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Folding studies of protamine 1, a sperm DNA condensing protein

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**Folding studies of protamine 1, a sperm DNA condensing
protein**

Natu, Vanita Sanjiv, M.S.

San Jose State University, 1991

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**FOLDING STUDIES OF PROTAMINE 1, A SPERM DNA
CONDENSING PROTEIN**

**A Thesis
Presented to
The Faculty of the Department of Chemistry
San Jose State University**

**In Partial Fulfillment
of the Requirements for the Degree
Master of Science**

**By
Vanita Natu
August, 1991**

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

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ABSTRACT

FOLDING STUDIES OF PROTAMINE 1, A SPERM DNA CONDENSING PROTEIN

by Vanita Natsu

Protamines are small, highly charged proteins that package the DNA of most vertebrate sperm in a highly condensed, genetically-inactive state. Bull Protamine 1 (50 amino acids in length) contains a central polyarginine (R) domain proposed to be responsible for DNA binding. The two terminal domains each contain one tyrosine (Y). A total of seven cysteine (C) are interspersed within the polypeptide.

Reduced and oxidized, chemically synthesized N- and C-terminal peptides of bull protamine were examined by acid-urea gel electrophoresis and intrinsic tyrosine fluorescence measurements. The oxidized peptides showed reduced fluorescence whereas the reduced peptides showed an enhanced fluorescence. It was observed that the N-terminal peptides were easier to unfold whereas the C-terminal peptides were difficult to fold and unfold.

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LIST OF ABBREVIATIONS

Amino acid one letter code designations:

A: alanine

R: arginine

N: asparagine

D: aspartate

C: cysteine

E: glutamate

Q: glutamine

G: glycine

H: histidine

I: isoleucine

L: leucine

K: lysine

M: methionine

F: phenylalanine

P: proline

S: serine

T: threonine

W: tryptophan

Y: tyrosine

V: valine

Other abbreviations used:

BME: bismaleimidoethane

C-terminal peptide: carboxy terminus of bull protamine

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

F_{rel}: relative fluorescence

GuHCl: guanidine hydrochloride

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC: high pressure liquid chromatography

IAA: iodoacetic acid

N-terminal peptide: amino terminus of bull protamine

TCA: trichloroacetic acid

Tris: Tris-[hydroxymethyl]-aminomethane

INTRODUCTION

The non-germ cells of the body (somatic cells) have developed a method to package DNA (the genetic material of the cell) into a small volume which is still accessible for the expression of the genetic information using proteins called histones. The organization of sperm DNA is considerably different. The sperm DNA is highly condensed (50 fold more than somatic cells) (23) and is genetically inactive. Further, the DNA is inaccessible for repair of damage.

Research has revealed that the DNA inside each mammalian sperm is packaged by an equal weight of small, positively charged proteins that bind to the negatively charged phosphates that outline the surface of DNA. These proteins, which are called protamines, are not present in any other kind of cell. The entire length of the sperm DNA appears to be coated by protamines, and the positive charges on the protamines are presumed to neutralize the negative charges on the phosphates. The resulting complex of DNA and protamine is neutral and therefore appears to collapse into a volume that is only slightly greater than the physical volume of the DNA molecules. Models describing the interactions between DNA and protamines have been proposed by Balhorn (2), and by Warrant and Sung-Hou (27).

The presence of large sperm heads (apparently due to incorrectly condensed DNA) has been identified as a problem in infertile males (6). Additionally, chemical damage to sperm may modify the DNA, the protamines, or their specific interactions. This damage may be responsible for the transmission of heritable mutations from male workers to their offspring and may lead to birth defects (12).

Protamine Structure

Proteins are composed of amino acid residues connected in a linear chain. As shown in Figure 1, two very different classes of protamines have been isolated from human and other mammalian sperm. Protamine 1, the smaller of the two proteins, contains 50 amino acids and has been found in all mammalian sperm. The second class, protamine 2, has been isolated from a number of species including mouse, human, vole, chinchilla and hamster sperm but appears to be absent from a number of species including bull. The sperm of humans and certain primates contain a third protamine that has been designated protamine 2b. Protamines 2a and 2b are larger than protamine 1 and each has a composition and primary sequence that differs dramatically from the known sequences of protamine 1. Protamine 2 also contains large amounts of histidine, an amino acid that binds metal atoms, such as zinc. (The presence of zinc in human sperm is known to be essential for fertility). Studies of the genes that

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Figure 1.

Amino acid sequence of protamine 1 and 2 (Fig 1, Ref 17) given as one letter code designations.

PROTAMINE 1

BULL	ARYRCCLTHSGSR
RAM	ARYRCCLTHSR
BOAR	ARYRCCTSHSR
STALLION	ARYRCCTRSQS
MOUSE	ARYRCCTRSKSR
HAMSTER	ARYRCCTRSKSR
HUMAN	ARYRCCTRSQS
RHESUS	ARYRCCTRSQS

PROTAMINE 2

HUMAN 2a	RTHGQSHYRRRHCS
HUMAN 2b	GQSHYRRRHCS
MOUSE	RGHHHHHRRC
HAMSTER	RGQHHHRRC

produce the two types of protamines have revealed that the synthesis and metabolism of the two proteins are quite different, suggesting that protamine 1 and protamine 2 may perform different functions (3).

The amino acid sequences of protamine 1 from a number of species are shown in Figure 1 (17). Comparisons of these protein sequences have demonstrated that the majority of the amino acid sequence in each molecule is highly conserved. The protamine is 50 amino acids in length with 26 arginine amino acids. This overrepresentation of arginine is very unusual for a protein. (Usually a protein contains 1 to 10% of any of the twenty amino acids). Of all the amino acids, arginine has the strongest affinity for DNA (2). Figure 2 is a schematic showing various peptide domains. The amino terminus of each protein begins with the same sequence, ARYRCC. The central half of protamine contains tandemly repeated polyarginine stretches and resembles the entire molecule of fish protamine, both in sequence and size (9). Protamine 1 also contains carboxy- and amino-terminal domains that are relatively deficient in arginine and appear to interact with other protamines or sperm nuclear proteins.

Mammalian protamines are extremely rich in cysteine (10-20%). There are seven cysteine (C) amino acids (Figure 3) in bull protamine 1. The bull protamine molecule contains two folded regions in vivo, one involving the

Figure 2.

General structure of bull protamine 1.

Schematic of bull protamine 1 which indicates three domains, the amino-terminal domain, proposed DNA binding site, and carboxy-terminal domain (Fig 2, Ref 4).

Figure 3.

Schematic representation of the amino acids found in bull protamine.

R represents the 26 arginine amino acids in bull protamine. S-S represents intramolecular crosslinks between amino acids 6 and 14, and 39 and 47. Amino acids 5, 22, and 38 are involved in intermolecular crosslinks designated by arrows (Fig 3, Ref 4).

amino-terminus of the molecule and one involving the carboxy-terminus (4). Each fold is stabilized by a single intramolecular disulfide bridge, while adjacent protamine molecules are crosslinked together around the DNA helix through three additional intermolecular disulfide crosslinks. These crosslinks help define the protamine structure and play a role in the condensation of the sperm DNA (4).

Balhorn and coworkers (4) have identified each inter- and intraprotamine disulfide crosslink in bull sperm chromatin by progressively reducing the sperm disulfides with dithiothreitol (DTT) and following the modification of each cysteine residue in bull protamine with tritiated iodoacetic acid (IAA) by HPLC peptide mapping and protein sequencing. The carboxymethylcysteine containing tryptic peptides were isolated by HPLC and the reduction of each cysteine determined by the appearance of tritium in the peptides as a function of the concentration of reducing agent. Five peptides were obtained that contained cysteine. Three of the five contained a single cysteine residue. By plotting the percent of cysteine modified in each peptide as a function of DTT/protamine cysteine, the DTT/cys ratio at which each cysteine modification (i.e. reduction) was completed could be estimated. These results were then used to identify which cysteines were paired as disulfides.

The results of these experiments revealed that the amino- and carboxy-terminal ends of the bull protamine molecule were folded inward toward the center of the molecule and were locked in place, each by a single intramolecular disulfide bridge. Three intermolecular disulfides were formed between neighboring protamine molecules. As sperm were treated with increasing amounts of DTT, the two equivalent intermolecular disulfides formed between C5 and C22, and C22 and C5 were reduced at a DTT/cys ratio of 0.2 to 0.28. The second disulfide reduced by DTT was an interprotamine crosslink between the C38 residues of two adjacent molecules. Reduction of this disulfide was complete at a DTT/cys ratio of 0.48. Once the DTT/cys concentration reached 0.6-0.63, the intramolecular disulfide locking the amino-terminal fold in place (a crosslink between C6 and C14) was reduced. The final disulfide to be reduced is the intraprotamine disulfide that crosslinks C39 and C47 in each molecule, locking the carboxy-terminal fold of the protein in place. Hence analysis of radiolabeled tryptic peptides isolated from progressively reduced and carboxymethylated protamine revealed that the opening of individual disulfides was completed at DTT/protamine cysteine molar ratios equal to 0.2, 0.5, 0.6, and above 2.0.

Spontaneous Folding as Visualized by Gel Electrophoresis

Gel electrophoresis has been used routinely to compare the apparent size and charge density of proteins. The acid-urea system of Panyim and Chalkley has been well suited for separating basic proteins such as histones and protamines (20). Fully reduced bull protamine migrated toward the cathode in this system as a single, well defined band (Figure 4). In the presence of excess DTT or mercaptoethanol, the sulfhydryl groups remained reduced and the protamine molecules migrated (at pH 2.4) as if they were in an extended conformation. Within minutes after solubilization of reduced protamine in 0.1M Tris-HCl buffer at pH 8, five new electrophoretic species were formed, each with an electrophoretic mobility greater than that of the reduced extended protamine molecule (Figure 4). As they appeared, the band corresponding to the extended reduced protamine (position marked with an arrow) disappeared. With increasing time, the quantitative distribution of protein within each band shifted progressively toward increased mobility. Those species with apparent molecular weights only slightly smaller than extended bull protamine appeared rapidly, while the relative proportion of the higher mobility bands increased slowly. The apparent molecular weights of the five folded protamine monomers were calculated using their increase in electrophoretic mobility

Figure 4.

Acid-urea gel electrophoresis of bull protamine.

Fully reduced bull protamine was dissolved in 0.1M Tris-HCl buffer, pH 8 and the appearance of folded forms monitored by electrophoresis (from left to right) in acid-urea gels (Fig 1, Ref 4b). The arrow marks the position of the extended (unfolded) protamine molecule.

relative to the fully reduced extended bull protamine molecule (Figure 5).

Chromatography of folded bull protamine (incubated at 21°C for 24 hr) on a Nucleosil RP-C18 column resulted in the fractionation of the mixture into two major peaks (Figure 6). Electrophoresis of the lyophilized fractions revealed that fractions one and two contained the higher mobility (monomer) protein species while fraction three contained the lower mobility (dimer) species. Following treatment with excess DTT at pH 8, the electrophoretic mobility of all three fractions returned to that of the extended fully reduced bull protamine molecule. This demonstrated that the higher mobility species were differentially folded, individual protamine molecules and not degradation products of protamine. The lower mobility species appeared to represent dimers of folded protamine monomers. These conformations were stable in the 2.5M urea used in the separating gel and appear to be stabilized by disulfide crosslinks.

In the Balhorn model of protamine binding to DNA, the arginine, with its positive charge, neutralizes the negative charge on the DNA phosphate backbone. Whether the placement of the arginine residues is the result of the protamine existing in an alpha helix conformation which

Figure 5.

Schematic representation of possible folded bull protamine structures stabilized by disulfide crosslinks.

The electrophoretic mobility of the folded molecule should relate directly its apparent molecular length; these values are listed for each structure (Fig 6, Ref 4b).

Figure 6.

HPLC separation of folded forms of bull protamine.

The panel on the left shows the chromatogram obtained upon HPLC of reduced protamine (24 hr) on a Nucleosil RP-C18 column. The panels on the right, labeled Fraction 1 through 3, show the protein species present in each HPLC fraction. Total folded protamine is shown in the top right panel for comparison. The dashed line showed the mobility of the proteins in each fraction after reduction with excess DTT at pH 8. In all three cases, the reduced sample had an electrophoretic mobility identical to that of the fully extended protamine molecule (Fig 2, Ref 4b).

interacts with the DNA in the major groove (Warrant and Sung-Hou model) (27) or in an extended conformation which interacts with the DNA in the minor groove (Balhorn model) (2) remains to be determined. The charge neutralization is what appears to allow the DNA to be so tightly packaged.

Fluorescence

The various types of luminescence can be classified according to the means by which energy is supplied to excite the luminescent molecule. When molecules are excited by interaction with photons of electromagnetic radiation, the form of luminescence is called photoluminescence. If the release of electromagnetic energy is from the singlet state, the process is called fluorescence.

Any fluorescent molecule has two characteristic spectra: the excitation spectrum (the relative efficiency of different wavelengths of exciting radiation to cause fluorescence) and the emission spectrum (the relative intensity of radiation emitted at various wavelengths).

Every molecule possesses a characteristic property that is described by the quantum yield, or quantum efficiency, ϕ . This is the ratio of the total energy emitted per quantum of energy absorbed:

$$\phi = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} = \text{quantum yield}$$

The higher the value of ϕ , the greater the fluorescence of a compound. Fluorometric methods can detect concentrations of substances as low as one part in ten billion, a sensitivity 1000 times greater than that of most spectrophotometric methods. The main reason for this increased sensitivity is that in fluorescence the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy (26).

Fluorescence is much more sensitive than absorbance. However, one of the disadvantages of fluorescence as an analytical tool is its serious dependence on the environment (temperature, pH, ionic strength, etc.). The fluorescence of a compound is affected by the viscosity of the medium, increasing with increasing viscosity. Thus the fluorescence of most compounds can be increased by using a more viscous solvent, such as glycerol or gelatin (13). Quenching sometimes causes a reduction in fluorescence. This can result from a competing deactivating process resulting from the specific interaction between a fluorophor and another substance present in the system.

Extrinsic And Intrinsic Protein Fluorescence

Aromatic amino acids present in proteins endow them with an intrinsic ultraviolet fluorescence. The fact that proteins have an intrinsic ultraviolet fluorescence has

been known only since about 1957, and this fluorescence has been used as a tool to study protein structure (13). The intrinsic fluorescence of proteins can be discussed in terms of contributions from tyrosine, tryptophan and, some minor contributions from phenylalanine too. Proteins conjugated with other fluorescent molecules may be said to have an extrinsic fluorescence.

Tyrosine seems to have a very low quantum yield in most proteins (0.01-0.03) so that emission from tyrosine is normally greatly overshadowed by tryptophan fluorescence (13). Since a variety of mechanisms exist for the quenching of tyrosine fluorescence, changes in protein structure can be expected to be reflected in the nature of tyrosine emission. Teale (24) found that acid, urea, and detergents greatly increased the tyrosine fluorescence of the albumins as well as of other proteins and suggested that this was due to changes in secondary and tertiary structure. An important quenching mechanism for tyrosine fluorescence in proteins was uncovered by Cowgill (10), who found that both disulfides and sulphydryl groups were quenching agents.

Cowgill has performed a number of studies relevant to tyrosine fluorescence in specific proteins. Pancreatic trypsin inhibitor, which has four tyrosines and no tryptophans, was completely quenched by the iodination of only two of the tyrosines (10). The curve of fluorescence

versus the degree of iodination suggested that only two of the tyrosines were fluorescent in the native protein and that their iodination resulted in the quenching.

Unlike tyrosine emission, tryptophan fluorescence in proteins was found by Teale (24) to have quantum yields that could be either higher, lower, or about the same as that of the free amino acid. In addition, the emission maxima of tryptophan fluorescence in native proteins were observed to vary from 332 to 342nm depending on the protein but shifted to about 350nm on denaturation.

White (30) studied the pH dependence of the fluorescence of phenol and its derivatives including tyrosine. It was clear in her results that loss of the phenolic proton resulted in loss of fluorescence. Quenching occurred not only at alkaline pH, where the phenolic group ionized, but also under conditions where the hydrogen can be transferred to an acceptor such as the ionized carboxyl or the amine group of tyrosine. She also performed fluorometric titrations of tryptophan (30). The results indicate that tryptophan fluorescence was rather constant between pH 3.5 and 7.5, decreased at lower pH due to the formation of an un-ionized carboxyl group (which is a quencher) and increased at higher pH due to deprotonation of the NH_3^+ group (also a strong quencher). At pH values higher than 10, quenching again occurred, presumably due to abstraction of a

proton from the indole imino nitrogen in the excited state.

Protamines have tyrosine and no tryptophan. Protamine concentration can be determined by fluorescence of the tyrosines in protamines. It has been found in this study that bull protamine fluoresces maximally at 305nm when excited by 275 or 280nm light.

In 1975 it was found that the widely used fluorogenic compound fluorescamine (7) reacts with protein amino groups only if they are not involved in stable salt linkages. This property can be used to quantitate protein-DNA interactions and to construct binding curves for these mixtures at equilibrium.

The binding parameters of protamine to DNA at equilibrium were determined through the use of fluorescein-labeled clupeine (a fish protamine) (28, 29) or dansyl-labeled mammalian protamines (25). The binding of fluorescein-labeled protamine to DNA was accompanied by quenching of the fluorescence, whereas the fluorescence of dansyl-labeled protamine was enhanced upon its binding to DNA. This quenching or enhancement permitted the convenient determination of the concentrations of free or bound protamines in a mixture.

Nakano and coworkers (18) have described a method

for obtaining the co-operative binding constant of protamine to DNA and other equilibrium parameters without the use of a fluorescent probe. A probe might affect the binding behavior. The binding of DNA-protamine complexes was determined by the measurement of the fluorescence of the tyrosine residues of protamine remaining in the supernatant after mixing and centrifugation. The concentrations of bound protamine (C_b) were obtained by subtraction of free protamine (C_f) from concentration of the total. The fluorescence was measurable without interference due to scattering since at a certain protamine to DNA ratio, the input DNA was completely precipitated and removed by centrifugation. The co-operative binding constants (K_C) found in this study were:

$3.3 \times 10^7 \text{ M}^{-1}$ for fowl protamine, $4.1 \times 10^6 \text{ M}^{-1}$ for boar protamine and $8.7 \times 10^5 \text{ M}^{-1}$ for a fluorescein labeled fish protamine (18).

Watanabe and Schwarz (28) showed that the protamine binding parameters were sensitive to ionic strength, which reflected the electrostatic character of the binding of protamine to DNA.

The most challenging questions to be discussed are the underlying reasons for and the biological relevance of cooperativity in protamine-DNA interaction. Cooperativity of binding had been deduced by authors (1, 14, 19) from thermal

denaturation and equilibrium dialysis studies, and was recently demonstrated by Bode and Willmitzer (8) using the fluorescamine assay. It was unlikely that the fluorescent fluorescamine label caused cooperativity, as these labeled protamine solutions (without DNA) do not show any interactions of the chromophores (at least in the concentration range tested). Moreover, cooperativity was also shown with unlabeled protamines by Nakano and coworkers (18).

The biological function of protamines would suggest a role in DNA condensation. An ordered condensation of DNA might involve parallel alignment of DNA molecules as a consequence of repeating crosslinking by many protamine molecules. On the basis of this ordered interaction, protamine-protamine interactions could be important.

Objective of Study

The objective of the research reported here was to determine the extent of folding of bull protamine 1 in vitro by using acid-urea gel electrophoresis and intrinsic tyrosine fluorescence. Preliminary experiments focused on intact protamines folded in vitro. The complexity and heterogeneity of these samples made interpretation of the fluorescence data difficult. As a result, the N-terminal and C-terminal peptides were used as model compounds to

determine the extent of fluorescence and thus structural changes in both the oxidized and reduced state of these domains.

MATERIALS AND METHODS

Protamine

The source of HPLC purified protamine was through our collaboration with Rod Balhorn (Lawrence Livermore National Laboratory). The protamine was isolated from bull semen purchased from American Breeder's Service (DeForest, WI) (5). The protamine was purified by reversed-phase high pressure liquid chromatography (17). The purified material migrated as a single band upon acid-urea polyacrylamide gel electrophoresis (20).

Peptides

The N-terminal (positions 1-16) and C-terminal (positions 34-50) peptides of bull protamine 1 were obtained from Lawrence Livermore National Laboratory. These were chemically synthesized from derivatized amino acids and purified by reversed phase HPLC. The peptides had the following sequences:

N-terminal: $\text{NH}_2\text{-ARYRACLTHSGSRCRR-CO}_2\text{H}$ (16 Aa)

C-terminal: $\text{NH}_2\text{-RRRVACRRYTVIRCTRQ-CO}_2\text{H}$ (17 Aa)

Note that there were changes from the bull sequence including an alanine for cysteine in position 5 in N-terminal peptide and position 38 in the C-terminus. By this

change, only one intramolecular disulfide can form.

PREPARATION OF REDUCED AND OXIDIZED SAMPLES

Reduction of native protamine

Fully reduced protamine was prepared by dissolving the protein in 6M guanidine hydrochloride (GuHCl), 0.05M Tris-HCl buffer pH 8, 0.002M ethylenediamine tetraacetic acid (EDTA) containing a 50-fold molar excess of dithiothreitol (DTT) and allowing the protein to be reduced overnight at room temperature (23°C). The protein was then dialyzed against 10mM HCl and subsequently lyophilized. The reduced protamine sample was dissolved in 10mM HCl. Further dilutions were carried out in 20mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5.

Disulfide formation during oxidation of reduced protamine

Two different conditions were used to obtain oxidized protamine samples.

In the first case, the reduced protamine sample at a concentration of 1.53 mg/mL was dissolved in 20mM HEPES, pH 7.5 and incubated at room temperature for 24 hours. In the second case, the reduced protamine sample at a concentration of 847 µg/mL was allowed to fold in the presence of 0.1M Tris-HCl buffer pH 7.9 by incubation at

23°C for 64 hours. Subsequent treatment of both samples involved TCA precipitation, washing, and drying under N₂. The samples were resuspended in 10mM HCl to maintain established disulfide bonds. Further dilutions were carried out in 20mM HEPES, pH 7.5. The extent of folding was determined by acid-urea gel electrophoresis (20).

Peptide oxidation

The HPLC purified N- and C-terminal peptides were dissolved in 0.1% ammonium bicarbonate pH 9 at a concentration of 1 mg/mL and allowed to oxidize at 20°C for 3 days. The samples were subsequently lyophilized to remove the buffer and checked for extent of oxidation by gel electrophoresis in 10cm acid-urea gels containing 2.5M urea, 0.9N acetic acid. Electrophoresis was performed at 130V, 20mA for 45 min. The folded (oxidized) form of the peptides migrated ahead of the unfolded (reduced) peptides and were clearly resolved. The treated samples were supplied by Lawrence Livermore National Laboratory.

MODIFICATION OF REDUCED PROTAMINE

The protamine samples were all reduced with dithiothreitol, followed by chemical modification with an alkylating agent.

Aminoethylation of protamine

One mg of protamine was dissolved in 1 mL of 0.5M Tris pH 8.0 containing 7.5 mg DTT. Fifteen μ L of ethylenimine was added and the mixture was incubated at 37°C for 1 hour. The remaining ethylenimine was hydrolyzed upon addition of a tenth volume of 5N HCl. The modified protamine was precipitated with a final concentration of 20% TCA at 4°C, the protamine pellet was washed with acidified acetone, and dried under N₂ (4). The aminoethylated samples were provided by Dr. P. Stacks.

CONCENTRATION DETERMINATION

Protamine concentration was determined by UV absorbance at 230nm using an extinction coefficient of 5.8 cm²/mg for unmodified protamine and 5.08 cm²/mg for carboxymethylated protamine in 10mM HCl (Balhorn, personal communications). A Hewlett Packard Diode Array UV spectrophotometer was used.

The following assumptions were made to calculate the molarity of peptides based on absorption measurements. The molecular weight of the amino terminal peptide was 1920 daltons and the molecular weight of the carboxy terminal peptide was 2221 daltons. The extinction coefficient of the unmodified protamine was 5.8 cm²/mg. The calculated ϵ_{280} for protamine was 1.068 cm²/mg. Since the N-terminal and C-

terminal peptides each have half as many tyrosines (the residue responsible for absorption at this wavelength), it was assumed that ϵ_{280} for each peptide was 1.5 cm²/mg (this will have to be confirmed by measurements). Accordingly, after absorption measurements of each peptide were obtained the ϵ_{230} for each was calculated

$\epsilon_{230} = [(A_{230}/A_{280}) \epsilon_{280}]$. The resulting extinction coefficients $\epsilon_{230} = 11.13$ cm²/mg N-terminal peptide and $\epsilon_{230} = 7.7$ cm²/mg C-terminal peptide were used to determine the concentration of peptides.

INSTRUMENTATION

The instrument used was the Perkin Elmer LS-3 fluorescence spectrophotometer. Fluorescence of the samples was measured at 18°C. The cell temperature was regulated by means of a water bath. (Water bath setting was at 16.9°C). The fluorescence of the standards and the samples was measured at excitation and emission wavelength of 275 and 305nm, respectively.

FLUORESCENT ASSAY

The relative fluorescence is defined as (26):

$$F_{rel} = \frac{F_{unk}}{F_{stand}} \cdot \frac{A_{stand}}{A_{unk}}$$

where F is the fluorescence and A is the absorbance measured at the same wavelength as the excitation wavelength.

Concentration effects are corrected by use of absorption measurements at a wavelength equal to the excitation wavelength.

The fluorescence of an unknown was compared to a standard of known quantum yield. Either quinine sulfate with excitation and emission wavelength of 350 and 450nm or tyrosine (pH 7.4) with excitation and emission wavelength of 275 and 305nm were used as standards. Quinine sulfate in 0.1N sulfuric acid has a known quantum yield (26). The quantum yield of tyrosine has not been precisely measured but has been used as a reference for protein tyrosine fluorescent measurements (26).

BUFFER SELECTION

Initial studies were performed to determine optimal buffer conditions. Buffers such as Tris-HCl, Borate and HEPES were used to determine fluorescence as a function of protamine concentration. It was then decided to perform all our experiments with 20mM HEPES pH 7.5 buffer. Determinations were also made in 10mM HCl.

GuHCl: Method of preparation

The following procedure was used to determine the change in volume when GuHCl was dissolved in water. A stock solution of 5M GuHCl in 20mM HEPES was prepared. Solutions

were prepared in 10 mL volumetric flasks. The weight of the 5M GuHCl and water added was determined and temperature noted. The increase in the volume was calculated by dividing the weight of the water by the density of the water at that temperature. This allowed the increase in volume to be taken into consideration in experiments using GuHCl.

FREE TYROSINE FLUORESCENCE

A 1mM stock tyrosine solution (pH 7.4) was prepared in 20mM HEPES buffer. All samples were incubated for at least 30 min at 18°C. A series of control experiments using free tyrosine were performed as follows: To measure the effect of DTT on tyrosine fluorescence, a stock solution of 100mM DTT was freshly prepared, appropriate dilutions of DTT were used, and measured after incubation. To determine the effect of GuHCl on free tyrosine fluorescence, appropriate dilutions of GuHCl were incubated at 18°C until temperature equilibration was established. A tyrosine sample (4µM) was then added, incubated and the fluorescence measured.

PROTAMINE FLUORESCENCE

Effect of GuHCl in the absence of DTT

Appropriate dilutions of GuHCl were incubated at 18°C until temperature equilibration was established.

Protamine samples were then added and incubated at 18°C for 30 min. The fluorescence was measured at 18°C.

Effect of DTT and GuHCl

Experiments were performed with 0.5, 1.5 and excess molar ratios of DTT/protamine cysteines. (This was an equivalence of DTT-SH/cysteine of 1.0 and 3.0). The molarity of protamine in a sample was determined by absorbance measurements at 230nm, an associated extinction coefficient of 5.1 cm²/mg and a MW of 6654 daltons. The molarity of cysteine was then determined to be seven fold higher than the protamine molarity. (There are 7 cysteines in bull protamine (4)).

A fresh stock of DTT was used with every experiment. The appropriate amount of DTT was added to each protamine sample which was incubated at 18°C for 30 min. The DTT treated protamine sample was added to the temperature equilibrated GuHCl solutions and incubated at 18°C for an additional 30 min prior to fluorescence measurements.

PEPTIDE FLUORESCENCE

N-terminal peptide (reduced and oxidized) and C-terminal peptide (reduced and oxidized) samples were treated with GuHCl in the presence and absence of DTT as described for the protamine samples.

RESULTS

STANDARDIZATION

Quinine sulfate

The instrument needed to be standardized and set to a constant sensitivity each day because of differences in lamp sensitivity and other instrumental variables. Generally a solution of a stable fluorophor of known concentration can be used to set the instrument. One of the most widely accepted standards is quinine sulfate due to its known quantum yield ($\phi = 0.55$) at 350nm (26). Quinine sulfate in 0.1N sulfuric acid has an excitation and emission wavelength of 350 and 450nm, respectively. There was a linear relationship between concentration and fluorescence in the range of 0 to 10 μ M (Figure 7).

FLUORESCENT ASSAY

Bull protamine with a molecular weight of 6654 daltons, contains a central polyarginine (R) core, an amino-terminal domain and a carboxy-terminal domain. The two terminal domains each contain one tyrosine (Y) residue (2). Protamine concentration can be determined by fluorescence of the tyrosines in boar and galline protamines (18). In this study it was determined that bull protamine fluoresces maximally at 305nm when excited by 275nm light and that there was a linear relationship between protamine

Figure 7.

Quinine sulfate standard curve

Linear least square regression analysis gave:

$y = 1.105 + 3.306x$ $R^2 = 0.995$ where R^2 is the correlation coefficient.

concentration and fluorescence in the range of 0 to 23.75 μ M (Figure 8).

BUFFER SELECTION

Initial studies were performed with Tris-HCl, Borate and HEPES to determine optimal buffer conditions. The fluorescence was determined as a function of protamine concentration and best results were obtained with 20mM HEPES pH 7.5 buffer (Figure 8). A decrease in the fluorescence as a function of time was observed with Tris-HCl buffer. Lower fluorescence values were observed with Borate buffer, whereas very stable and higher fluorescence values were observed in HEPES buffer.

TYROSINE MODEL SYSTEM

It was important to use a standard with its fluorescent properties close to the substance being determined. Hence free tyrosine standards were employed to determine relative fluorescence of bull protamine. Its quantum yield was not known accurately. Reports on tyrosine's quantum yield ranged from 0.01 to 0.03 (13).

Tyrosine had an excitation and emission wavelength of 275 and 305nm, respectively. There was a linear relationship between concentration and fluorescence in the range of 0 to 20 μ M (Figure 9A and 9B). The standard curves

Figure 8.

HEPES-folded bull protamine standard curve

Linear least square regression analysis gave:

$y = 3.880 + 2.660x$ $R^2 = 0.998$ where R^2 was the correlation coefficient.

Figure 9A.

Tyrosine standard curve

Old Lamp

Four repeats (done on different days) shown of the tyrosine standard curve.

Figure 9B.

Tyrosine standard curve

New Lamp

Four repeats shown (done on separate days) of the tyrosine standard curve.

which resulted from the tyrosine samples demonstrated a good fit of the data. The correlation coefficients (R^2) of a linear least square regression analysis of each curve were very close to one. (A range of 0.998-1.00 for Figure 9A and 0.999-1.000 for Figure 9B). Because of differences in lamp intensity and photomultiplier sensitivity, as well as other instrumental variables, it was important to standardize the instrument to a constant sensitivity each day. Figures 9A and 9B represented the effect of lamp intensity on fluorescence intensities. The fluorometer had a new bulb installed which increased the sensitivity of the instrument four fold and resulted in different fluorescence values between the old and the new lamp.

Tyrosine standards were employed to determine relative fluorescence of bull protamine, and to evaluate the effects of various agents like DTT and GuHCl on tyrosine fluorescence in the absence of any contributions from protein structure.

A range of DTT concentration (0-100 μ M) did not affect a 12 μ M tyrosine fluorescence (Table 1). At higher concentrations, 1 and 5mM, DTT did quench tyrosine fluorescence. Therefore, our studies employed DTT, a reductant, at concentrations less than 100 μ M.

GuHCl was a denaturant used which also increased the viscosity of solutions. The effect of viscosity on free

Table 1.

Dithiothreitol concentration and tyrosine
fluorescence

	DTT (μ M)	F (275nm)
1	0	145.0
2	10	147.8
3	15	145.9
4	20	145.1
5	25	145.6
6	30	144.9
7	35	146.1
8	40	145.9
9	100	144.1
10	1000	130.8
11	5000	62.2

tyrosine fluorescence in the absence of any protein structural effects was determined (Figure 10). Quinine sulfate was used as a standard for relative fluorescence tyrosine measurements. A slight increase (10%) was observed, presumably a reflection of viscosity changes (13).

PROTAMINE RESULTS

Effect of GuHCl on protamine fluorescence

Bull protamine, dissolved in aqueous buffers at alkaline pH, spontaneously formed disulfide bonds as determined by acid-urea gel electrophoresis and intrinsic tyrosine fluorescence measurements. Disulfide formation required the ionization of the sulfhydryl group of cysteine which was facilitated at alkaline pH.

GuHCl was considered to compete for H-bonds and thus, break protein hydrogen bonds. GuHCl was also proposed to increase the solubilities of non-polar molecules, including those of the amino acid chains, in proportion to their accesible surface area. This diminished the magnitude of the hydrophobic effect by up to one third, thus bringing about unfolding of proteins (See Figure 4-6 in Ref 11).

Ethylenimine modification of protamine cysteines was thought to eliminate the appearance of folded structures (5). The aminoethylated protamine appeared to migrate as a single band upon acid urea gel electrophoresis (Figure 11,

Figure 10.

Tyrosine Fluorescence in 0-2M GuHCl

Linear least square regression analysis gave:

$$y = 1.080 + 5.170 \times 10^{-2}x, R^2 = 0.588$$

(correlation coefficient).

Figure 11.

Acid-urea gel electrophoresis of
aminoethylated protamine.

The direction of electrophoresis was from
right to left.

Lane 1. Aminoethylated bull protamine.
Lane 2. HPLC purified bull protamine.

Lane 1), which indicated the protamine cysteines were completely alkylated. The HPLC purified protamine was a more heterogeneous sample (Figure 11, Lane 2).

Figure 12 showed a comparison of the relative fluorescence of the aminoethylated protamine, HEPES-folded and the Tris-folded protamine as a function of increasing GuHCl concentration. The aminoethylated protamine showed higher fluorescence compared to the HEPES-folded and the Tris-folded protamine. When GuHCl concentration was increased from 0 to 2M the aminoethylated protamine showed a 24% increase in fluorescence, the Tris-folded protamine showed a 23% increase and the HEPES-folded protamine showed a 19% increase in fluorescence. All of these showed greater increase than free tyrosine showed. Although the protamine was folded under different conditions, the fluorescence of the folded samples was determined in 20mM HEPES, pH 7.4.

The aminoethylated material which was not capable of establishing disulfide bonds had the highest fluorescence even in the absence of any denaturant. This indicated that the tyrosine fluoresced more in an unfolded conformation as expected. However, it was possible that either the aminoethylated modification or the lack of disulfides may have caused the increase in fluorescence. Although there was clearly less fluorescence in the Tris and HEPES folded

Figure 12.

Relative fluorescence of Aminoethylated, HEPES-folded and Tris-folded protamine.

Linear least square regression analysis gave:

AE Protamine: $y = 0.294 + 3.007 \times 10^{-2}x$
 $R^2 = 0.918$,

HEPES-fold Protamine: $6.970 \times 10^{-2} + 6.625 \times 10^{-3}x$
 $R^2 = 0.869$,

and Tris-fold Protamine: $0.184 + 1.843 \times 10^{-2}x$
 $R^2 = 0.858$,

where R^2 was the correlation coefficient.

protamine compared to the aminoethylated, comparison between the two oxidized samples is difficult due to the complex mixture found in each (Figure 13A and 13B).

Figure 13A and Figure 13B showed the separation of folded forms as monitored by electrophoresis in acid-urea gels. By comparison to the results in Figure 4 (taken from 20), electrophoresis of the HEPES-folded protamine and the Tris-folded protamine samples, it was revealed that the first peak contained the higher mobility (monomer) protein species while the second peak contained the lower mobility (dimer) species. The lower mobility species appeared to represent dimers of folded protamine monomers. These conformations appeared to be stable in the 2.5M urea used in the separating gel and apparently were stabilized by disulfide crosslinks. These gels demonstrated the heterogeneity of these samples.

Effect of DTT and GuHCl on protamine fluorescence

Protamine fluorescence was monitored in the presence of a reducing agent (DTT) and a denaturant (GuHCl).

The disulfides in protamine can be cleaved reversibly by treating them with a reagent such as DTT, which forms mixed disulfides with cysteine side chains. In the presence of a large excess of DTT, the mixed disulfides are reduced, so that the final product is a protein in which

Figure 13A.

Gel electrophoresis of HEPES-folded protamine.

A densitometer scan of bull protamine electrophoresed on acid-urea gel is demonstrated that the fastest migrating species (on the right) was the most folded form and accounted for 67% of the total sample. The y-axis was the absorbance of the amido-schwarz stained protein bands and the x-axis was the mobility (mm).

Figure 13B.

Gel electrophoresis of Tris-folded protamine.

A densitometer scan of bull protamine electrophoresed on acid-urea gel is demonstrated that the fastest migrating species (on the right) was the most folded form and accounted for 42% of the total sample. The y-axis was the absorbance of the amido-schwarz stained protein bands and the x-axis was the mobility (mm).

the disulfides (cystines) are fully converted into sulfhydryls (cysteines).

Native protamine (reduced form)

Figure 14 showed the relative fluorescence of the reduced protamine in the presence of various concentrations of DTT and GuHCl.

The reduced protamine showed higher fluorescence in the absence of GuHCl and presence of 10 μ M and 100 μ M of DTT compared to the HEPES-folded and Tris-folded protamine (Figure 12).

Figure 14 suggested that some folding existed even with the completely reduced protamine at 0M GuHCl compared to the protamine sample in the presence of 2M GuHCl. There was a 30% increase with both the 10 μ M and 100 μ M DTT samples which meant that some unfolding might be taking place.

HEPES-folded protamine

Figure 15 showed a comparison of relative fluorescences of HEPES-folded protamine incubated with varying concentrations of DTT and GuHCl. In the absence of DTT, disulfide bonds would be retained and thus the HEPES-folded protamine (Figure 15, line a) would show slightly lower fluorescence readings compared to the DTT treated samples (Figure 15, lines b-d). In the absence of multiple

Figure 14.

Relative fluorescence of reduced protamine in various [DTT]

The samples were all reduced protamine in the following DTT concentrations: 0, 10 or 100μM.

Linear least square regression analysis gave:

RED. PR -DTT: $y = 0.323 + 1.475 \times 10^{-2}x$
 $R^2 = 0.911.$

RED.PR 10μM DTT: $y = 0.333 + 4.384 \times 10^{-2}x$
 $R^2=0.946.$

RED.PR 100μM: $y = 0.323 + 3.287 \times 10^{-2}x$
 $R^2 = 0.774.$

where R^2 was the correlation coefficient. The standard deviation of three measurements was shown by the error bars.

Figure 15.

Relative fluorescence of HEPES-folded protamine in various [DTT]

[HEPES-folded protamine] = 9.20 μM
64 μM cysteine and yielded molar ratios of 0.5, 1.5 and excess DTT/protamine cysteines.

Linear least square regression analysis gave:

NO DTT: $y = 6.946 \times 10^{-2} + 6.901 \times 10^{-3}x$
 $R^2 = 0.890$.

32 μM DTT: $y = 7.442 \times 10^{-2} + 3.836 \times 10^{-3}x$
 $R^2 = 0.374$.

96.6 μM DTT: $y = 7.878 \times 10^{-2} + 5.402 \times 10^{-3}x$
 $R^2 = 0.309$.

500 μM DTT: $y = 8.999 \times 10^{-2} + 8.183 \times 10^{-3}x$
 $R^2 = 0.550$.

where R^2 was the correlation coefficient.

measurements, it was not clear if this was a significant decrease in fluorescence. Under the conditions employed here, it was not sufficient to obtain complete reduction as compared with data shown in Figure 14.

Tris-folded protamine

Solubilization of lyophilized reduced protamine in Tris-HCl buffer at pH 8, gave rise to spontaneous folding and formation of very stable disulfide crosslinks.

Figure 16 showed the relative fluorescence of Tris-folded protamine alone and Tris-folded protamine treated with dithiothreitol (3.6 or 10.8 μ M). When these samples were treated with increasing concentrations of GuHCl from 0-2M, a 23%, 51% and 72% change of relative fluorescence was observed, respectively. However, there was significant scatter in this data. Again, there appeared to be incomplete reduction and denaturation under these conditions (see Figure 14).

PEPTIDE RESULTS

Figure 17A and B showed the acid-urea gel profiles of the N- and C-terminal peptides. The N-terminal peptide showed greater than 90% oxidation (Figure 17A). The C-terminal peptide was only 50% oxidized (Figure 17B). The use of the individual peptides allowed the dissection of the two

Figure 16.

Relative fluorescence of Tris-folded protamine in various [DTT]

[Tris-folded protamine] = 1.02 μM
 7 μM cysteine and molar ratios of 0.5 and 1.5
 DTT/protamine cysteines.

Linear least square regression analysis gave:

NO DTT: $y = 0.184 + 1.846 \times 10^{-2}x$ $R^2 = 0.859$.

3.6 μM DTT: $y = 0.148 + 4.123 \times 10^{-2}x$
 $R^2 = 0.893$.

10.8 μM DTT: $y = 0.178 + 4.574 \times 10^{-2}x$
 $R^2 = 0.744$.

where R^2 was the correlation coefficient.

Figure 17A.

Acid-urea gel of oxidized and reduced N-terminal peptides.

The direction of electrophoresis was from right to left.

Lane 1-2. Amino-terminal peptide (- β mercaptoethanol).

Lane 3. Amino-terminal peptide (+ β mercaptoethanol).

Figure 17B.

Acid-urea gel of oxidized and reduced C-terminal peptides.

The direction of electrophoresis was from right to left.

Lanes 1-2. Carboxy-terminal peptide (50% oxidized).

Lanes 3-5. HPLC purified carboxy-terminal peptide.

domains which contained tyrosine, the N- and C-terminal peptides. The effect of denaturation on the intrinsic fluorescence was measured. Reduced and oxidized peptides were compared.

Amino-terminal peptide

Figure 18 showed a comparison of the relative fluorescence of the oxidized N-terminal peptide in the presence and absence of DTT, and increasing concentrations of GuHCl. The oxidized N-terminal peptide in the absence of DTT was in a folded state as judged by gel electrophoresis. It showed a lower relative fluorescence with increasing concentrations of GuHCl as compared to the oxidized N-terminal peptide in the presence of DTT. A 83% increase was observed in the relative fluorescence with the oxidized N-terminal peptide in the presence of DTT as the GuHCl was increased from 0 to 2M. In the absence of GuHCl, the relative fluorescence was comparatively low in spite of an excess molar ratio of DTT/protamine cysteine used to bring about the reduction. It was not until an excess concentration of GuHCl was reached where denaturation or unfolding had occurred sufficiently to yield an increase in relative fluorescence (see Figure 19, reduced N-terminal peptide).

Figure 19 showed a comparison of the relative

Figure 18.

Relative fluorescence of oxidized N-terminal peptide in presence and absence of DTT.

[Oxidized N-terminalpeptide] = $1.96\mu\text{M}$
 $13.7\mu\text{M}$ of cysteine and molar ratio of excess
 DTT/protamine cysteines.

Control:[Oxidized N-terminal peptide] =
 $1.96\mu\text{M}$; NO DTT.

Linear least square regression analysis gave:

$$\text{N/Ox (+DTT): } y = 0.199 + 7.066 \times 10^{-2}x$$

$$R^2 = 0.995.$$

$$\text{N/Ox (-DTT): } y = 0.165 + 2.667 \times 10^{-2}x$$

$$R^2 = 0.936.$$

where R^2 was the correlation coefficient.
 Error bars were the standard deviations of
 three separate experiments.

Figure 19.

Relative fluorescence of
reduced N-terminal peptide (+ DTT)
and oxidized N-terminal peptide (-DTT).

[Reduced N-terminal peptide] = $1.96\mu\text{M}$
 $13.7\mu\text{M}$ of cysteine and molar ratio of excess
 DTT/protamine cysteines.

Control: [Oxidized N-terminal peptide] =
 $1.96\mu\text{M}$; NO DTT.

Linear least square regression analysis gave:

$$\text{N/Red (+DTT): } y = 0.255 + 4.943 \times 10^{-2}x$$

$$R^2 = 0.981.$$

$$\text{N/Ox (-DTT): } y = 0.165 + 2.667 \times 10^{-2}x$$

$$R^2 = 0.936.$$

where R^2 was the correlation coefficient.
 Error bars were the standard deviations
 observed for three separate experiments.

fluorescence of the reduced amino-terminal peptide in the presence of DTT and the oxidized amino-terminal peptide in the absence of DTT and increasing concentrations of GuHCl. The reduced N-terminal peptide (Figure 19) showed a higher relative fluorescence compared to the oxidized N-terminal peptide in the presence of DTT, even in the absence of GuHCl (Figure 18). The reduced N-terminal peptide had the tyrosine residues exposed which gave rise to an increase in fluorescence. The reduced N-terminal peptide showed a 30% increase when GuHCl was increased from 0 to 2M in the presence of DTT compared to the 83% increase in the oxidized N-terminal peptide in the presence of DTT and GuHCl. DTT and GuHCl conditions were sufficient to achieve the same extent of unfolding or exposure to the solvent in the oxidized N-terminal peptide and hence achieve the same relative fluorescence as the reduced N-terminal peptide in 2M GuHCl (Figure 18 and 19).

The oxidized N-terminal peptide, which served as the control, showed lower relative fluorescence in the absence of DTT compared to the reduced N-terminal peptide in the presence of DTT. In the absence of GuHCl, the control showed a lower relative fluorescence value compared to the reduced N-terminal peptide in the presence of DTT. The control still showed lower fluorescence values even with increasing concentrations of GuHCl. The control showed a

35% increase in fluorescence at 2M GuHCl.

Carboxy-terminal peptide

Figure 20 showed a comparison of the relative fluorescence of the oxidized C-terminal peptide in the presence and absence of DTT and increasing concentrations of GuHCl. In the absence of GuHCl, the oxidized C-terminal peptide in the presence and absence of DTT showed very similar relative fluorescence values. There was not a significant difference between them. In the absence of DTT, the oxidized peptide showed a 29% increase in the relative fluorescence when GuHCl concentration was increased from 0 to 2M. The oxidized C-terminal peptide in the presence of DTT showed a 60% increase in relative fluorescence when the concentration of GuHCl was increased from 0 to 2M. Thus even in the presence of DTT there was some resistance to the opening of the molecule.

Figure 21 showed a comparison of the relative fluorescence of reduced C-terminal peptide in the presence of DTT and oxidized C-terminal peptide in the absence of DTT and increasing concentrations of GuHCl. In the absence of GuHCl, the oxidized peptide showed a significantly lower relative fluorescence compared to the reduced C-terminal peptide in the presence of DTT. This may be due to an unfolded structure of the reduced peptide which would give

Figure 20.

Relative fluorescence of oxidized C-terminal peptide in presence and absence of DTT.

[Oxidized C-terminal peptide] = $2.85\mu\text{M}$
 $19.95\mu\text{M}$ cysteine and molar ratio of excess
 DTT/protamine cysteines.

Control: [Oxidized C-terminal peptide] =
 $2.85\mu\text{M}$; NO DTT.

Linear least square regression analysis gave:

$$\text{C/Ox (+DTT): } y = 0.146 + 4.035 \times 10^{-2}x$$

$$R^2 = 0.988.$$

$$\text{C/Ox (-DTT): } y = 0.149 + 1.974 \times 10^{-2}x$$

$$R^2 = 0.948.$$

where R^2 was the correlation coefficient.
 Error bars were the standard deviations
 observed for three separate experiments.

Figure 21.

Relative fluorescence of
reduced C-terminal peptide (+ DTT)
and oxidized C-terminal peptide (-DTT).

[Reduced C-terminal peptide] = $2.85\mu\text{M}$
 $19.95\mu\text{M}$ of cysteine and molar ratio of excess
 DTT/protamine cysteines.

Control: [Oxidized C-terminal peptide] =
 $2.85\mu\text{M}$; NO DTT.

Linear least square regression analysis gave:

$$\text{C/Red (+DTT): } y = 0.289 + 7.271 \times 10^{-2}x$$

$$R^2 = 0.944.$$

$$\text{C/Ox - DTT: } y = 0.149 + 1.974 \times 10^{-2}x$$

$$R^2 = 0.948.$$

where R^2 was the correlation coefficient.
 Error bars were the standard deviations
 observed for three separate experiments.

rise to an exposed tyrosine residue and yield an increase in the fluorescence. This was in contrast to the oxidized C-terminal peptide in the presence or absence of DTT which yielded comparable fluorescence values at 0M GuHCl (Figure 20).

A 61% increase in relative fluorescence was observed in the reduced C-terminal peptide when the concentration of GuHCl was increased from 0M to 2M in the presence of DTT. This was a significant change compared to oxidized peptide. This change was also significant when compared to the oxidized C-terminal peptide in the presence of DTT.

Figures 20 and 21 showed that DTT and GuHCl conditions were not sufficient to cause the oxidized C-terminal peptides in the presence of DTT to reach the same fluorescence as the reduced C-terminal peptides in the presence of DTT. These conditions were not sufficient to unfold the peptide or increase the exposure of its tyrosine to solvent and thus cause a corresponding increase in the fluorescence.

DISCUSSION

Analyses of protamine disulfide formation in vivo have indicated that approximately half of the disulfides formed in bull and mouse sperm represent intramolecular crosslinks that form in the testis and caput epididymis. By progressively reducing the disulfide crosslinks in intact chromatin and mapping the location of each disulfide using HPLC peptide mapping and protein sequencing techniques, it has been shown that both ends of the molecule are folded in toward its center and locked in place by a single disulfide bridge. The three remaining cysteines in each protamine crosslink adjacent protamine molecules together, around the DNA helix, through three specific intermolecular disulfide bridges. In this manner, bull sperm DNA is packaged in a highly condensed, biochemically inert state by a single chromosomal protein (4).

These studies demonstrated that protamine folding also occurs in vitro and suggested that the fundamental mechanisms driving this process in vitro may be the same ones that operate in vivo. The rapid rate at which folding and disulfide formation occurred was consistent with the extremely small size of protamine and short distances that separate cysteine residues within the molecule (6). Bull protamine is a small protein only 50 amino acids in length. At neutral pH, 86% of the amino acids in the central,

arginine-rich half of the molecule are positively charged. As a result, this region of the molecule should be maintained in a fully extended conformation in solution. Because of its length, this extension appears to prevent the formation of disulfide crosslinks between cysteine residues located in opposite ends of the molecule. The cysteines that participate in disulfide formation are only 7-15 amino acids apart and reside within amino and carboxyl-terminal domains that are 10-21 amino acids in length.

Tyrosine standards were employed to determine relative fluorescence of bull protamine. It was important to evaluate the effects of various agents like DTT and GuHCl on tyrosine fluorescence in the absence of any protein structure. Our studies employed DTT, a reductant, at concentrations less than 100 μ M. Only at higher concentrations (1 and 5mM) was quenching of tyrosine fluorescence observed. Therefore, only DTT concentrations less than 100 μ M were employed with protamine, and any change in the fluorescence thus could be attributed to protamine structural changes.

GuHCl is a denaturant which also increases the viscosity of solutions. The effect of viscosity on free tyrosine fluorescence in the absence of any protein structural effects was determined. A slight increase (10%) was observed and was thought to be a reflection of viscosity

changes. When GuHCl was mixed with protamine, whatever fluorescence change was observed could be interpreted to be due to the presence of protamine. Thus, higher changes in fluorescence with GuHCl and protamine may reflect structural changes such as unfolding. Another control which could be employed to test viscosity changes and the effect on protamine fluorescence would be to employ a non-denaturant such as glycerol and determine its effect upon tyrosine and protamine fluorescence.

Electrophoresis is frequently used as a tool for monitoring the formation of protein complexes and protein folding. In acid-urea gel where the mobility of the protein depends on both the physical size of the molecule and its charge density, protein folding may be visualized directly by changes in the electrophoretic mobility of the protein, since the charge density of the protein does not change significantly as a result of folding.

When mammalian protamines were dissolved in aqueous buffers at neutral or alkaline pH, these proteins folded spontaneously and formed both intra- and intermolecular disulfide crosslinks that stabilized several different structures. In the absence of reducing agents, the crosslinked, folded protamines may be visualized and quantitated by gel electrophoresis. Using this technique, it was demonstrated that bull protamine folded in solution, and

a variety of factors that affect this process was described (Balhorn et al submitted). Folding did not appear to occur within the central polyarginine domain of the molecule, but involved only the N-terminal and C-terminal ends of the protein. The appearance of folded structures was shown to be eliminated by alkylating the protein's cysteine residues.

The studies reported here employed ethyleneimine modification of protamine cysteines which eliminated the possibility of disulfide formation. Modification of the cysteine residues in the protein by ethylenimine, produced electrophoretic species of increased mobility upon electrophoresis in acid-urea gels (Figure 11). Conversion of the cysteine residues in the proteins to S-(2-aminoethyl) cysteine increased the cationic charge density on the protein species, thus increasing their electrophoretic mobilities.

A comparison of the relative fluorescence of the aminoethylated, HEPES-folded and the Tris-folded protamine was done in the presence of GuHCl (Figure 12). In our studies the amino-ethylated protamine which was not capable of establishing disulfide bonds had the highest fluorescence even in the absence of any denaturant. This indicated that the tyrosine may fluoresce more in an unfolded conformation. However, it may be possible that either the aminoethylation itself or the lack of disulfides may have caused the

increase in fluorescence. Tris-folded and HEPES-folded protamine showed less fluorescence compared to the aminoethylated protamine. Comparison between the two oxidized samples was difficult due to the complex mixture found in each (Figure 13A and Figure 13B). It was apparent that different folded species in the Tris-folded and the HEPES-folded samples existed. This heterogeneity was in agreement with results in the literature (Figure 4).

Progressive disulfide reduction and crosslinking experiments have revealed that each protamine molecule in bull sperm participates in the formation of two intramolecular and three intermolecular disulfide crosslinks. All five crosslinks have been identified by titrating the disulfides of intact bull sperm with dithiothreitol and following the progressive carboxymethylation of paired cysteine residues with radiolabeled sodium iodoacetate. The derivatization of each cysteine was monitored as a function of DTT concentration by a combination of HPLC peptide mapping and protein sequencing. The least-stable bull protamine disulfide was reduced at a DTT/cysteine ratio of 0.3 while the most-resistant disulfide required a DTT/cys ratio in excess of 2.0 (4).

Hence in our studies, we wanted to see how fluorescence was affected by varying DTT/cys concentrations

in both HEPES-folded and Tris-folded protamines. It was observed that under these conditions both protamines showed incomplete reduction and denaturation (Figures 15 and 16). The Tris-folded (Figure 16) data showed much scatter. Chemically synthesized peptides (both N-terminal and C-terminal) obtained from Lawrence Livermore National Laboratory were used to obtain homogeneous samples for fluorescence analysis rather than repeat the experiments with heterogeneous and complex protamine samples. There were two changes from the bull sequence which included an alanine for cysteine in position 5 in N-terminal peptide and position 38 in the C-terminal peptide. By this change, only one possible intramolecular disulfide could form.

The acid-urea gel profiles (Figure 17A and B) of the peptides revealed that the N-terminal peptide was >90% oxidized whereas the C-terminal peptide was only about 50% oxidized.

At least a 2-fold excess of the DTT/cysteine concentration was used to ensure complete reduction. The oxidized N-terminal peptide in the absence of DTT was folded as judged by gel electrophoresis (Figure 17A) and showed lower relative fluorescence with increasing concentrations of GuHCl when compared to the oxidized N-terminal peptide in the presence of DTT (Figure 18). In the absence of denaturant, the relative fluorescence of the oxidized N-

terminal peptide was comparatively low in spite of an excess molar ratio of DTT/protamine cysteine used to bring about the reduction. It was not until an excess concentration of GuHCl was reached that denaturation or unfolding had occurred sufficiently to yield an increase in relative fluorescence. The reduced N-terminal peptide showed a higher relative fluorescence compared to the oxidized N-terminal peptide in the presence of DTT, even in the absence of denaturant (Figure 19). This indicated that the reduced N-terminal peptide was already partially unfolded, exposing the tyrosine residues giving rise to an increase in fluorescence. Yet additional unfolding (30% increase in fluorescence) appeared to take place as the concentration of denaturant was increased (Figure 19). A much larger change in fluorescence and presumably unfolding occurred in the oxidized N-terminal peptide (83%). It was important to note that both reduced and oxidized N-terminal peptide achieved the same relative fluorescence after addition of DTT and denaturant, suggesting that the same unfolded state was achieved.

The analysis of the C-terminal peptide was complicated by incomplete oxidation; only 50% of the peptides were oxidized as judged by gel electrophoresis (Figure 17B). Under the same reaction conditions over 90% of the N-terminal peptides were oxidized. Yet these two

oxidized samples yielded very similar relative fluorescence in the absence of reductant (Figure 20 and Figure 18) (about 29% and 34% increase in 2M GuHCl, respectively). Even though only half the C-terminal peptide sample was oxidized and stabilized by disulfide bonds, the sample as a whole was quite resistant to structural changes. The presence of DTT and 2M GuHCl was not sufficient to open the structure to the same extent as the reduced C-terminal peptide (Figure 21).

In the absence of denaturant and reductant, the relative fluorescence of the oxidized C-terminal peptide was very close to the relative fluorescence of the C-terminal peptide in the absence of denaturant and presence of reductant. It was not until an excess concentration of GuHCl was reached that denaturation or unfolding had occurred sufficiently to yield an increase in relative fluorescence (Figure 20).

The oxidized C-terminal peptide showed lower fluorescence than the reduced C-terminal peptide (Figure 21) and both oxidized and reduced N-terminal peptides (Figures 18 and 19), which indicated a strong resistance to the opening of the molecule even in the presence of DTT. The C-terminal peptide appeared to be a tighter domain compared to the N-terminal peptide.

The DTT and GuHCl conditions were not sufficient

to cause oxidized C-terminal peptides to reach the same fluorescence as the reduced C-terminal peptides. This indicated that the conditions were not sufficient to completely unfold or increase the exposure to the solvent. Yet in the case of the N-terminal peptide the conditions were sufficient.

CONCLUSION

In conclusion, the results of our experiments revealed that the C-terminal peptide appeared to be a tighter domain compared to the N-terminal peptide. In the absence of reducing agents, the folded peptides were visualized and quantitated by gel electrophoresis. It was observed that the C-terminal peptide was only 50% oxidized, whereas under the same reaction conditions over 90% of the N-terminal peptides were oxidized. Yet both of these oxidized samples in the absence of reductant yielded very similar relative fluorescence.

The oxidized C-terminal peptide showed lower fluorescence than the reduced C-terminal peptide and both the oxidized and reduced N-terminal peptides in the presence of both DTT and GuHCl. This indicated that even though only half the sample was oxidized and stabilized by disulfide bonds, the sample as a whole was quite resistant to structural changes. This meant that there was a strong resistance in the opening of the molecule and that both the reductant and denaturant conditions were insufficient to completely unfold or increase the exposure of its tyrosine to the solvent. Yet these conditions were sufficient to open the structure in the case of the N-terminal peptide.

Work in progress has indicated that oxidation of

the C-terminal peptide under different conditions (methanol/water mixtures) may yield a higher amount of oxidized peptide. Future studies could involve

1. Purifying by HPLC a completely folded C-terminal peptide.
2. Trying the new methanol water mixture folding technique on intact protamine molecules.
3. Trying these experiments by changing the incubation conditions for exposure to denaturant and reductant.
4. Using another technique like circular dichroism to analyze the type of structural changes which may be occurring under various reductant and denaturant concentrations.

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