sequencing of Radiolabeled Peptides. Both the intact protamines and the peptides isolated from each protamine sample were subjected to automated protein sequence analysis on an Applied Biosystems 470A sequencer. Each cleavage cycle was collected and counted in a scintillation counter to identify the amount of tritium released during successive sequencing cycles. The amount of label present in each cycle was corrected by using an observed repetitive yield of 94% (this was experimentally determined by analyzing the release of amino acids from carrier myoglobin added to the sequenced samples).

RESULTS

Titration of Disulfides in Intact Sperm. Each bull protamine molecule (Figure 1, top) contains seven cysteine residues (Mazrimas et al., 1986). The number of these residues that participate in disulfide bond formation in mature sperm was determined by treating aliquots of ejaculated sperm with increasing concentrations of DTT and subsequently carboxymethylating the reduced free sulfhydryls with tritiated sodium iodoacetate. The protamines were isolated and recarboxymethylated with unlabeled sodium iodoacetate, and the number of modified cysteine residues per protamine (relative to a fully reduced sample treated at a DTT:cysteine ratio of 25:1) was determined by scintillation counting. As the results in the bottom panel of Figure 1 show, all seven of the bull protamine cysteines are cross-linked as disulfides in mature sperm. In the absence of DTT (and the presence of the chelating agent EDTA), less than 5% of the cysteines are available for carboxymethylation with iodoacetic acid. The reduction of these

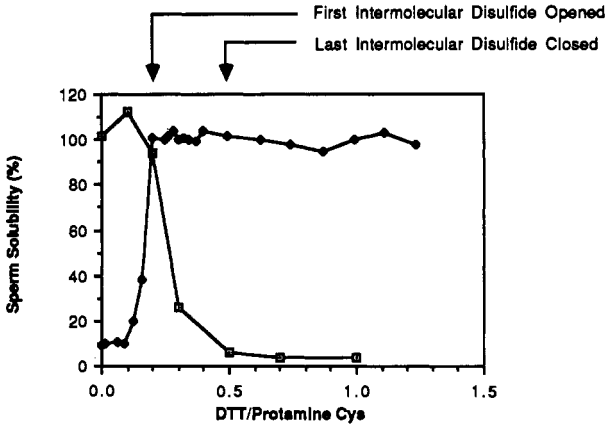

FIGURE 2: Identification of the first and final inter-protamine disulfides reduced by DTT. Sperm solubility (and protamine dissociation) in 5 M GuCl and 0.01 M Tris, pH 8, as a function of DTT:cys ratio, ◆, identifies the first inter-protamine disulfide to be opened. The solubility of sperm (and protamine dissociation) following reduction with DTT and treatment with *N,N'*-ethylenedimaleimide to re-cross-link the disulfides, □ (see Materials and Methods), identifies when the final inter-protamine disulfide is reduced.

disulfides occurs over a small range of DTT concentrations, approaching completion at a DTT:cysteine ratio of 2.0.

Discrimination between Inter- and Intra-Protamine Disulfides. The protamines of mature bull sperm are known to be cross-linked around the DNA helix by disulfide bridges. The sperm are insoluble in 5 M guanidine hydrochloride, and the protamines cannot be dissociated from DNA unless the protamine disulfides are first reduced with DTT or 2-mercaptoethanol (Figure 2). The DTT concentration (DTT:cys ratio) at which the first inter-protamine disulfide is reduced in intact bull sperm was determined by monitoring the solubility of the sperm chromatin in 5 M guanidine hydrochloride and 10 mM Tris-HCl as a function of the DTT concentration. As the results in Figure 2 show, the reduction of the first disulfide that permits protamine dissociation and chromatin dispersal is complete at a DTT:cysteine ratio of 0.2.

The DTT concentration required to complete the reduction of the last inter-protamine disulfide was determined by treating each reduced sperm sample in the titration series with *N,N'*-ethylenedimaleimide (EDM). Intermolecular disulfides reduced by DTT and subsequently re-cross-linked with EDM are resistant to cleavage by reducing agents. If only a single type of inter-protamine cross-link is present in bull sperm chromatin (e.g., the disulfide reduced at a DTT:protamine Cys ratio of 0.2), the DTT-reduced EDM-re-cross-linked sperm chromatin would be expected to remain insoluble in 5 M guanidine hydrochloride at all higher DTT concentrations. If, on the other hand, additional inter-protamine cross-links are present, the reduced EDM-re-cross-linked sperm chromatin should remain soluble in GuCl until a DTT concentration is reached that begins to reduce the last inter-protamine cross-link. At this and higher DTT concentrations, the reclosure of this final intermolecular cross-link by EDM would complete the DTT-resistant cross-linking of protamine around the DNA and render the chromatin insoluble in GuCl. As the results in Figure 2 show, bull sperm contain at least two different inter-protamine disulfide cross-links that interlock the protamines around DNA. The reduction of the second group of disulfides begins at a DTT:cysteine ratio of 0.2. Reduction of the inter-protamine disulfides is complete at a DTT:cysteine ratio between 0.5 and 0.6.

Intra-Protamine Disulfides. Disulfide re-formation experiments performed with sperm treated at increasing DTT concentrations have provided additional information about the DTT concentrations required to open the intra-protamine disulfides. After the protamine disulfides of intact sperm are reduced with various concentrations of DTT, the rate of re-formation of the intermolecular disulfides may be followed by monitoring the solubility of the sperm chromatin in 5 M GuCl. The inter-protamine disulfides of sperm treated at DTT:cysteine ratios of 0.2, 0.3, 0.4, and 0.5 all re-form at the same rate, and the sperm become insoluble in 5M GuCl after 120 min at pH 8 in Tris–saline (see supplementary material for a figure showing these data).

As the DTT concentration is increased above a DTT:cysteine ratio of 0.6 (DTT:cysteine = 0.7, 0.8, 0.9, and 1.0), the first of the intramolecular disulfides begins to be reduced. Once this happens, intermolecular disulfide re-formation appears to require longer times, presumably because the additional intramolecular disulfides must re-form before the inter-protamine cross-links can re-form and the sperm again become insoluble. The reduction of a second group of intramolecular disulfides appears to occur in sperm treated at DTT:cysteine ratios above 2. The opening of this disulfide delays the re-formation of the inter-protamine disulfides and the associated reversion to GuCl insolubility by several additional hours.

Isolation of Tryptic Peptides of Bull Protamine Containing Cysteine. Digestion of fully carboxymethylated bull protamine with trypsin yields sixteen peptides that are resolved by HPLC on a RP-C18 column (Figure 3, top panel). Analyses of these peptides by amino acid analysis and sequencing (Mazrimas et al., 1986) have shown that the seven cysteine residues of the molecule are distributed among four different peptides (Figure 3, bottom panel): Cys5 and Cys6 in peptide 10, Cys14 in peptide 4, Cys22 in a separate peptide that coelutes with peptide 4, Cys38 and Cys39 in peptide 8, and Cys47 in peptide 5.

Identification of Disulfide Cross-Links. The progressive reduction of individual cysteine residues in bull protamine was followed by treating intact bull sperm with increasing concentrations of DTT and quantitating the carboxymethylation of specific cysteines in each of the four peptides by using tritiated sodium iodoacetate (IAA). Following carboxymethylation with the radiolabeled IAA, the sperm were washed and reduced and the protamines isolated. The additional cysteine residues released upon complete reduction were subsequently carboxymethylated with unlabeled IAA. Each protamine sample was digested with trypsin, peptides 4, 5, 8, and 10 were isolated by HPLC, and the extent of disulfide reduction (cysteine carboxymethylation with [^{3}H]IAA) was determined for each peptide (Figure 4).

The disulfide containing the single cysteine residue of peptide 5 (Cys47) was found to be extremely resistant to reduction. This cysteine did not approach 100% reduction until the DTT concentration was increased to a DTT:cysteine ratio above 2.

To differentiate between the reductions of individual cysteine residues in each of the remaining three peptides (each of these three peptides contains two cysteine residues), the DTT:cysteine ratio required for complete reduction of the first of the two cysteines was estimated as the point of 50% reduction. Clearly, these values only represent an estimate, since the reductions of the different disulfides must certainly overlap. The point of complete reduction of the second residue was identified as the point of 100% reduction for both residues in the peptide.

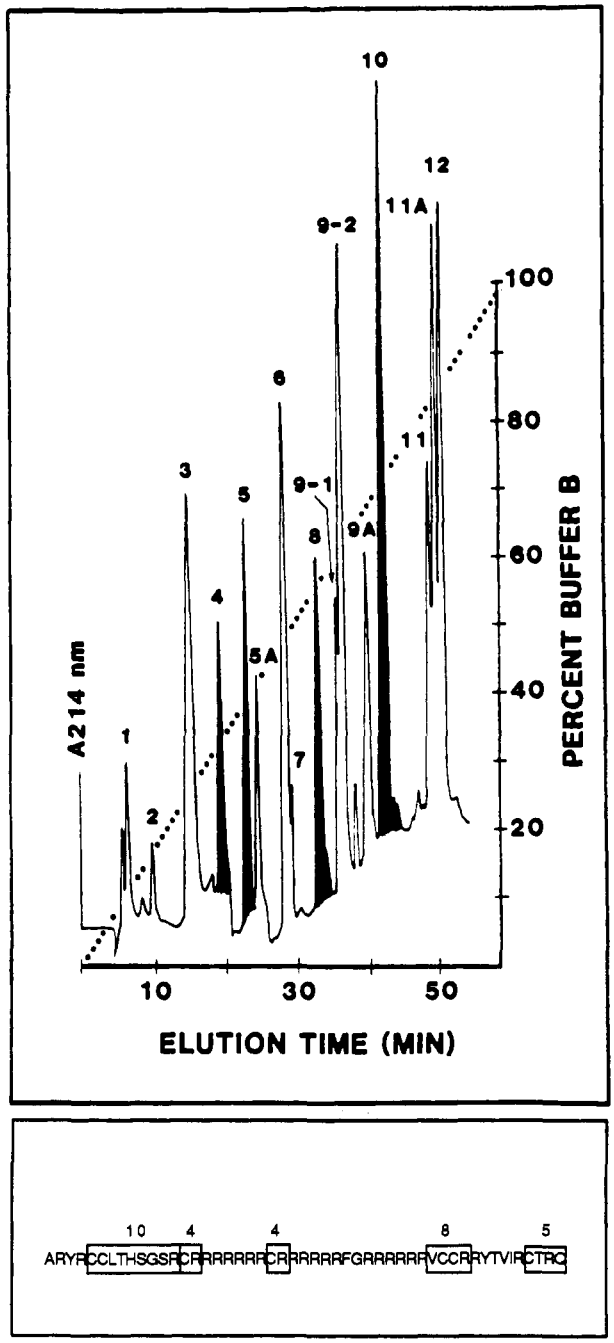

FIGURE 3: (Top) HPLC fractionation of the bull protamine tryptic peptides on a Nucleosil RP-C18 column using an acetonitrile gradient in 0.1% TFA (Mazrimas et al., 1986). The cysteine-containing peptides of interest are colored in black. (Bottom) Cysteine-containing peptides produced by digestion of bull protamine with trypsin. The numbered peptides correspond to the HPLC peaks 4, 5, 8, and 10 in the top panel.

By using the results obtained from these experiments, shown in Table I, four disulfide pairs were identified. The first disulfide, reduced at a DTT:cysteine ratio of 0.2–0.3, involved a cross-link between cysteines in peptides 4 and 10. At a DTT:cysteine ratio near 0.5, only peptide 8 was radiolabeled, indicating that this cysteine residue must be cross-linked to the same peptide in another molecule. The other cysteine residue in peptide 8 was completely reduced at a DTT:cysteine ratio above 2.0. The reduction of this cysteine correlated with the reduction of the single cysteine residue found in peptide 5. The remaining disulfide, reduced at a DTT:cysteine ratio near 0.6, involved a second cross-link between peptides 4 and 10.

These results compare remarkably well with the data obtained from the GuCl solubility, EDM cross-linking, and disulfide re-formation experiments. As shown in Figure 5, the first intermolecular disulfide to be opened by DTT correlates with the first disulfide to be reduced between peptides 4 and 10. The last intermolecular disulfide to be reduced (and subsequently re-cross-linked closed by EDM) involves the disulfide formed between peptide 8 molecules on two different proteins. The first intramolecular disulfide to be reduced corresponds to the second cross-link reduced between peptides 4 and 10 at a DTT:cysteine ratio of 0.6. A second and final intramolecular disulfide between cysteine residues in peptides 5 and 8 is reduced at a much higher DTT concentration (DTT:Cys above 2.0).

To determine the order of reduction of adjacent cysteines in peptides 8 (Cys38 and Cys39) and 10 (Cys5 and Cys6) and the two cysteines coisolated as peptide 4 (Cys14 and Cys22), radiolabeled intact protamines and peptides 8 and 10 were sequenced and the amino acid derivative from each cycle was collected and counted to determine the extent to which each cysteine residue was carboxymethylated as a function of DTT:cys ratio. The relative labeling of the cysteine pairs in the three peptides is shown in Figure 6. A comparison of the reductions of Cys38 and Cys39 in peptide 8 show that the disulfide containing Cys38 is reduced before the disulfide containing Cys39 (Figure 6, panel A). In peptide 4, the disulfide involving Cys22 is reduced before the disulfide involving Cys14 (Figure 6, panel B). The discrimination between the reduction of disulfides involving Cys5 and Cys6, shown in Figure 6, panel C, was less obvious. The reduction of the disulfide involving the first cysteine residue in this pair, Cys5, appears to permit considerable disulfide interchange to occur between the Cys5 free sulfhydryl and the Cys6 residue participating in a disulfide bond.

These results, combined with the order of cysteine reduction identified by DTT titration, provide the information required to identify the disulfides present within and between protamine molecules. The first disulfide to be reduced in intact sperm treated with DTT is an intermolecular cross-link connecting Cys5 and Cys22 in two different, neighboring molecules (Figure 7). Two equivalent inter-protamine disulfides must be present. The second disulfide to be reduced, also an intermolecular cross-link, interconnects the Cys38 residues of two neighboring molecules. The first intra-protamine disulfide to be reduced occurs between Cys6 and Cys14. The last disulfide to be reduced is the intramolecular cross-link that connects Cys39 with Cys47.

Discussion

We have identified the pairs of cysteines in bull protamine that are cross-linked as disulfides in mature bull sperm by using the reducing agent dithiothreitol to titrate the disulfides of bull protamine, the bivalent disulfide cross-linker *N,N'*-ethylenedimaleimide to identify inter-protamine disulfides, and radiolabeled iodoacetate to carboxymethylate individual cysteine residues as they are reduced. The results of these studies show that all seven of the cysteine residues in bull protamine participate in the formation of disulfide cross-links. Both the amino- and carboxy-terminal ends of the protamine are folded inward toward the polyarginine-rich center of the molecule, and each is locked in place by a single disulfide bridge (Figure 7). Three additional intermolecular disulfides cross-link adjacent protamine molecules together around the DNA helix.

Complete reduction of the protamine disulfides in intact bull sperm requires only a 2-fold molar excess of reducing agent, unlike the results that have been reported for boar sperm,

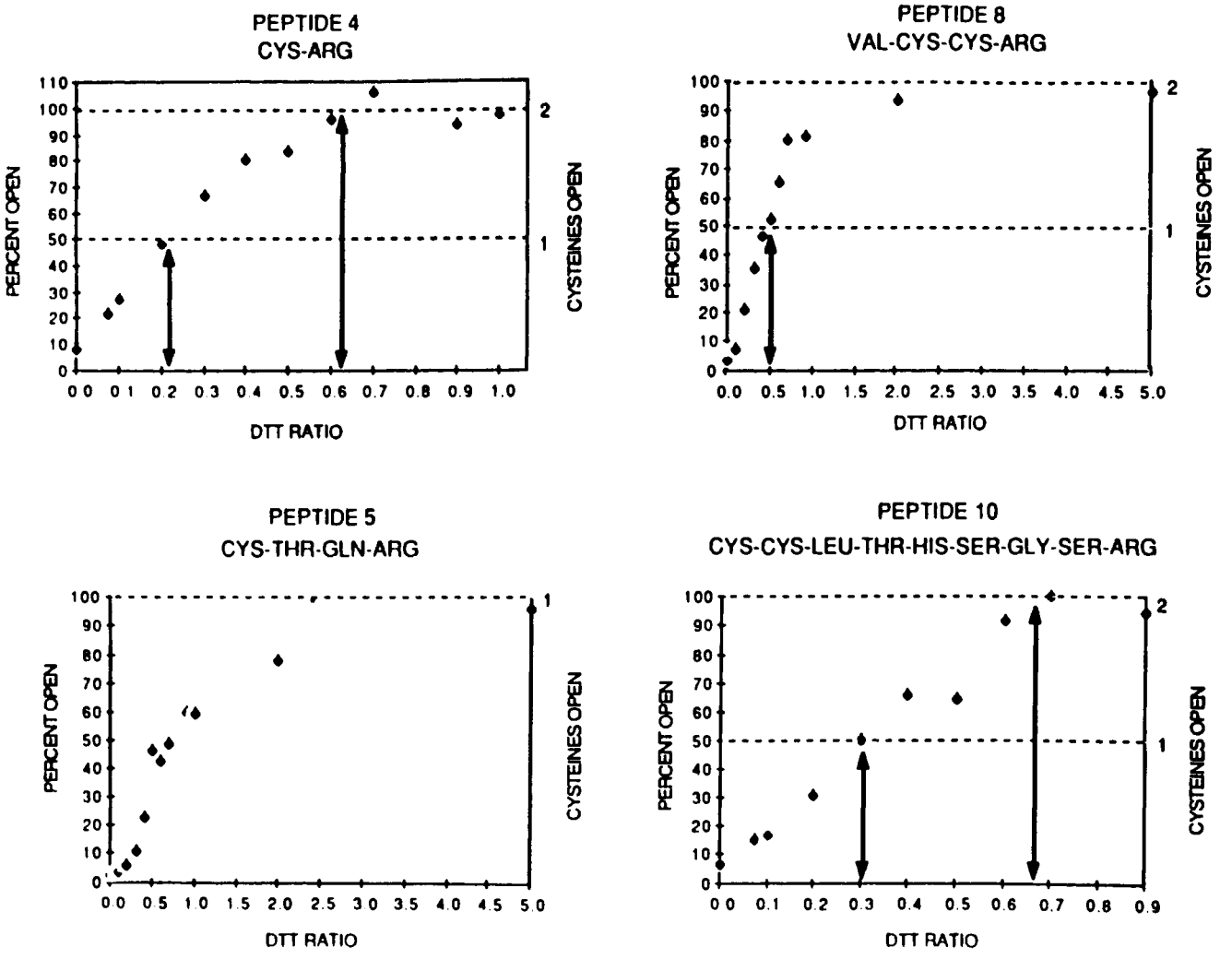

FIGURE 4: Carboxymethylation of peptides 4, 5, 8, and 10 as a function of DTT:Cys ratio. Dashed lines show 50% and 100% reduction. Arrows identify the DTT:Cys ratios that correspond to 50% and 100% reduction of the cysteine residues in those peptides containing more than one cysteine residue.

where a 54-fold molar excess of mercaptoethanol did not reduce all the boar protamine disulfides (Tobita et al., 1983). As others have demonstrated for the intermolecular disulfides of rabbit immunoglobulin G (Markus, 1962; Utsumi & Karush, 1964) and other proteins (Cecil & Loening, 1960; Cecil & Wake, 1962; Cecil & Weitzman, 1964), the inter-protamine disulfides in bull sperm are the first to be reduced, and these reductions occur over a narrow range of DTT concentrations. Intermolecular disulfides, in general, are most accessible to solvent and reducing agent are are typically under a great deal more strain than intramolecular disulfides. The two intramolecular disulfides in protamine, by comparison, behave quite differently to reduction. Following reduction of the Cys5–Cys22 intermolecular disulfide, the Cys6–Cys14 disulfide located in the amino-terminal domain of the protamine exhibits considerable disulfide interchange. As a result, the preferential labeling of Cys5 is barely detectable over the labeling observed for Cys6 by sequencing. This would suggest that the position of the amino-terminal end of the molecule is not rigidly constrained by other intramolecular interactions.

The intramolecular disulfide that cross-links the carboxy-terminal peptide in place (Cys39–Cys47), on the other hand, is much more resistant to reduction. The location of two valines and an isoleucine adjacent to the cross-linked cysteines may form a hydrophobic pocket that excludes water and inhibits the reaction of the disulfide with the mercaptide ion of thiols (Rosenthal & Oster, 1954; Hird, 1962). This is one of several mechanisms that have been proposed to account for observed differences in the reducibility of intramolecular disulfides in other proteins (Torchinsky, 1981). The Cys39–Cys47 disulfide also exhibits substantially less disulfide interchange. While the presence of the proposed hydrophobic pocket would also be expected to minimize exchange, an alternative explanation may involve the proximity of the Cys38 and Cys39 residues to the DNA-binding domain and the attendant constraints that could be placed on the flexibility of these residues by neighboring arginine residues that are tightly bound to DNA. Cys5 and Cys6, in contrast, are located near the end of the protein chain, further away from the central DNA-binding domain. Consequently, the binding domain should not affect the flexibility of these residues.

Although the identification of the cysteines that are cross-linked as disulfides has revealed that the amino-terminal end of one protamine molecule must be cross-linked to the amino-terminal end of its neighbor while its carboxy-terminal end is cross-linked to the carboxy-terminal end of another protamine, these results do not identify how the interacting protamines must be positioned along the DNA strand (the term "strand" is used here to describe the DNA double helix). The disulfide cross-linking patterns identified in this study are compatible with two very different intermolecular disulfide cross-linking models (Figure 8). One model involves the formation of interstrand disulfide cross-links between adjacent protamine molecules positioned along the same strand of DNA,

Table I: DTT:Cys Ratio at Which the Cysteines in Each Peptide Are Carboxymethylated by Reaction with Sodium Iodoacetate

peptide	no. of cysteines[a]	DTT:Cys ratio at completion of reduction
4	2	1 at 0.20
		1 at 0.63
5	1	above 2.0
8	2	1 at 0.48
		1 above 2.0
10	2	1 at 0.28
		1 at 0.60

[a] In peptides containing two different cysteine residues, the point of complete reduction of each peptide is estimated from the points of 50% and 100% reduction.

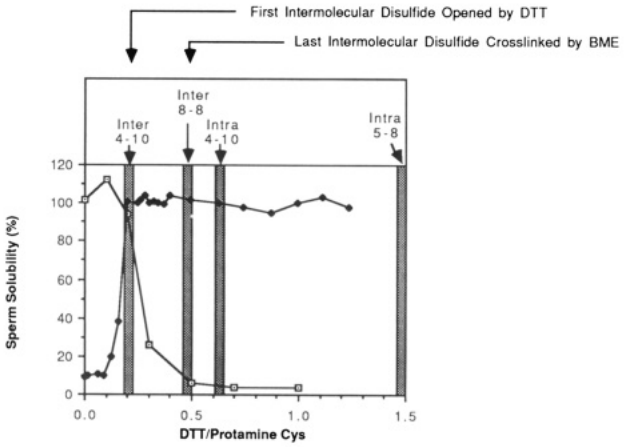

FIGURE 5: Composite of disulfide reduction and EDM re-cross-linking/guanidine hydrochloride solubility data. Vertical bars identify cross-linked cysteines in specific peptides and the type of disulfide cross-link (intra- or intermolecular).

as shown in Figure 8A. In this model, the presence of the Cys39–Cys39 cross-link between the carboxy-terminal domains of neighboring molecules and the two Cys5–Cys22 cross-links between the amino-terminal domains of neighboring molecules requires that the protamines be positioned in alternating (amino–carboxyl, carboxyl–amino) orientations along the DNA strand. The other model, which yields identical inter-protamine disulfides, is one that involves the formation of disulfide cross-links between protamine molecules on different DNA strands. Interestingly, in this second model, either orientation of protamine molecules is consistent with the identified disulfides. If the protamines bind along the DNA strand in the same orientation (Figure 8B), the observed intermolecular disulfides can only be formed between molecules on neighboring DNA strands oriented in the same direction. If adjacent protamines alternate orientation along the strand (Figure 8C), the correct disulfide cross-links can be achieved with protamines on neighboring DNA strands running in either direction.

Clearly the interactions hypothesized in Figure 8, panels B and C, must take into account the three-dimensional packing of multiple DNA strands and the possibility that protamine molecules on any one strand may form disulfide bridges to protamines on a number of different, neighboring strands. This could result in the formation of cross-links that interconnect protamines across the entire sperm chromatin complex. The figures as presented, however, have been intentionally simplified to emphasize only the directionality of protamines bound to DNA and the relative orientation of neighboring DNA strands. The current results do not provide information

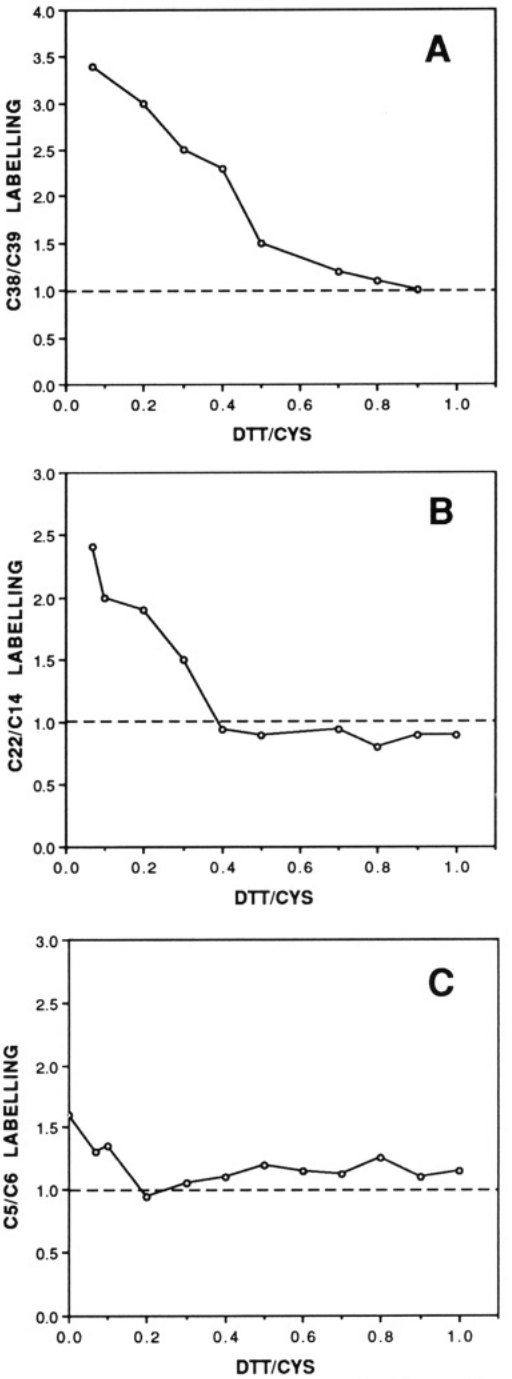

FIGURE 6: Relative labeling of cysteines paired in peptides as determined by radiolabeling and sequencing. (A) Cys38 and Cys39; (B) Cys22 and Cys14; (C) Cys5 and Cys6. The peptides containing the two cysteine residues carboxymethylated with tritiated IAA were sequenced, and the amino acid derivative from each cycle was collected and counted. The counts were corrected for the efficiency of repetitive yield (94%) and then plotted as the appropriate ratio to identify which cysteine residue in the peptide is reduced first. To provide an example, the actual tritium counts (above background) obtained for each cysteine residue treated at the DTT:Cys ratio 0.07 were 443 cpm (Cys38), 131 cpm (Cys39), 1443 cpm (Cys22), 591 cpm (Cys14), 487 cpm (Cys 5), and 367 cpm (Cys6).

about the location of DNA strands to which cross-linked protamines may be bound.

There are no experimental data available, at present, that exclude either model. Both appear feasible from examinations of two-dimensional models. Observations of apparent changes in sperm chromatin viscosity as a function of inter-protamine disulfide reduction in the current experiments suggest that interstrand cross-links may exist. As the DTT:cys ratio is

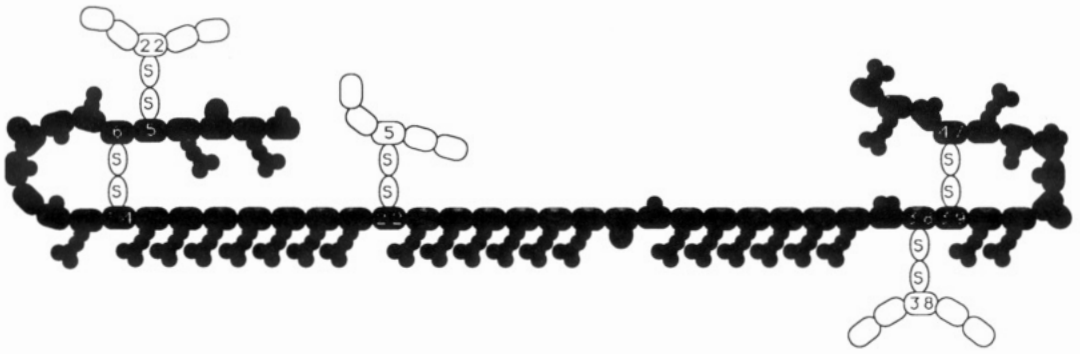

FIGURE 7: Intra- and intermolecular bull protamine disulfides. S represents the sulfur of cysteine residues contributing to disulfide cross-links. Cysteines 6, 14, 39, and 47 form intramolecular disulfides, while cysteines 5, 22, and 38 form intermolecular disulfides. Segments of neighboring protamine molecules participating in the intermolecular disulfides are designated as chains of white amino acids.

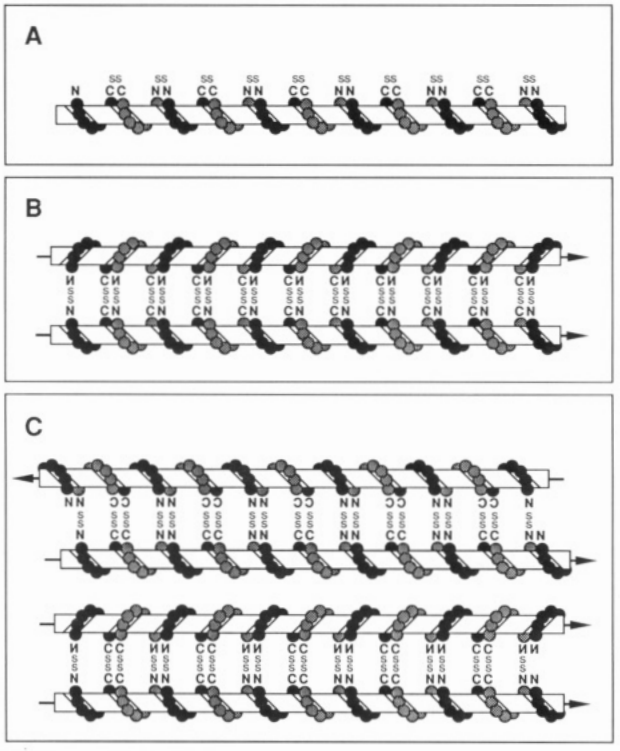

FIGURE 8: Intermolecular protamine disulfide cross-linking models. (A) The orientation of each protamine molecule alternates along the same DNA strand. Cross-links are formed between neighbors along the entire length of the DNA. (B) Each protamine is oriented in the same direction along the DNA strand, and the intermolecular disulfides are formed between protamines on two different strands running in the same direction. (C) The orientation of each protamine molecule alternates along the same DNA strand. Correct intermolecular cross-links can form between protamines on neighboring DNA strands running in either direction. Individual protamine molecules are schematically represented with only the carboxy- (C) or amino-terminal (N) intermolecular disulfides.

increased from 0 to 1.0 in the progressive reduction experiments monitoring the solubility of bull sperm chromatin in GuCl, the viscosity appears to increase to a maximum and then decrease to a somewhat lower level and plateau. Such changes in viscosity would be consistent with the progressive reduction of inter-protamine disulfide cross-links linking neighboring DNA strands. Incomplete reduction of these inter-protamine disulfide cross-links would result in the formation of partially cross-linked DNA strands with a larger hydrodynamic volume than fully reduced samples containing only individual, unlinked DNA molecules. Actual measurements demonstrating detectable changes in viscosity have not been made, however, and the presence of such cross-links is speculative at best.

Supplementary Material Available

One figure showing re-formation of protamine disulfides in intact bull sperm following reduction at different DTT:Cys ratios (1 page). Ordering information is given on any current masthead page. A copy of this material will be provided by the author upon request.

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