1998

DNA-Protamine interactions

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DNA-PROTAMINE INTERACTIONS

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

Sujata Singh

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ABSTRACT
DNA-PROTAMINE INTERACTIONS
by Sujata Singh

Methodology was developed to study protamine-DNA interactions using 1,1'-carbonyldiimidazole (CDI) activated resin. The method involves immobilization of protamine onto CDI activated resin by carbamate linkage, followed by interaction of ³²P-labeled 12 bp DNA oligomer with protamine. The effect of pH on the immobilization reaction was monitored by a functional DNA binding assay. The relative binding affinity of bull P1 (mammal) and clupeine Z (fish) protamine with DNA has been determined by measuring the dissociation rate constant. A fast dissociation of DNA from bull P1 in an initial hour of dissociation assay indicates the presence of more than one type of complex. The dissociation rate constants for one and four hours were calculated as 10.23×10^{-3} and $2.47 \times 10^{-3} \text{ min}^{-1}$, respectively. However, dissociation rate measurements for DNA-clupeine Z complexes suggest the existence of only one type of complex.

Dedicated

to

My Grandmother

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LIST OF ABBREVIATIONS

One Letter Symbols of the Amino Acids

A	Alanine	M	Methionine
C	Cysteine	P	Proline
F	Phenylalanine	Q	Glutamine
G	Glycine	R	Arginine
H	Histidine	S	Serine
I	Isoleucine	T	Threonine
K	Lysine	V	Valine
L	Leucine	Y	Tyrosine

LIST OF ABBREVIATIONS

Reagents and Other Common Terms

ATP	Adenosine 5'-triphosphate
AFM	Atomic force microscopy
bp	Base pair
CDI	1,1'-Carbonyldiimidazole
cpm	Counts per minute
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IR	Infrared
k₂	Dissociation rate constant
NaCl	Sodium chloride
ODMR	Optical detected magnetic resonance
TAPS	N-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid
TP	Transition protein
TEM	Transmission electron microscope
TRIS	Tris[hydroxymethyl]aminomethane
UV	Ultraviolet

DNA-PROTAMINE INTERACTIONS

INTRODUCTION

Nuclear Proteins of Somatic Cells

Most of the genetic material in a eukaryotic cell nucleus is packaged into chromatin, a form of nucleoprotein. More than a century ago, Friedrich Miescher successfully isolated a material rich in phosphorus from the pus cell nuclei which he termed as "nucleins" (1). Later, "nuclein" was established as nucleic acid due to its strong acidic nature. All living cells, regardless of their origin, require deoxyribonucleic acid (DNA) as genetic material to replicate themselves.

In somatic cell nuclei, the packaging of DNA is directed by a group of five basic nuclear proteins (H1, H2A, H2B, H3 and H4) commonly known as histones (2). The discovery of histones is associated with the work of Kossel (1, 3) who isolated these basic proteins from goose erythrocytes in 1884. Histones are lysine and/or arginine rich small proteins made up of 100-200 amino acid residues. They lack tryptophan residues in their primary structure. The primary function of these binding proteins is to package DNA in a more compact and manageable form in the nucleus (2).

In chromatin (2), two polypeptide chains of each group, except H1 histone, organize as an octamer around which double stranded DNA encircles. The DNA-histone complex forms a nucleosome core which is attached to another nucleosome by linker DNA. H1 histone either binds tightly to the nucleosome in transcriptionally inactive chromatin or is attached loosely or is absent in transcriptionally active chromatin (4).

Nuclear Proteins of Sperm Cells

The genetic material in the nuclei of mature sperm of most vertebrates is packaged in a highly condensed state by a variety of basic proteins different than normal histones of somatic cells (5). It was also Miescher (1, 6) who isolated these DNA binding proteins more than a century ago as a nitrogenous base from the sperm nuclei of Rhine salmon along with "nuclein" and named them as protamine. Later, Kossel (1, 7) proved polypeptide nature of the protamines based on their behavior in the presence of proteolytic enzymes. The normal histones of sperm nuclei are replaced by protamines in the late stages of spermatogenesis (8), a process of sperm maturation. Protamines are sperm specific highly basic proteins, rich in arginine. These are found to have the simplest amino acid composition as compared to other proteins. Since their isolation by Miescher (6), protamines have been identified in most vertebrates including fishes (1), birds (9), reptiles (10) and mammals (11).

Expression of Protamines

In the testis of sexually mature vertebrates, spermatogenesis involves a series of highly complex processes in which haploid spermatozoa are produced from a diploid stem cell (8). The production of spermatozoa occurs mainly in three steps: (i) stem cell differentiation into germ cells and replication; (ii) meiosis, a process of chromosome pairing; and (iii) spermiogenesis. During spermiogenesis, a haploid round shaped nucleus of sperm cell changes into a transcriptionally inactive, elongated structure containing highly condensed chromatin (8). The packaging of nucleoprotein in sperm cell nuclei is approximately 40-fold more as compared to the chromatin of somatic cell nuclei (12). This is an extremely complicated process guided by several basic nuclear

condensation of genetic material and changes in the shape of the sperm nucleus, there is a formation of a tail in the late step of spermiogenesis (8).

In the initial phase of mammalian spermatogenesis, the normal histones of stem cell nucleosome are first replaced by a group of testis-specific nuclear proteins known as transition proteins (TPs) (8, 13). TPs are the first group of nuclear proteins which are expressed during spermatogenesis and facilitate condensation of chromatin. Four types of transition proteins, namely TP1, TP2, TP3 and TP4, are expressed in the rat. The sequence analysis (14) of cDNA showed that rat TP1 is a small basic protein of 54 amino acids containing 50% arginine and lysine. A partial characterization of rat (8) TP2 and TP4 showed the presence of cysteine in contrast to the lack of cysteine in TP1 in addition to about 30 % basic amino acids. No amino acid sequencing data is available for rat TP3. TPs are eventually replaced by newly synthesized protamines. TPs are not identified in lower vertebrates, e.g. rainbow trout (15), where the transition of histones to protamines occurs directly.

Studies on mouse and rat nucleoprotamine complexes (16,17) showed resistance to sonication in late spermiogenesis which indicates expression of protamines in late stages of spermiogenesis. It was also discovered in case of rainbow trout (18), and several mammals, e.g. rat (19), bull (20) and human (21), that protamines are postsynthetically modified by phosphorylation shortly after expression. The phosphorylation of protamines occurs by binding of phosphate to hydroxyl groups of serine and threonine residues. It was suggested that phosphorylation increases the negative charge of protein and thus it allows correct binding of protamine to the DNA backbone (5).

Electron microscopic (EM) studies carried out by Pogany and coworkers (12) demonstrated that the volume of sperm DNA and sperm nuclei is almost equal. This finding indicates that the packaging of DNA in sperm cell is highly condensed. Later, analyses of bull and mouse sperm using atomic force microscopy (AFM) (22) showed that chromatin in sperm nuclei is extremely hydrated and the volume of the nucleus may be more than that found in previous studies (12). More recent AFM studies (23) of reconstituted sperm chromatin determined that DNA is closely packed as nodules of DNA-protamine aggregates. When these nodules were further imaged by transmission electron microscope (TEM) to get insight about arrangement of DNA-protamine complex, the nodules were distinguished as 200 Å thick "DNA-protamine toroids" having a hole of 150 Å in diameter. It was suggested that these DNA-protamine toroids are the basic packing unit of sperm chromatin. The unique compact packaging of DNA in sperm nuclei by protamines makes it metabolically and transcriptionally inert (24) and protects it from enzymatic cleavage (25). It is found that almost 85 % of DNA is packed in human sperm chromatin by protamines (26).

Types of Protamines

Although the presence of protamines with DNA in sperm nuclei was described a century ago, most of the earlier work was limited to fish protamine. Until the 1950s, very few reports were cited in the literature in which protamines were found in sperm of other organisms. Fish protamines are the simplest basic proteins containing a limited amino acid composition with high arginine content. As depicted in Figure 1, amino acid sequences of various fish protamines (1, 5) show that these are small proteins containing 5-7 types of amino acids with no cysteine residues. The N-terminus is more

	1	10	20	30
IRIDINE 1a (Rainbow Trout) (5)	PRRRRASRRV	RRRRRPRVSR	RRRRGGRRRR	
SALMINE (Chum Salmon) (5)	ARRRRSSSRP	VRRRRRPRVS	RRRRRRAGRR	RR
CLUPEINE Y I (Herring) (1)	ARRRRSSSRP	IRRRRPRRRT	TRRRRAGRRR	R
CLUPEINE Y II (Herring) (1)	PRRRTRRASR	PVRRRRPRRV	SRRRRARRRR	
CLUPEINE Z (Herring) (1)	ARRRRSRRAS	RPVRRRRPRR	VSRRRRARRR	R

Figure 1: Complete amino acid sequences of different species of fish protamines.
(Numbers in parenthesis are reference numbers)

or less conserved either with an alanine or a proline followed by a cluster of 3-4 arginine residues.

Initially, fish protamines (1) were classified into three groups depending on the types of basic amino acids present in the primary sequence. For example, clupeine, iridine and salmine protamine contain only arginine as the basic amino acid (Figure 1), and they are known as monoproamines. However, diproamines contain arginine and either lysine (e.g. cyprinine and barbine) or histidine (e.g. percine and lacustrine). Sturine, an example of triproamines, contain both basic amino acids in addition to arginine.

The research work carried out on these remarkable basic proteins in last two decades has been focused primarily on mammalian protamines. At least, two types of protamines, namely P1 and P2, are found in mammals (27-30). Protamine P1 is present in all the mammals and is rich in cysteine and tyrosine in addition to arginine. P2 protamine is rich in cysteine and histidine. Electrophoretic and chromatographic studies carried out on bull, ram, rabbit, boar and rat showed the presence of only P1 in their sperm chromatin. However, sperm cells of mouse, hamster and human contain both P1 and P2 (29).

Complete amino acid sequences of P1 protamine found in several mammal species (11, 29, 31-36) are summarized in Figure 2. It is evident from Figure 2 that P1 protamine is only 50 amino acids long with an arginine rich central region. The another noticeable feature is the presence of a highly conserved N-terminal hexapeptide sequence (ARYRCC) and a nonconserved carboxyl terminus in all the cases. It is also revealed from primary sequence analysis that P1 protamines have three distinct structural domains (29), a basic central core flanked both sides with less basic amino-

	1	10	20	30	40	50
BULL (11)	ARYRCCLTHS	GSRCRRRRR	RCRRRRRRFG	RRRRRVCCR	RYTVIRCTRQ	
RAM (31)	ARYRCCLTHS	RSRCRRRRR	RCRRRRRRFG	RRRRRVCCR	RYTVIRCTRQ	
GOAT (32)	ARYRCCLTHS	RSRCRRRRR	RCRRRRRRFG	RRRRRVCCR	RYTVIRCTRQ	
RAT (32)	ARYRCCRSKS	RSRCRRRRR	CRRRRRCCR	RRRRCCRRR	RSYTFCKRY	
HAMSTER (29, 33)	ARYRCCRSKS	RSRCRRRRR	CRRRRRCCR	RRRRCCRRR	RTYTLRCKRY	
MOUSE (34)	ARYRCCRSKS	RSRCRRRRR	CRRRRRCCR	RRRRCCRRR	RSYTIRCKY	
BOAR (35)	ARYRCCRSKS	RSRCRPRRR	CRRRRRCCP	RRRRRAVCCR	RYTVIRCRRC	
HUMAN (36)	ARYRCCRSQS	RSRYRQRQR	SRRRRRRSC	TRRRAMRCCR	PRYRPRCRRH	

Figure 2: Complete amino acid sequences of different species of mammalian P1 protamines.

and carboxyl-termini (Figure 3). The central arginine rich core is proposed by Balhorn (37) as a DNA binding site due to the high positive charge. The N- and C-termini are comparatively arginine deficient and contain polar hydroxyl amino acid residues, such as serine, threonine and tyrosine residues, prone to phosphorylation. There are several inter-dispersed cysteine residues responsible for intra- and intermolecular disulfide cross linking (38).

Protamines P2 are larger in size with variable length and are not as conserved as P1. P2 protamines are found to have 50 % homology with P1. A literature search showed that considerable less work was carried out with P2 as compared to P1 protamines. Very few complete amino acid sequences (mouse and human) for P2 are known (29).

A comparison of Figure 1 and 2 shows that although fish protamines contain several polyarginine clusters, these are considerably simple and different from mammalian protamines in both sequence and size. Unlike fish protamines, several cysteine residues are present in mammalian protamines. Table 1 also illustrates various physical and structural differences between fish and mammalian protamines.

Most of our current knowledge, pertaining to protamines and their interaction with DNA, came from the studies of clupeine Z and bull P1, a representative of fish and mammalian protamine, respectively. These two protamines were used in the present research to study DNA-protamine interactions as well as to compare their interactions with DNA.

1 10 20 30 40 50

ARYRCCLTHS GSRCCRRRRR RCRRRRRRFG RRRRRRVCCR RYTVIRCTRQ

(--Amino-terminal domain--)(-----Arginine rich central domain*-----)(--Carboxy-terminal domain--)

Figure 3: Complete amino acid sequence of bull protamine P1 showing three structural domains (11).

Note: *proposed site for DNA binding by Balhorn (37)

Table 1: A comparison of fish and mammalian protamines.

CHARACTERISTICS	FISH PROTAMINES	MAMMALIAN PROTAMINES
MOLECULAR WEIGHT	~4200	~7200
ARGININE CONTENT	80 %	50 %
CYSTEINE CONTENT	0 %	10-20 %
PROLINE CONTENT	~10 %	0 - 4 %
HISTIDINE CONTENT	0-7 %	~20%
TYPES	Only one type per species	Two types, namely P1 and P2 (P2 has sub types: P2, P3 and P4)
DISULFIDE BONDING	Absent	Present (both intra- and intermolecular)
N- AND C- TERMINI	Not differentiated	Well differentiated

Clupeine Z

Clupeine, a monoprotonated, is a small protein of 30-31 amino acid residues containing arginine as the only basic residue (Figure 1). The 20-21 arginines are present either as a single residue or as a cluster of a maximum of four residues separated by 1-3 neutral amino acid residues. It is a heterogeneous protein of three similar components, namely Z, YI and YII. The complete amino acid sequence of all the three components was determined by Ando et al. (39-41). Clupeine Z and YI contain a primary amino group at their N-termini, whereas, the YII component carries an imino group (Figure 1). Clupeine Z is the simplest of all three components containing only five types of amino acids as compared to YI (seven types) and YII (six types). Moreover, it was the first sperm basic protein which was sequenced more than three decades ago (39).

Bull P1

Bull P1 is a small protein of 50 amino acid residues. The complete amino acid sequence (11) is given in Figure 3 which shows three structural domains. Disulfide reduction and crosslinking studies carried out by Balhorn and coworkers (38) showed that all seven cysteine residues of bull P1 lead to the formation of two intramolecular and three intermolecular disulfide bonds. In their experiments, the disulfide crosslinks were confirmed by reducing with dithiothreitol followed by carboxymethylation using iodoacetate. The intermolecular crosslinks were identified by correlating the dissociation of protamine from DNA between N,N'-ethylenedimaleimide treated sperm with partially reduced sperm. The findings based on this experiment suggest that both N- and C-terminal of each protamine molecule are folded towards the center and fastened through intramolecular disulfide bonding (Figure 4). The three intermolecular

Figure 4: Schematic representation of intra- (–) and intermolecular (→) disulfide cross-linking in bull protamine P1 (38).

Cysteine residues located at positions 6 and 39 form two intramolecular disulfides with 14 and 47, respectively. Cysteines present at positions 5, 22, and 38 are involved in three intermolecular disulfides with cysteines 22, 5 and 38, respectively, of neighboring protamines (represented as gray chain). All cysteine residues are shown as white in color.

disulfides cross-link neighboring protamines tightly around the DNA. Therefore, the key step to separate protamines from DNA, is to reduce interprotamine disulfides. Three models have been proposed to describe disulfide crosslinking between neighboring protamine chains as depicted in Figure 5.

Recently, *in vitro* studies (42) were carried out by dissolving reduced bull protamine in Tris-HCl buffer to examine the formation of intramolecular disulfide bridges. It has been observed that the N- and C-termini of protamine fold inwards within a minute by forming intramolecular disulfides between terminally located reduced cysteines at pH 8. The formation of intraprotamine disulfides were found to be catalyzed by air oxidation at alkaline pH and inhibited drastically by decreasing the pH below 7. These findings also strengthen the results of a previous study carried out by Balhorn and coworkers (38) for protamine disulfide bridges.

Proposed Models for DNA-Protamine Interactions

Over the past four decades, the field of sperm nucleoproteins has witnessed a two sided effort. Some groups were engaged in determining the primary structure of various protamines, while others were interested to learn about their interaction with the DNA helix. Atomic force microscopy (22, 23), spectroscopic techniques (43, 44), X-ray diffraction studies (45-47) and chemical modification methods (48, 49) were used in the past in an attempt to get insight about nucleoprotamine structure in sperm nuclei. Several models have been proposed which suggest protamine binding either in the major or minor groove of DNA in different conformations or simply the wrapping of protamine by DNA in supercoiled conformation. Although these models differ in important details, they share some fundamental aspects.

Figure 5: Proposed models showing intermolecular disulfide cross-linking among neighboring protamine molecules (38).

(A) Each protamine molecule aligned in alternate direction along the same DNA strand. (B) The orientation of each protamine molecule is same along both DNA strands. The formation of intermolecular disulfides between protamine molecules bound to two different DNA strands showing same direction. (C) Each protamine molecule aligned in alternate direction along the same DNA strand. The formation of intermolecular disulfides between protamine molecules bound to two different DNA strands showing either same or opposite directions. Each protamine is represented by N (amino-) and C (carboxyl-) terminus only.

Warrant and Kim (45) proposed a nucleoprotamine structure based on their X-ray diffraction studies on the tRNA-protamine complex. According to this model the protamine adopts an α -helical conformation and forms three or four domains separated by either proline, alanine or tyrosine residues. These α -helical domains occupy the major groove of double stranded DNA. This model was supported by methylation studies (48) in which the N3 atom of adenine present in the minor groove was methylated, whereas, the N7 atom of the guanine located in the major groove was protected. The α -helical configuration of protamine was not verified by deuterium exchange studies (43) which showed the protamine in an extended form.

In 1982, another model was proposed by Balhorn (37) which suggests interaction of protamine in an extended conformation with DNA to change the normal B form of DNA to the "modified B form". The neutralization of the negatively charged DNA backbone occurs by the central polyarginine region of protamine which is proposed to lie in the minor groove of DNA. Moreover, he also postulated that the individual protamine molecules are interlocked by forming inter- and intramolecular disulfides due to the presence of cysteines mostly at the N- and C-termini. The proposed binding of protamine in the minor groove of DNA was additionally supported by X-ray diffraction studies (46).

A new study by Bianchi and collaborators (49) in 1994 contradicted protamine binding in the minor groove. They investigated interactions of human protamines with DNA fragments of various sizes ranging from 48-802 bp in the presence of a well known minor groove binding dye (Hoescht 33258), followed by observing fluorescence of the complexes. The increase in fluorescence intensity suggests the absence of

protamine in the minor groove of DNA. They further confirmed their results by the DNase I footprinting assay. The same digestion pattern obtained by endonuclease in the presence and absence of protamines, suggested no change in endonuclease activity which is known to act in the minor groove of DNA. Based on both of these experiments, they proposed that protamine binds at the surface of DNA without any sequence or groove specificity. Moreover, the salt dependent binding of protamine to DNA showed that interactions are electrostatic in nature.

In 1997, Prieto and coworkers (50) employed optical detected magnetic resonance (ODMR) to study the binding location of protamine on DNA. For this purpose, they prepared a 14 amino acid long peptide model (R_6WGR_6) similar to the central protamine domain (proposed binding site for DNA) where phenylalanine was replaced with tryptophan. Double stranded poly(dABrU) in which thymine was replaced with 5-bromouracil to fit bromine in the major groove and calf thymus DNA were utilized to make a complex with the model peptide. They examined the "heavy atom effect" (HAE) of a bromine atom located in the major groove on tryptophan of the model peptide. The heavy atom induces short range interactions if a chromophore is present approximately at van der Waals distances. Therefore, if the model peptide binds in or a little above the major groove, the HAE can be observed. They determined the location of tryptophan in the peptide-poly(dABrU) complex by phosphorescence yields, radiative decay lifetimes and ODMR measurements at low temperature before and after complex formation. Based on the appearance of the HAE which is only possible when tryptophan is present in the major groove, they proposed that protamine binds in the major groove of DNA. This study supports the model for protamine binding in the major groove.

Since various species ranging from fish, fowl and bull to human have diversity in protamine size, sequence, as well as the presence of more than one type, neither model is fully applicable to the nucleoprotamine structure of all species. Further work is needed to explore different nucleoprotamine organization in sperm cells.

Methods Employed to Study DNA-Protamine Complexes

The participation of protamine in DNA organization makes *in situ* structural and conformational analysis of sperm nuclei very difficult. In spite of the productive and imaginative contributions of multiple research groups, the actual structure of the DNA-protamine complex still remains unsolved.

The analysis of DNA-protamine complexes in most of the previous studies were done by the formation or dissociation of these species in solution, followed by characterization using a physical or chemical method. Often, the DNA-protamine complexes were prepared either by mixing (51-53) DNA and protamines in buffer solutions at biological pH or by gradient dialysis (51, 52). In gradient dialysis, the protamine and DNA were dissolved in buffer containing a high salt concentration (2 M) which was gradually reduced. Various chemical techniques used to analyze interactions of protamine with DNA in their complexes were methylation of DNA bound to protamine by dimethyl sulfate(48), DNase I footprinting assay (49) and gel mobility shift assay (49). The characterization of the DNA-protamine was done using a number of physical techniques. The limitations of these techniques are now discussed.

EM (12, 53) has been used to examine packing of DNA in the sperm chromatin. However, the dense nature of nucleoprotein in sperm chromatin restricts the resolution in these studies.

AFM (22, 23), which does not depend on density, was used to study native and reconstituted mammalian sperm chromatin. Since, this technique measures surface topology, it can not be used to examine secondary structures in DNA-protamine complexes. However, as discussed earlier, AFM images (23) prove the existence of closely packed toroids of DNA-protamine aggregates.

The analysis of DNA-protamine complexes by X-ray diffraction (45-47) also provides limited information regarding secondary structures since a high resolution determination requires a single crystal of the complex.

Raman Spectroscopy (54) has been used to study complexes of DNA with salmine at 98 % relative humidity. The appearance of an amide bond at 1683 cm^{-1} has indicated the presence of an "unusual secondary structure" when protamine binds to DNA. This finding suggests the formation of a $1\rightarrow 3$ hydrogen bond inside a γ -turn and does not verify the formation of either a β -sheets or α -helix as proposed in earlier studies (45).

It is evident from the discussion above that the use of all these sophisticated physical techniques to analyze DNA-protamine complexes are restricted due to several reasons. The most noteworthy reasons are: (i) the high degree of compaction of sperm DNA by protamines, (ii) heterogeneity in types and structure of protamines in different species, (iii) the aggregation of DNA-protamine complexes in solution, (iv) the aggregation of protamines in solution, (v) difficulty in getting a single crystal of DNA-protamine complexes, and (vi) change in protamine conformation in solution and upon binding with DNA.

It is notable that in most of the previous studies, the DNA and protamines were free in solution. Therefore, both were flexible in changing conformations, interacting

with each other in different orientations, and forming aggregates, either individually (protamine-protamine) or as complexes (DNA-protamine). These difficulties can be overcome by developing a method in which either protamine or DNA is immobilized to some solid support (e.g. resin) and keeping the other component free in the solution. At least, the aggregation of one component could be prevented if sparsely attached to the resin. Mammalian protamine has aggregation problems in solution due to intermolecular disulfide crosslinking. This problem could be minimized by binding protamine to the moderately activated resins. In the past, activated resins have been used as a coupling agent to isolate proteins in affinity chromatography (55-57). 1,1'-carbonyldiimidazole (CDI) activated resin was used successfully for the isolation of trypsin (55), immunoglobins (56) and thyroid proteins (57).

Previously, activated sepharose resins, which would only bind to thiol groups, were employed as a solid support (58) to immobilize bull P1 protamine. The DNA oligomers of variable length (10-14 bp) were used to make complexes with immobilized protamine. A novel protocol was developed by Wren (58) to study DNA-protamine interactions by employing centrifugation and filtration methods. There are two main disadvantages in using thiol activated resins as solid support for immobilization of protamines. Since bull P1 contains six cysteine residues, it can couple with the resin in more than one binding location. Also, thiol resins could interfere with the process of forming native intramolecular disulfide bonds in protamines.

Later, CDI activated resins (59) were used to couple bull P1 protamine with an aim to study DNA-protamine interactions. The CDI activated resin couples smoothly with primary amino groups (55) and is comparatively inert towards O-nucleophiles (56). The CDI activated resins have two advantages over thiol activated resins. First, bull

protamine has only one N-nucleophile, a primary amino group at the N-terminus; therefore, it could bind to the CDI-activated resin with only one attachment site. Secondly, the proposed binding site of protamine and DNA is a centrally located highly positively charged arginine core which is at a distance from the binding site of protamine and CDI-activated resin. Therefore, it might be easier to study the interactions of DNA and protamine.

Specific Goals for Current Research

Poenisch (59) used CDI activated resins as support matrix for binding of protamines at pH 8.6. Unfortunately, he got only ~13 % bound DNA with immobilized protamine. Therefore, in the present study, the protamine binding to an activated resin was carried out at a higher pH. The aim was to increase the amount of attached protamine to an activated resin to get at least 30% to 50 % bound DNA.

A worthy goal of the present research was to develop a methodology to study DNA-protamine interactions by optimizing the reaction conditions for the immobilization of bull P1 onto the CDI activated resin. The use of a CDI activated resin as solid support was extended to clupeine Z, a fish protamine, to compare their binding (fish vs mammalian) with the resin as well as with DNA oligomers. A series of coupling experiments were carried out by varying protamine concentration. The amount of coupled protamine was indirectly determined by a radiolabeled DNA binding assay (58). The DNA-protamine complexes were separated by a combination of centrifugation and filtration methods. Dissociation assays were performed to determine dissociation rate constants. Finally, the nonspecific binding of DNA oligomers with resin, nitrocellulose filters and Eppendorf tubes were also examined.

The formation of a high degree of immobilized protein yielding ~ 90 % DNA binding at pH 9.0 at some protamine concentrations prompted an extension of this research to conditions at pH 8.6. The results were compared with the binding experiments carried out at pH 9.0 and with the previous study (59).

MATERIALS AND METHODS

Instrumentation

The UV absorption measurements to quantify protamine concentrations were performed on a HP 8452A Diode Array Spectrophotometer. ^{33}P radioactive decay measurements were made on a Packard 1900CA Tri-Carb Liquid Scintillation Analyzer. An IEC Centra-8R Centrifuge was used to centrifuge gel permeation columns at 1000 x g in DNA labeling procedures. Centrifugation carried out in protamine-resin immobilization and in dissociation experiments was performed on an Eppendorf Centrifuge 5415 at 14,000 x g.

Buffers

Commonly used buffers, e.g. borate and phosphate, could not be used for this study since protamine aggregates in these solutions due to its limited solubility. Since protamines are soluble in sulfonate-based buffers, TAPS and HEPES were used. Acids and sodium salts of TAPS, HEPES and TRIS were ordered from Sigma. Guanidine-HCl was purchased from ICN Biomedicals. Sodium azide (0.01 % w/v) was added in all buffers to avoid bacterial contamination.

TAPS (50 mM, pH 8.6 and 9.0) and TAPS-NaCl (50 mM NaCl, 50 mM TAPS, pH 8.6 and 9.0) were used for the substitution of protamines onto the activated resin. After protamine substitution, HEPES buffer (50 mM, pH 7.4) was used to establish the reaction medium to biological pH. TRIS buffer (2.0 M, pH 8.0) was used to deactivate any remaining carbonyldiimidazole groups of the resin. HEPES-Guanidine HCl (6M Guanidine HCl, 50 mM HEPES, pH 7.4), was used to remove uncoupled protamine

from the resin after the binding reaction. HEPES-NaCl (100 mM NaCl, 50 mM HEPES, pH 7.4) was used as the dissociation buffer.

Protamines

HPLC reversed phase purified bull protamine P1 was generously supplied by Dr. Balhorn (LLNL). Clupeine Z protamine was purchased from Sigma. The stock solutions of both protamines were made by dissolving the solid in 10 mM HCl. The concentration of protein stock solutions was determined by measuring absorptions at 230 nm for bull P1 and at 190 nm for clupeine Z. The molar extinction coefficients (1, 58), used to calculate bull P1 ($\text{MW } 6654 \text{ gm mol}^{-1}$) and clupeine Z ($\text{MW } 5000 \text{ gm mol}^{-1}$) protamine concentration, were $3.85 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $0.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively, at the above wavelengths.

Protamine has a tendency to aggregate in solution and/ or with aging of the sample. The absorption values at 280 nm and 320 nm were used to measure protein aggregation in the bull P1 stock solution. Bull P1 has UV absorption at 280 nm due to the presence of tyrosine in its primary structure. The absorption at 320 nm would be due to light scattering, mainly caused by the presence of large particles in the solution. In the case of high protein aggregation, the absorption value at A_{320} should increase which would result in a decrease in the A_{280}/A_{320} value. On the other hand, if there is minimal protein aggregation, the ratio of A_{280} and A_{320} should be high. Previously, it was observed that bull P1 with little aggregation in solution had a A_{280}/A_{320} value between 5-10, whereas it was nearly one at high aggregation (59). Since clupeine Z does not have absorption at 280 nm due to the absence of tyrosine residues, absorption at 320 nm

nm was compared with the bull P1 spectrum to measure protein aggregation in the solution.

CDI Activated Resin

The solid substrate used to bind protamines was 6 % cross-linked agarose beads activated with 1,1'-carbonyldiimidazole (CDI). The commercially available CDI activated resin beads (Reacti-Gel®, 6X) stabilized in acetone slurry were purchased from Pierce. The material has a particle size ranging from 45 to 165 μm . Figure 6 shows the activation reaction of an agarose gel matrix with 1,1'-carbonylimidazole (55, 60). The activation level of agarose beads is about 50 μmoles per mL of gel. The activated resins suspended in acetone are stable and keep full activity for several months, when stored at 5 °C. The stability of the CDI groups, covalently attached to the agarose matrix, make washing and filtration easy. At basic pH 8.5-9.0, the complete hydrolysis of the CDI groups need at least 30 hours (Reaction A, Figure 7). All specifications and conditions reported here are suggested by the vendor.

Bull protamine P1 contains one N-nucleophile as a primary amino group at the N-terminus, two O-nucleophiles as hydroxyl groups of tyrosine at positions 3 and 42, and seven S-nucleophiles as thiol groups of cysteine at positions 5, 6, 14, 22, 38, 39 and 47 in its primary sequence (Figure 3). Therefore, a good electrophile is needed to make an attachment between protamine and the resin. The imidazolyl carbamate group of CDI activated resin acts as an electrophile which smoothly couples with N-nucleophiles (55, 56) through a N-alkylcarbamate linkage between the vendor's suggested pH range 8.5-11.0. This covalent linkage is also relatively stable towards

Figure 6: The activation of agarose matrix with CDI.

Figure 7: Hydrolysis (Reaction A) and primary amine coupling (Reaction B) of CDI activated resin.

O-nucleophiles (56). No information is available regarding reactivity of the CDI group towards the S-nucleophile as compared to N-nucleophile. The coupling reaction (55, 56) of the carbonyl-diimidazole group of an activated agarose matrix with a primary amino group is given in Figure 7 (Reaction B).

Immobilization of Protamine onto CDI Activated Resin

Each protamine immobilization reaction used 200 μ L of Reacti-Gel® suspension containing CDI activated resin. Each aliquot was centrifuged for one min in a 1 mL Eppendorf tube™ and the acetone was removed by pipette. The resin paste was kept at room temperature for 15 min to evaporate much of the acetone. The volume of the moist resin paste was about 120 μ L after acetone removal.

TAPS buffer containing salt was used for initial washings. The resin was washed three times with 1.0 mL of TAPS-NaCl buffer, followed by centrifugation for 15-20 seconds. The supernatant was removed by pipette. The resin was washed three additional times with 1.0 mL TAPS buffer. Finally, ~700 μ L of TAPS buffer was added to the tube to bring the total volume to 800 μ L. At this point, 200 μ L of protamine solution in 10 mM HCl was added. The tubes containing 1.0 mL of protamine-resin solution were kept in a vertical position at 4 °C for 48 hours. The optimum pH range for coupling is 9-10 accompanied with overnight incubation (60). However, coupling at pH 7.5-9.0 can be used for the proteins sensitive to high pH. It has been observed that if less basic conditions (pH 8.0) are employed (59) coupling takes place within 42 hours.

In initial dissociation experiments (discussed later), variable amounts of bound DNA to immobilized protamine was found in duplicate runs. Therefore, in later immobilization experiments, the position of the Eppendorf tube was changed from

vertical to horizontal position in order to expose more surface area and obtain even protamine binding to the resin. Also, in later experiments, addition of protamine solution to the resin was followed by one hour of shaking in order to induce more interaction of protamine and resin. It is assumed that most of the protamine coupling with CDI-activated resin takes place within one hour of mixing both reactants.

After a 48 hour incubation, the protamine-resin solutions were centrifuged, followed by the removal of supernatant. The samples were further incubated for one hour at 4 °C with 1.0 mL of 2M Tris buffer to remove any remaining active sites (carbonyldiimidazole groups). The samples were again centrifuged and supernatant removed. Unreacted protamine was removed by treating protamine-coupled resin with 1.0 mL of HEPES-guanidine HCl buffer for five minutes at room temperature. The resulting solutions were centrifuged and the supernatant removed. The protamine-immobilized resin was washed twice with 1.0 mL of HEPES buffer. Finally, HEPES buffer was added to bring the final volume of the sample to 500 μ L. The total volume of the resin was between 100-110 μ L. The protamine-immobilized resin samples were stored at 4 °C and used within 20 days.

A series of protamine concentrations was used to vary the amount of immobilized protamine onto the activated resin. Bull P1 concentrations from 0.16 μ M to 2.56 μ M (1.06 μ g/mL to 16.96 μ g/mL) were used for immobilization reaction. The concentration of clupeine Z was varied from 0.16 μ M to 6.05 μ M (0.76 μ g/mL to 30.25 μ g/mL). The 200 μ L of CDI resin provides more than 10 μ mol of activated sites for bull P1 binding yet only 0.03-0.5 nmol of bull P1 were added to the reaction. In the case of clupeine Z, 0.02-1.21 nmol were used for the immobilization reactions. The purpose of

regulating protamine concentration was to obtain the amount of protamine at the resin surface which could yield at least 30 to 50% DNA binding (~150-250 nM bound DNA). Highly protamine-immobilized resins lead to irreversible binding of protamine-DNA complexes (58).

The coupling reaction at each protamine concentration was carried out in duplicate to check the reproducibility. One control reaction was carried out with each duplicate set by adding only 10 mM HCl to the washed resin instead of protamine solution to determine binding of DNA with the resin itself. The immobilization of protamine at each concentration was carried out at pH 8.6 and 9.0 to determine the effect of pH during the coupling of protamine with CDI-activated resin.

DNA Oligomer

A synthetic 12 base pair double stranded DNA oligomer was used in this study. It has been shown previously (58) that DNA oligomers containing 10 or less base pairs form less stable complexes with protamine. It was also observed that bull protamine P1 typically binds to about 10-12 bp long DNA segments (58).

Reverse phase high performance liquid chromatography purified single stranded DNA oligomers having a blocked 5' end were synthesized by the vendor Clontech. The nucleotide sequences of two 12 bp long complementary DNA strands were 5'TCGAATGTACAA3' and 5'TTGTACATTCTGA3' with a molecular weight of 3970 and 3954 gm mol⁻¹, respectively. As can be seen, the ratio of purine and pyrimidine bases are equal. The complementary single stranded DNAs were deblocked, annealed, and purified by reverse phase HPLC in Dr. Balhorn's lab (LLNL).

Single stranded DNA absorbs 12-40 % more UV light than double stranded DNA. This criterion was used to confirm the double stranded character of DNA oligomers by an UV hyperchromicity experiment (58). The concentration of DNA stock solution was calculated as 2.47×10^{-4} M by UV absorbance using a molar extinction coefficient of $20 \text{ cm}^2 \text{ mg}^{-1}$ ($1.58 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) at 260 nm. The stock solution was diluted with HEPES buffer to get DNA concentration of 50 pmol per μL .

DNA Labeling

$[\gamma \text{ } ^{33}\text{P}]\text{-ATP}$ was obtained from NEN Research Products (DUPONT). The specific activity of $[\gamma \text{ } ^{33}\text{P}]\text{-ATP}$ was 0.25 mCi / 0.025 ml in 10 mM tricine at a specified date given by the vendor. The ^{33}P isotope has a half life of 24.5 days. Therefore, fresh ATP was used for DNA labeling within a month.

Purified T4 polynucleotide kinase (M410A) expressed in *E. coli* cells was supplied by Promega. The concentration of enzyme was given as 10 units/ μL , where one unit of activity is defined as the amount of enzyme required to catalyze the transfer of one nmol of phosphate from $[\gamma \text{ } ^{32}\text{P}]\text{-ATP}$ to the 5'-OH end of a polynucleotide. The enzyme storage buffer contained 20 mM Tris-HCl (pH 7.5), 25 mM KCl, 2 mM DTT, 0.1 mM EDTA, 0.1 μM ATP and 50% (v/v) glycerol. Kinase buffer, 10X (Promega) which contained 700 mM Tris-HCl (pH7.6), 100 mM MgCl_2 and 50 mM DTT, was diluted 10 fold to use as the reaction buffer.

Ready-made Quickspin® columns (Nu-Clean D25 disposable spun columns) manufactured by Eastman Kodak Company were obtained from VWR. These columns contained Sephadex G-25 gel in a buffer (pH 8.0) containing 10 mM Tris HCl, 0.1 M NaCl and 1.0 mM EDTA. According to the vendor's specifications, these columns have

an exclusion limit of 10 bp DNA oligomers. Moreover, the retention of unincorporated nucleotides as well as sample recovery is more than 90 %. Prior to use, columns were washed four times with HEPES buffer (pH 7.4) followed by centrifugation (1000 x g) for two minutes to replace the buffer.

Cytoscint™ Liquid Scintillation Cocktail, purchased from ICN Biomedicals, was used for counting ³³P radioactive decay. Cytoscint™ cocktail, a mixture of linear alkylbenzene, phenyl xylylene, 2,5-diphenyloxazole, p-bis(o-methyl styryl) benzene and non-ionic surfactants, is designed especially for counting small volume aqueous solutions and nitrocellulose filters.

A modified T4 polynucleotide kinase method (61) was used for DNA labeling. The free 5'-OH ends of DNA oligomer were labeled by mixing the following contents in an Eppendorf™ tube to get 20 µL of total volume in deionized water: 2.5 µM (50 pmol/µL) DNA oligomer, 1X kinase buffer (7 µM Tris-HCl (pH7.6), 1.0 µM MgCl₂ and 0.5 µM DTT), 1.0-2.0 x 10⁻² mCi [γ ³³P]-ATP, and 15 units T4 polynucleotide kinase enzyme. The reaction mixture was incubated for 45 min in a water bath set at 37 °C. After incubation, the reaction mixture was placed in an ice bath for 30 min to reduce the enzymatic reaction. At this point, 30 µL of HEPES buffer was added to the reaction mixture to yield 50 µL. The reaction mixture was loaded onto the Quickspin® column placed on a collection vial and centrifuged. A pre and post centrifugation 1.0 µL aliquot was removed into a 20 mL scintillation glass vial containing 7.0 mL of Cytoscint™ to evaluate efficiency of the labeling of DNA oligomers. The efficiency of the labeling was calculated between 47-58 % (samples A-E, Table 2). The labeled DNA sample was collected in a clean vial and stored at 4 °C until needed.

The specific activity of the [^{33}P]DNA (samples A-E) used in all experiments was between 6.8×10^2 to 14×10^2 cpm/ng on the day DNA oligomers labeled. The specific activities of the [^{33}P]DNA samples on the specified day are given in Table 2.

Dissociation Experiment

Dissociation experiments were carried out by preparing protamine-DNA complexes in equilibrium with free DNA and free protamine, followed by a 100 fold dilution and monitoring the complex dissociation. Incubation and dissociation experiments were performed by placing an Eppendorf™ tube in a controlled water bath at 25 ± 1 °C. Each experiment was carried out in duplicate. One control reaction containing unsubstituted resin preparation was also carried out with each experiment to measure nonspecific binding. Millipore nitrocellulose filters (pore size $0.45 \mu\text{m}$) were used in association with a vacuum to filter DNA-protamine complexes from the free DNA.

Protamine-DNA complexes were prepared by incubating $120 \mu\text{L}$ of protamine immobilized resin sample with $1.0 \mu\text{L}$ of labeled DNA in a one mL Eppendorf™ tube for 60 min. Previously, It has been observed (58) that complex formation attains equilibrium within 5 min when 63 nM bull P1 and 13.7 nM DNA are used.

After an hour incubation, $10 \mu\text{L}$ of the protamine-DNA complex preparation was measured for each time point and placed in a separate Eppendorf™ tube. In each tube, $990 \mu\text{L}$ of HEPES-NaCl buffer was added followed by mixing with the help of a pipette. All the tubes were kept in a controlled water bath at 25 ± 1 °C until processed. In the first 60 min of dissociation, tubes were processed in 15 min time intervals (i.e. at t_0 ,

Table 2: Specific activities of [³³P]DNA oligomers used in all experiments.

Reference experiment No.	Specific activity on the day [³³ P]DNA oligomers labeled		Specific activity on the day [³³ P]DNA oligomers used	
	Sample No.	(cpm/ng) x 10 ²	Days	(cpm/ng) x 10 ²
1	A	18.00	7	14.97
2	A	18.00	9	14.08
3	A	18.00	14	12.29
4	A	18.00	16	11.63
5	A	18.00	16	11.63
6	B	9.80	1	9.53
7	C	6.80	2	6.47
8	D	14.00	5	12.21
9	D	14.00	5	12.21
10	A	18.00	28	8.39
11	A	18.00	28	8.39
12	B	9.80	3	9.03
13	D	14.00	6	11.89
14	D	14.00	6	11.89
15	A	18.00	3	16.58
16	A	18.00	23	9.61
17	A	18.00	6	15.28
18	A	18.00	23	9.61
19	B	9.80	10	7.46
20	B	9.80	14	6.69
21	C	6.80	7	5.64
22	E	8.80	3	8.13
23	E	8.80	3	8.13
24	C	6.80	8	5.49

t_{15} , t_{30} , t_{45} and t_{60}). The processing time length was increased to 30 min between 60 to 240 min (i.e. t_{90} , t_{120}and t_{240}).

At each time point, the corresponding tube was centrifuged for 15 seconds. An aliquot of 100 μ L was removed to a scintillation vial containing Cytoscint™ to measure total unbound radioactivity. The rest of the sample was filtered through a Millipore nitrocellulose filter and the filtrate was discarded. Filter paper containing protamine-DNA complex was transferred to another scintillation vial to get radioactive counts per minute for protamine bound DNA.

Calculations and Data Analysis

The efficiency of the DNA labeling was determined by using cpm from aliquots of pre and post centrifugations of the DNA labeling mixture as given below:

$$\% \text{ incorporation of } ^{33}\text{P} = \{ \text{Post cpm} / (\text{Pre cpm} + \text{Post cpm}) \} \times 100 \quad \dots \text{Eqn. 1}$$

In dissociation experiments, % DNA binding to protamine and background % binding to resin and filter (nonspecific binding as in control experiment) at a time point was calculated according to the following equation:

$$\% \text{ Bound DNA} = [\text{cpm filter} / \{ \text{cpm filter} + (10 \times \text{cpm supernatant}) \}] \times 100 \quad \dots \text{Eqn.2}$$

Percent DNA binding gives a measurement of both nonspecific and specific binding of DNA to resin and protamine, respectively. Therefore, corrections have been made to calculate % specifically bound DNA to protamine by subtracting background % binding from the value of % binding calculated for protamine immobilized resin.

Incubation of protamine attached resin and DNA for a sufficient time allows the system to reach equilibrium as shown by the following equation:

where R-P corresponds to protamine immobilized resin.

If the R-P-DNA complex is diluted to 100 fold, complexes dissociate according to first order kinetics:

$$-d[\text{R-P-DNA}]/dt = k_2[\text{R-P-DNA}]$$

$$[\text{R-P-DNA}]_t = [\text{R-P-DNA}]_0 e^{-k_2 t}$$

$$\ln ([\text{R-P-DNA}]_t \backslash [\text{R-P-DNA}]_0) = -k_2 t \quad \dots \text{Eqn. 4}$$

Therefore, if we plot a graph between $\ln ([\text{R-P-DNA}]_t \backslash [\text{R-P-DNA}]_0)$ and time according to equation 4, the value of the slope would give the dissociation rate constant. The dissociation rate constant was calculated for both bull P1 and clupeine Z protamines and compared with literature results.

RESULTS

Protamine Concentration

Protamines containing aromatic amino acids generally show a peak of UV absorption around 280 nm which coincides with the UV spectrum shown in Figure 8 for bull P1. This spectrum was recorded at a high concentration (31.5 μM) to see the peak at 280 nm due to the presence of tyrosine in its primary structure. The concentration of bull P1 stock solution in 10 mM HCl was determined from Figure 9 as 5.13 μM (33.9 $\mu\text{g/mL}$) using the absorption at 230 nm. The ratio of A_{230}/A_{320} was calculated as 7.5 indicating little protein aggregation in solution. Figure 10 shows the UV spectrum of clupeine Z in 10 mM HCl. It does not exhibit any significant amount of absorption at 280 nm due to the absence of aromatic amino acids in its primary structure. Since the absorption due to light scattering increases with decreasing wavelength, the low absorption at 280 nm as compared to 320 nm is more likely due to light scattering. The concentration of clupeine Z was calculated by using A_{180} as 12.1 μM (60.5 $\mu\text{g/mL}$).

Effect of Bull P1 Concentration During Immobilization Reaction at pH 9.0 as Determined by DNA Binding Studies

The results of DNA binding assays for previous work noted very little DNA binding to protamine immobilized on CDI activated resin when immobilization at pH 8.6 was carried out using 0.5 μM (3.375 $\mu\text{g/mL}$) bull P1 protamine (59). Therefore, initial experiments were carried out by coupling bull P1 with CDI activated resin at pH 9.0 for the purpose of increasing the amount of immobilized protamine by increasing the pH of the immobilization reaction. The extent of the covalent attachment of bull P1 onto resin at pH 9.0 was tested by the labeled DNA binding assay. The protamine-DNA

Figure 8: Ultraviolet-absorption spectrum of bull P1 protamine solution in 10 mM HCl. Absorption values at desired wavelengths are recorded as given below:
 A_{230} : 1.2115; A_{280} : 0.17276; A_{320} : 0.01987

Figure 9: Ultraviolet-absorption spectrum of bull P1 protamine stock solution in 10 mM HCl.

Absorption values at desired wavelengths are recorded as given below:

A_{230} : 0.19374; A_{280} : 0.02768; A_{320} : 0.00369

Figure 10: Ultraviolet-absorption spectrum of clupeine Z protamine stock solution in 10 mM HCl.

Absorption values at desired wavelengths are recorded as given below:

A_{190} : 0.08475; A_{205} : 2.1100; A_{220} : 2.0798; A_{280} : 0.031080; A_{320} : 0.02808

complexes were prepared and processed at the t_0 time point to find out if adequate immobilization had occurred.

Table 3 shows the effect of bull P1 concentration during the immobilization reaction and its resulting binding with labeled DNA at t_0 . The concentration range of bull P1 used during immobilization reactions at pH 9.0 was 0.16 to 2.56 μM . In initial experiments (1-5), when the same resin batch (termed as 'old resin') and same reaction conditions (except an increase in pH during the immobilization) were used as reported by the previous researcher (59), the specifically bound DNA was between 140 and 667 nM. In the control reactions, the non-specifically bound DNA with unsubstituted resin was found to range between 6-11 nM. These data show that the amount of specifically bound DNA with bull P1 attached resin is clearly distinguishable from background. An increase in binding of DNA with bull P1 immobilized resins compared to unsubstituted resins suggests the presence of protamine on CDI activated resins.

The duplicate reactions performed in experiments 2 to 5 have shown significantly different % DNA binding with bull P1 immobilized resin (Table 3). In experiments 2 and 3, the same resin preparation was used and the dissociation assays were performed five days later. However in experiments 4 and 5, different protamine concentrations were used, although, bull P1 immobilization reactions were carried out same day (i. e. had exactly same experimental conditions). Therefore, this discrepancy in results is attributed to uneven attachment of bull P1 at the resin surface. It was thought that at some places resin has more coupled protamine than others, since the resin has a tendency to settle down at the bottom of a 'standing' Eppendorf tube. Therefore, later experiments were carried out with two changes made in immobilization protocol. The addition of protamine solution to resin was followed by one hour manual

Table 3: Effect of [bull P1] on DNA binding at t_0 * (immobilization reaction was carried out at pH 9.0).

Exp. No.	[Bull P1] [†] (μM)	Non-specifically bound DNA with unsubstituted resin (%) (nM)		Specifically bound DNA with bull P1 immobilized resin (%) (nM)		Standard deviation [‡]
Old resin without one hour shaking before 48 hours incubation						
1	2.56	4.9	8	91.7 91.2	667 548	0.3
2	1.28	3.2	11	89.2 73.5	555 346	11.1
3	1.28	3.7	6	80.3 60.3	364 353	14.1
4	0.64	2.9	8	69.2 64.5	345 376	3.3
5	0.32	2.9	8	38.0 30.0	140 155	5.6
Old resin with one hour shaking before 48 hours incubation						
6	0.64	3.7	10	80.3 78.4	429 387	1.3
New resin without one hour shaking before 48 hours incubation						
7	0.32	3.5	9	44.4 41.1	146 124	2.3
8	0.32	3.2	9	61.1 63.7	330 337	1.9
9	0.16	3.2	9	56.2 51.5	215 214	3.3

Notes:

Old resin: Same resin batch used by previous researcher (59).

*Zero dissociation time after one hour incubation of labeled DNA with unsubstituted or bull P1 immobilized resin samples.

[†]Bull P1 concentration at the time of immobilization onto CDI activated resin at pH 9.0.

[‡]The standard deviation of duplicate % specifically bound DNA samples as calculated by using relationship $\sqrt{[n\sum x^2 - (\sum x)^2 / n(n-1)]}$.

shaking at room temperature. Secondly, the Eppendorf tubes containing the protamine-resin solution were kept for 48 hours incubation at a horizontal position (without shaking). As can be seen from the data of experiments 6 to 9, the percent specifically bound DNA of two duplicate sets is closely matched. The resin used in later experiments, corresponds to an identical CDI activated resin but a different shipment of material.

DNA binding is the criterion used to indirectly determine amount of immobilized protamine onto the resin in this study. Therefore, if the resin has higher amount of attached protamine, more DNA should be bound to it as compared to the resin having less protamine. It can be observed from Table 3, when high protamine concentration (0.64 to 2.56 μM) was used during the immobilization reaction, the amount of specifically bound DNA was greater than 300 nM. However, by decreasing protamine concentration, less DNA was bound to the bull P1 immobilized resins. When 0.32 μM bull P1 immobilized on the old resin was used, less than 200 nM specifically bound DNA was observed. These results show that the amount of coupled protamine onto a resin can be monitored by changing the bull P1 concentration. By comparing the results of experiments 4 and 6 (Table 3), it can be observed that DNA binding was increased to about 80 % (~400 nM) due to the protocol changes although the same concentration of protamine was used at the time of immobilization. Therefore, bull P1 concentration during binding reaction was decreased further with the new resin batch. In experiments 7 and 8, two different resin preparations were used with the same bull P1 concentration at the time of coupling and a somewhat different % DNA binding was observed. This discrepancy in results might be due to a change in the buffers used in the immobilization as well as in dissociation protocols. Experiment 7 was performed with

the old buffer solutions stored for a month, whereas, experiment 8 was carried out with new buffer solutions. When bull P1 concentration was reduced to 0.16 μM during binding reaction (experiment 9, Table 3), DNA binding was further decreased as compared to experiment 8. All these results again suggest that protamine immobilization onto CDI activated resin is a direct function of protamine concentration, although it is sensitive to small changes in protocol used for the resin preparation.

Effect of pH

Results of bull P1 immobilization onto CDI activated resins at pH 9.0 showed a significant amount of bound DNA as compared to unsubstituted resins (Table 3). Therefore, experiments with bull P1 immobilized resins at pH 8.6 were also performed to see the effect of pH on protamine coupling with the resin. The same bull P1 concentration range (0.16 to 2.56 μM) and other experimental conditions as employed at pH 9.0 were used to vary protamine immobilization at pH 8.6.

Table 4 depicts the results of DNA binding with protamine covalently attached to resin at pH 8.6. Again, the nonspecifically bound DNA with unsubstituted resin was found to be not more than 11 nM at t_0 . When protamine attached resin at pH 8.6 was used, the specifically bound DNA measured considerably higher (>250 nM) as compared to unsubstituted resins. These data again indicate bull P1 attachment onto CDI activated resin. It is noteworthy that the previous researcher (59) obtained very little DNA binding using 0.51 μM bull P1. The protamine attachment was also found to be a direct function of its concentration at pH 8.6 since by decreasing bull P1 concentration, less bound DNA was observed.

Table 4: Effect of [bull P1] on DNA binding at t_0^* (immobilization reaction was carried out at pH 8.6).

Exp. No.	[Bull P1] [†] (μM)	Non-specifically bound DNA with unsubstituted resin (%)	(nM)	Specifically bound DNA with bull P1 immobilized resin (%)	(nM)	Standard deviation [‡]
Old resin without one hour shaking before 48 hours incubation						
10	2.56	3.0	11	69.1 68.3	407 380	0.6
11	1.28	3.0	11	68.0 63.9	406 383	2.8
Old resin with one hour shaking before 48 hours incubation						
12	0.64	3.9	10	81.8 83.1	401 354	0.9
New resin with one hour shaking before 48 hours incubation						
13	0.32	3.6	9	71.4 75.3	364 324	2.7
14	0.16	3.6	9	66.8 57.5	289 260	6.6

Notes:

Old resin: Same resin batch used by previous researcher (59).

*Zero dissociation time after one hour incubation of labeled DNA with unsubstituted or bull P1 immobilized resin samples.

†Bull P1 concentration at the time of immobilization onto CDI activated resin at pH 8.6.

‡The standard deviation of duplicate % specifically bound DNA samples as calculated by using relationship $\sqrt{[n\sum x^2 - (\sum x)^2 / n(n-1)]}$.

The results given in Tables 3 and 4 were compared to analyze the effect of pH on covalent attachment of protamine with activated resin. In both the cases, the nonspecifically bound DNA was measured to be less than 12 nM. By comparing data of experiments 1 and 2 (pH 9.0) with 10 and 11 (pH 8.6), initially it was believed that an increase in pH could result in an increase in the amount of protamine binding on resin. When the new protocol was carried out, the results showed more or less the same % DNA binding at pH 9.0 and 8.6 as can be observed from experiments 6 and 12 of Tables 3 and 4, respectively. The same trend of DNA binding was obtained when 0.16 μ M bull P1 immobilized resins were created at both pHs. In experiments 7 and 8 (Table 3) performed at pH 9.0, the % DNA found was even less than that obtained at pH 8.6. All these results suggest that other factors such as the protocol for the protamine immobilization, age of the resin and age of the protamine may have a more profound effect on DNA binding than the pH of the immobilization reaction. Since the pH change has apparently no effect on protamine binding onto CDI activated resins, a pH between 8.6 to 9.0 can be used for immobilization reaction, although pH may affect the structure of the protamine (alter its disulfide content) and thus affect DNA binding capability.

Determination of Dissociation Rate Constant (k_2) for Bull P1

Bull P1 immobilized resins, prepared by varying protamine concentration during immobilization reaction, were used to make protamine-DNA complexes. Dissociation studies have been performed on these complexes at pH 7.4 for a period of four hours to determine dissociation rate constant at 25 °C. In the case of DNA complexes with high protamine immobilized resin, the amount of specifically bound DNA should not decrease with time after the addition of dissociation buffer or by increasing salt

concentration with 100 fold dilution (58). Protamine has a high affinity towards DNA due to its negatively charged backbone. Therefore, when DNA molecules dissociate from protamine, they can again be attracted to densely immobilized protamine on the resin surface due to the high positive charge density. However, if less protamine molecules are immobilized, the amount of bound DNA should decrease as a function of time.

An example of the raw data collected in a typical four hour dissociation experiment is given in Table 5. As can be seen in Table 5, there is a marked drop in % DNA binding with increasing time from the bull P1 attached resin. These data suggest reversible binding due to dissociation of DNA from its complex with bull P1. However, the % DNA binding in the case of unsubstituted resin showed a slight increase with time. These results indicate either some modification at the resin surface over the time due to processing at high pH or another association mechanism. This aspect will be discussed later based on additional experiments. The data for overall % specifically bound DNA also showed a decrease in binding with time elapsed as illustrated in Figure 11.

Each dissociation protocol was performed in duplicate at each bull P1 concentration used to prepare immobilized resin. The dissociation rate constants were calculated from the slope of the plot between $\ln B_t/B_0$ (specific binding at t time per specific binding at zero time) and the time of one and four hours as depicted in Figures 12 and 13. The calculated k_2 values are tabulated in Tables 6 and 7 at pH 9.0 and 8.6, respectively. The results show that the dissociation rate constant is not dependent on the protamine concentration used during the immobilization reaction. These values also fall in the same range as determined previously (59) ($k_2 = 2.47 \pm 0.51 \times 10^{-2} \text{ min}^{-1}$) for protamine-DNA complexes. Moreover, experiments 7-9 and 12-14 carried out with

Table 5: Dissociation experiment with 0.64 μ M bull P1 immobilized resin^v at pH 9.0.

Time [†] (Min)	Bull P1 immobilized resin				Unsubstituted resin (control)			
	Cpm x10 (100 μ L)	Cpm (Filter)	Total Cpm ^{...}	% Binding	Cpm x10 (100 μ L)	Cpm (Filter)	Total Cpm ^{...}	% Binding
0	6460	33895	40355	83.99	19960	770	20730	3.71
15	10740	30044	40784	73.67	16050	756	16806	4.50
30	8500	20793	29293	70.98	12818	877	13687	6.41
45	6780	15886	22666	70.09	12500	695	13195	5.27
60	5460	12449	17909	69.51	13680	793	14473	5.48
90	1450	2187	13637	60.13	9160	876	10036	8.73
120	4600	11242	15842	70.96	6420	690	7110	9.70
150	4740	9075	17815	73.39	6820	527	7347	7.17
180	3960	9148	17108	76.85	12050	857	12907	6.64
210	3380	9741	13121	74.24	6310	876	7186	12.19
240	3360	8157	11517	70.83	5160	876	6036	14.51
								56.31

Notes:

^vBull P1 immobilization was carried out with old resin with one hour shaking before 48 hours incubation (Exp. 6, Table 3).

[†]Dissociation time of protamine-DNA complexes.

^{*}Cpm of free labeled DNA in 1.0 mL solution.

^{..}Cpm of bound labeled DNA.

^{...}Sum of cpm of free and bound labeled DNA.

[†]Calculated by subtracting % DNA binding with control from % DNA binding with bull P1 immobilized resin.

Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4.

Specific activity of ³³P labeled DNA was 9.54 x 10² cpm/ng.

Figure 11: Plot of % specifically bound ^{33}P labeled DNA to bull P1 immobilized resin versus time.

Bull P1 immobilized resins were prepared by using ($\square$) $0.64 \mu\text{M}$, (Δ) $0.32 \mu\text{M}$ and ($\circ$) $0.16 \mu\text{M}$ bull P1 at pH 9.0 (Table 3, Exp. 6, 8 and 9).

Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4. Specific activity of ^{33}P labeled DNA was 9.54×10^2 (Exp. 6) and 12.21×10^2 cpm/ng (Exp. 8 and 9).

Figure 12: Plot of $\ln B_t/B_0$ versus time for determination of k_2 for one hour. Bull P1 immobilized resin were prepared by using ($\square$) 0.64 μM , (Δ) 0.32 μM and ($\circ$) 0.16 μM bull P1 at pH 9.0 (Table 5, Exp. 6, 8 and 9). Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4. Specific activity of ^{33}P labeled DNA was 9.54×10^2 (Exp. 6) and 12.21×10^2 cpm/ng (Exp. 8 and 9).

Figure 13: Plot of $\ln B_t/B_0$ versus time for determination of k_2 for four hours. Bull P1 immobilized resin were prepared by using ($\square$) 0.64 μM , (Δ) 0.32 μM and ($\circ$) 0.16 μM bull P1 at pH 9.0 (Table 5, Exp. 6, 8 and 9). Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4. Specific activity of ^{33}P labeled DNA was 9.54×10^2 (Exp. 6) and 12.21×10^2 cpm/ng (Exp. 8 and 9).

Table 6: Determination of k_2 for dissociation of bull P1-DNA complexes at 25 °C (immobilization reaction was carried out at pH 9.0).

Exp. No.	[Bull P1] (μM)	k ₂ × 10 ³ (1 hour) min ⁻¹	R ² (1 hour)	k ₂ × 10 ³ (4 hour) min ⁻¹	R ² (4 hour)
Old resin without one hour shaking before 48 hour incubation					
1	2.56	-	-	0.4	0.55
		0.7	0.96	0.2	0.65
3	1.28	11.1	0.90	4.1	0.34
		2.3	0.98	3.8	0.76
4	0.64	7.7	0.88	4.4	0.84
		4.2	0.97	6.2	0.29
5	0.32	3.2	0.99	5.9	0.32
		5.0	0.99	4.9	0.43
Old resin with one hour shaking before 48 hour incubation					
6	0.64	1.5	0.68	0.3	0.06
		1.2	1.00	0.7	0.16
New resin with one hour shaking before 48 hour incubation					
7	0.32	6.7	0.34	2.0	0.13
		14.4	0.86	5.1	0.33
8	0.32	14.4	0.96	3.1	0.36
		10.6	0.98	2.8	0.32
9	0.16	4.6	0.51	1.3	0.23
		10.7	0.91	0.5	0.08

Notes:

Old resin: Same resin batch used by previous researcher (59).

^{*}Bull P1 concentration at the time of immobilization onto CDI activated resin at pH 9.0.

Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4.

k_2 is a negative slope of the plot of $\ln B_t/B_0$ versus time (Figures 12 and 13).

R^2 is a linear regression done by using trendline of Microsoft excel™.

Table 7: Determination of k_2 for dissociation of bull P1-DNA complexes at 25°C (immobilization reaction was carried out at pH 8.6).

Exp. No.	[Bull P1] (μM)	k ₂ x10 ³ (1 hour) min ⁻¹	R ² (1 hour)	k ₂ x10 ³ (4 hour) min ⁻¹	R ² (4 hour)
Old resin without one hour shaking before 48 hour incubation					
10	2.56	1.5	0.62	5.9	0.57
		-	-	1.8	0.86
11	1.28	-	-	3.2	0.95
		6.1	0.94	2.0	0.65
Old resin with one hour shaking before 48 hour incubation					
12	0.64	4.90	0.99	0.5	0.08
		0.70	0.35	2.1	0.23
New resin with one hour shaking before 48 hour incubation					
13	0.32	19.3	0.88	2.7	0.34
		14.1	0.83	1.0	0.13
14	0.16	9.10	0.73	2.2	0.06
		8.60	0.85	3.6	0.50

Notes:

Old resin: Same resin batch used by previous researcher (59).

^{*}Bull P1 concentration at the time of immobilization onto CDI activated resin at pH 8.6.

Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4.

k_2 is a negative slope of the plot of $\ln B_t/B_0$ versus time (plots not shown).

R^2 is a linear regression done by using trendline of Microsoft excel™.

modified protocol show that the dissociation rate constants calculated for one hour are higher than the values at four hours (Tables 6 and 7). This is an indication of the presence of more than one population of DNA-protamine complexes. The fast dissociation of specifically bound DNA in the initial hour can be clearly observed in experiments 8 and 9 (Figure 11) where protamine immobilized resin at pH 9.0 was used. Actually, the dissociation of DNA was very rapid in the initial 15 minutes, however due to the lack of experimental data points, the rate of dissociation for that period was not calculated.

Effect of Clupeine Z Concentration during Immobilization Reaction at pH 9.0

Clupeine Z does not contain any cysteine residues in its primary structure (Figure 1). Therefore, it can not make any disulfide bond (inter- or intramolecular) or an attachment with the CDI activated resin via a S-nucleophile. It has a N-terminal N-nucleophile and O-nucleophiles due to the presence of three serine residues. Since CDI activated resins preferentially react with N-nucleophiles (55, 56), clupeine Z would be an ideal candidate to study how it interacts with the carbonyl diimidazole group. There is a chance that it could make only one attachment with the resin through its N-terminal primary amino group.

The effect of clupeine Z concentration during the immobilization reaction was studied by using 0.16 to 6.05 μM at pH 9.0. The extent of coupling was determined by the same labeled DNA binding assay as carried out for bull P1. Table 8 contains the results of a series of experiments showing % DNA binding with and without clupeine Z immobilized resin at t_0 . These data show DNA binding between 2.64 to 4.43 % (6 to 22

Table 8: Effect of [clupeine Z] on DNA binding at t_0 * (immobilization reaction was carried out at pH 9.0).

Exp. No.	[Clupeine Z] (μ M)	Non-specifically bound DNA with unsubstituted resin		Specifically bound DNA with clupeine Z immobilized resin		Standard deviation†
		(%)	(nM)	(%)	(nM)	
Old resin without one hour shaking before 48 hours incubation						
15	6.05	2.6	6	91.6 72.5	438 396	13.5
16	5.00	3.7	15	53.9 49.5	288 307	3.1
17	3.02	3.2	6	85.3 84.0	438 524	0.9
18	2.50	3.7	15	46.9 42.3	255 208	3.2
Old resin with one hour shaking before 48 hours incubation						
19	5.00	3.5	22	81.7 81.5	501 430	0.1
20	2.50	4.4	13	80.8 78.9	399 353	1.4
New resin with one hour shaking before 48 hours incubation						
21	1.25	3.5	11	84.7 83.6	288 312	0.8
22	0.32	3.88	16	69.3 71.9	212 188	1.87
23	0.16	3.88	16	48.9 44.7	114 138	5.62

Notes:

Old resin: Same resin batch used by previous researcher (59).

*Zero dissociation time after one hour incubation of labeled DNA with unsubstituted or clupeine Z immobilized resin samples.

†Clupeine Z concentration at the time of immobilization onto CDI activated resin at pH 9.0.

*The standard deviation of duplicate % specifically bound DNA duplicate samples as calculated by using relationship $\sqrt{[n\sum x^2 - (\sum x)^2 / n(n-1)]}$.

nM) with unsubstituted resin although it is greater than the 11 nM as observed in previous experiments. The % DNA binding increased to 90 % when 6.05 μ M protamine was used for immobilization. This data suggests a covalent attachment of clupeine Z with the CDI resin. It is also clear from Table 8 that the amount of clupeine Z can be varied at the resin surface by decreasing the protamine concentration. Since experiments 15-18 were performed with the resin prepared using old protocol, the variation in % DNA binding between duplicates is noticeable. When experiments 19-21 were carried out with the immobilized resin prepared by new protocol, there was an increase in % DNA binding despite the decrease in protamine concentration during the immobilization reaction. However, when a low concentration of clupeine Z (0.16 μ M, experiment 22) was used for protamine immobilization, the % DNA binding decreased. This indicates that the covalent attachment of clupeine Z can also be optimized by varying its concentration at the time of immobilization.

Although, the % DNA binding with clupeine Z at t_0 is comparable with the values calculated for bull P1 (Table 3), no firm conclusion can be made from these data due to variability in old and new protocols, a change of resin batch and the aggregation problem due to disulfide crosslinking in bull P1. Therefore, further work is needed with constant experimental conditions to compare the interaction of bull P1 and clupeine Z with DNA.

Determination of Dissociation Rate Constant for Clupeine Z

A DNA binding assay was performed to calculate dissociation rate constants for DNA-clupeine Z complexes at 25 °C. The plots for the k_2 determinations for one and four hours (experiments 19-21) are illustrated in Figures 14 and 15, respectively, and

Figure 14: Plot of $\ln B_t/B_0$ versus time for determination of k_2 for one hour. Immobilized resin were prepared by using ($\square$) 5.00 μM , (Δ) 2.5 μM and ($\circ$) 1.25 μM Clupeine Z at pH 9.0.(Table 7, Exp. 19-21). Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4. Specific activity of ^{33}P labeled DNA was 7.46×10^2 (Exp. 19), 6.69×10^2 (Exp. 20) and 5.64×10^2 cpm/ng (Exp. 21).

Figure 15: Plot of $\ln B_t/B_0$ versus time for determination of k_2 for four hours. Immobilized resin were prepared by using ($\square$) 5.00 μM , (Δ) 2.5 μM and ($\circ$) 1.25 μM Clupeine Z at pH 9.0.(Table 7, Exp. 19-21). Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4. Specific activity of ^{33}P labeled DNA was 7.46×10^2 (Exp. 19), 6.69×10^2 (Exp. 20) and 5.64×10^2 cpm/ng (Exp. 21)

summarized in Table 9. No noticeable changes were found between the k_2 determination of one hour versus the four hour analysis. The k_2 values for clupeine Z are similar to the values obtained for bull P1 (Tables 4 and 7).

Role of Nitrocellulose Filter and Eppendorf Tube

The same amount of labeled DNA (1.0 μL) was added to incubation tubes containing protamine immobilized resin preparation (120 μL) for preparing DNA-protamine complexes and in control tubes containing unsubstituted resin preparation (120 μL). Therefore, the same recovery was expected at zero dissociation time. Raw data in Table 5 shows fewer total cpm with unsubstituted resin (20730 cpm) as compared to bull P1 immobilized resin (40355 cpm) at t_0 . Similar results were obtained with all the experiments carried out for this study at pH 9.0 and 8.6. This finding suggests that all the DNA was not recovered when incubated with unsubstituted resin. It was also observed from the results of DNA binding assays carried out for four hours with the control reaction that % nonspecific DNA binding increased with increasing dissociation time (Table 5). The same trend was obtained with all the controls (unsubstituted resin processed at pH 8.6 and 9.0) carried out for experiments 1-23. These results further suggest that besides nonspecific DNA binding with unsubstituted resin, DNA might be either nonspecifically binding to the nitrocellulose filter or to the Eppendorf tube.

To learn more about the role of filters and tubes, one more DNA binding assay (experiment 24) was carried out for four hours using only unsubstituted resin processed at pH 9.0. The control reaction used for this particular experiment was HEPES buffer (pH 7.4) in the absence of unsubstituted resin. In the case of the control reaction

Table 9: Determination of k_2 for dissociation of clupeine Z-DNA complexes at 25 °C (immobilization reaction was carried out at pH 9.0).

Exp. No.	[Clupeine. Z] (μM)	$k_2 \times 10^3$ (1 hour) min^{-1}	R^2 (4 hour)	$k_2 \times 10^3$ (4 hour) min^{-1}	R^2 (4 hour)
	Old resin without one hour shaking before 48 hours incubation				
16	5	1.5	0.78	2.2	0.80
		3.2	0.97	3.7	0.62
18	2.5	5.2	1.00	5.4	0.37
		5.5	0.84	5.4	0.80
	Old resin with one hour shaking before 48 hours incubation				
19	5	1.5	0.86	1.4	0.91
		0.8	0.73	1.4	0.89
20	2.5	1.2	0.79	1.2	0.96
		1.2	0.96	1.1	0.96
	New resin with one hour shaking before 48 hour incubation				
21	1.25	-	-	0.3	0.43
		1.7	0.98	2.7	0.80
23	0.16	2.6	0.58	2.0	0.73
		-	-	2.3	0.43

Notes:

Old resin: Same resin batch used by previous researcher (59).

*Clupeine Z concentration at the time of immobilization onto CDI activated resin at pH 9.0.

Dissociation assay was carried out in the presence of 100 mM NaCl at pH 7.4.

k_2 is a negative slope of the plot of $\ln B_t/B_0$ versus time (Figures 14 and 15).

R^2 is a linear regression done by using trendline of Microsoft excel™.

(without resin), empty Eppendorf tubes after processing at each time point were also kept in the scintillation vial for counting radioactive decay. Therefore, for controls, the total cpm were calculated by adding the cpm of 100 μ L aliquot, filter and Eppendorf tube. The results are delineated in Figure 16 as % nonspecific DNA binding versus time. Although nonspecific DNA binding with unsubstituted resin and nitrocellulose filter was increased with increasing dissociation time as shown in Figure 16, it is about 10-15 % in both cases except t_{120} with nitrocellulose filter. This finding shows that % nonspecific binding is mainly with nitrocellulose filter and appears to be insignificant with unsubstituted resin. However with Eppendorf tubes, there was a large increase in % nonspecific DNA binding. These data suggest an affinity of the DNA for Eppendorf tubes. However, when protamine immobilized resin are present in the dissociation tube, DNA binds to protamine preferentially apparently due to its higher affinity for the protamine than the Eppendorf tube. In the case of unsubstituted resin (absence of protamine), DNA appears to be more attracted to Eppendorf tube as compared to the nitrocellulose filter and the resin. These results explain the loss of cpm in the case of unsubstituted resin (control) at the zero time point as compared to protamine immobilized resin (Table 5).

Figure 16: Plot of % nonspecific DNA binding versus. time.

(■) Unsubstituted resin, (□) Nitrocellulose filters, (▨) Eppendorf tube[†].

[†]Eppendorf tubes used for cpm from DNA binding assay performed in the absence of unsubstituted resin (control reaction used for this experiment). Specific activity of ³³P labeled DNA was 5.49×10^2 cpm/ng (Exp. 24).

DISCUSSION

Although the presence of protamines in sperm nuclei was originally described in 1886, we have a limited understanding regarding their interactions with DNA. As discussed earlier, solution studies are restricted due to the highly aggregated nature of DNA-protamine complexes even in dilute solutions. The present work was devoted mainly to development of methodology to study DNA-protamine interactions by optimizing conditions for immobilizing protamine onto a solid support.

Selection of CDI Activated Resin as Solid Support

There are some requirements for protein immobilization onto a solid matrix. The protein should have some functionality, such as the presence of amino, sulphydryl, hydroxyl or any other functional group, which can be used to make a covalent attachment to a suitable support. As discussed earlier, both clupeine Z and bull P1 are devoid of lysine and contain only one free primary amino group at their N-terminus and several dispersed hydroxyl groups (Figures 1 and 3). Bull P1 also contains six sulphydryl groups which are absent in clupeine Z. Since the aim of the present study was to immobilize protamine onto a solid support by making only one attachment, the amino moiety was targeted. The advantage of using an amino group over hydroxyl and sulphydryl groups for immobilization of protamine is that all the protamines would couple to the resin in the same attachment through the sole amino group.

On the other end of the linkage, the suitable matrix should also have compatible reactive groups. The compatible reactive group can be covalently introduced onto the insoluble matrix by controlled activation. It is important that the covalent linkage formed between the activated group and protein, as well as the compatible group and insoluble

matrix must be stable at the working pH and the other experimental conditions employed. Moreover, the chemical linkage formed between the protein and the activated group should not interfere with the interaction of the protein and other biological substances (e.g. DNA in the present study). Lastly, the activated matrix should be devoid of other charged groups to minimize nonspecific electrostatic interactions.

The CDI activated, cross-linked agarose resin beads fulfill all these conditions. The imidazolyl carbamate group of the agarose matrix smoothly reacts with the ligands containing a primary amino group functionality by forming an urethane (N-alkyl carbamate) linkage (55, 60). It is also commercially available with a moderate level of activation and can be conveniently refrigerated in acetone for a period of six months (60). Since, the positively charged central domain of protamine is postulated to interact with the phosphate backbone of DNA (37), the protamine binding to resin through the N-terminus should not affect the interactions between DNA and protamine. Moreover, CDI activated resins do not carry any additional charged groups which could lead to nonspecific electrostatic binding of protamine or DNA.

Stability of Carbamate Linkage

The stability of the carbamate linkage of activated resin was evaluated by Bethell et al. (60) by coupling it with [^{14}C]glycine at several pH values (3, 5, 6, 7, 8, 10 and 11). The release of [^{14}C]glycine was measured over a six week period. Their data showed the loss of coupled ligand as 0.3 % and 1.1 % at pH 8.0, and 0.9 % and 2.0 % at pH 10 over a period of two and six weeks, respectively. These results indicate a good stability of the CDI resin coupled with an amino group at basic pH, although it was

was reported by Poenisch (59) that a 10 % loss of immobilized protamine occurred over a period of five days.

Conditions That Affect the Protamine Immobilization onto Activated Resin

By careful examination of the experimental data of Poenisch (59) and the initial experiments carried out for this study, it was determined that this method is very sensitive to the experimental conditions used to immobilize the protamine. There are some important factors which should be consider when following this method.

(i) Protamine Aggregation

When Poenisch (59) used CDI resin to immobilize bull P1, he encountered the problem of getting sufficient attachment of bull P1 onto the resin surface. As a result, a low level of specifically bound DNA in comparison to the background cpm was detected. When 0.5 μ M bull P1 was used for attachment, a maximum DNA binding of ~13% was observed. It was noticed that the protamine sample used had some aggregation since the A_{280}/A_{320} value was close to 5. When a protamine solution with an A_{280}/A_{320} value less than 5 was used, a more concentrated solution was required for the same amount of DNA binding on immobilized protamine. In the present study, when the initial experiments of protamine immobilization were carried out at pH 9.0 using 3.7 μ M bull P1 protamine with an A_{280}/A_{320} value of 1.3, no appreciable DNA binding was observed (data not shown). From these results it is apparent that the age and condition of protamine is very important to get adequate attachment. Therefore, a new preparation of bull protamine with an A_{280}/A_{320} value of 7.5 was used for immobilization in later experiments. Results given in Tables 3 and 4 show that when 1.28 and 2.56 μ M

bull P1 were utilized for immobilization, ~70-90 % DNA binding was achieved at pH 8.6 and 9.0.

(ii) Buffers

Buffers play an important role in biological studies. Since very small quantities (μL) were used in the present study, it is possible that a change of buffer solution might change the % binding. This statement is based on the fact that in experiments 7 and 8 (Table 3), the protamine immobilization conditions used were exactly the same except for a change in the buffer solution which resulted in a variable % binding. It should be noted that the two duplicates used in the above mentioned experiments were reproducible.

(iii) The Position of Eppendorf Tube

A difference of 4-20% DNA binding with immobilized protamine was observed in most of the duplicate sets with the old resin preparation and without a one hour shaking period. It was observed that the resin beads settled down in the tapered tube within five minutes when the tubes were kept vertical for incubation. It was assumed that the resin which was deposited near the bottom, would not be as fully exposed to the protamine as the top layer. As a consequence, the activated sites would be less available for reaction. Therefore, the Eppendorf tubes were kept horizontal in later experiments to expose more CDI groups of the activated resin. In addition, one hour of gentle manual shaking before the 48 hour incubation was carried out in order to attain a more even protamine immobilization. Experiments 6-9 (Table 3), 12-14 (Table 4) and 19-23 (Table 8) showed that the variation in % DNA binding of duplicate reactions at the same

protamine concentration was minimized by increasing the exposed surface area of the resin.

Effect of High Protamine Concentration During Immobilization Reaction

In the present study, several batches of protamine coupled resin were made by varying the protamine concentration at the time of immobilization at pH 9.0. The extent of protamine attachment was measured indirectly by the DNA binding assay. When a high concentration (2.56 μM) of bull P1 was used, more than 90 % specifically bound DNA (> 500 nM) was observed at t_0 after a 100 fold dilution by the buffer containing 100 mM NaCl (Exp. 1, Table 3). Even four hours after the addition of the buffer, very little DNA was dissociated and about 80 % was still bound to protamine (data not shown for four hour dissociation).

The high % DNA binding at t_0 was also observed when 6.05 μM clupeine Z was used for resin binding at pH 9.0 (Table 8). No significant difference in bound DNA was observed after four hours of dissociation. The four hour dissociation assay carried out for both fish and mammalian protamines supports previous results (58) that higher protamine concentrations result in irreversible binding of DNA.

A possible explanation for the irreversible binding of DNA from bull P1 over a four hours period is that the DNA may possibly be attached to more than one protamine molecule. Probably, at higher protamine concentration, some regions of the resin surface are highly modified which lead to an increased number of localized binding sites for DNA. The other 8-10 % DNA population, which may not be able to bind properly, dissociated quickly.

Another possible explanation is based on the studies carried out by Inoue and Ando (51) who prepared DNA-clupeine Z complexes by two methods. One method employed a simple mixing of protamine and DNA solutions which they considered as "rapid binding". A gradient dialysis was used in the other method to make complexes by "slow binding". In this procedure, the unfractionated clupeine and herring DNA were dissolved in 2 M NaCl and the salt concentration was gradually reduced to 0.03 M by dialysis. Thermal analysis showed that the complexes made with "rapid binding" had a lower T_m (93 °C) as compared to the "slow binding" complexes ($T_m > 100$ °C). In the later case, the complexes didn't show a thermal transition up to 100 °C. Moreover, melting profiles of "slow binding" carried out in the presence of a large amount of clupeine Z, showed the presence of both types of binding populations. In the present study, since only a small amount of DNA dissociated from the high level protamine immobilized resin after four hours of dissociation assay, there is a possibility of the formation of more than one type of complex.

Wren (58) also observed an irreversible binding of DNA with high level bull P1 immobilized thiol resin when 6.3 μ M bull P1 was used for immobilization reaction. It was suggested that the DNA binding might appear irreversible since the DNA oligomers which dissociate from bull P1 upon 100 fold dilution, may again be involved in complex formation due to a large number of available binding sites on the resin surface.

Protamine Immobilization onto Resin Surface Can Be Controlled

Both mammalian and fish protamine immobilization can be controlled by decreasing the input protamine concentration at the time of coupling reaction (illustrated in the Figure 17). Approximately the same amount of DNA (~300 nM) was

Figure 17: Plot of specifically bound DNA as a function of protamine concentration at the time of immobilization.

(A) Effect of Bull P1 concentration:

($\square$) Bull P1 immobilized resin at pH 9.0 (Table 3, Exp. 6, 8, 9)

(-) Unsubstituted resin at pH 9.0 (control for bull P1)

($\blacklozenge$) Bull P1 immobilized resin at pH 8.6 (Table 4, 12-14).

(Δ) Bull P1 unsubstituted resin at pH 8.6 (control reaction)

(B) Effect of Clupeine Z concentration:

($\circ$) Clupeine Z immobilized resin at pH 9.0 (Table 7, Exp. 19-23)

(-) Clupeine Z unsubstituted resin at pH 9.0 (control reaction)

initially bound to 1.25 μM clupeine Z immobilized resin (Exp. 14, Table 8) as compared to the bull P1 coupled resin, prepared by using four fold lower concentration (0.32 μM , experiments 8 and 13, Table 3 and 4) at pH 9.0 and 8.6. When resins bound with same concentration (0.16 μM) of either bull P1 or clupeine Z were used, less DNA was complexed with immobilized clupeine Z. There are two possible explanations for these observations. Either more input clupeine Z concentration was needed to get the same amount of protamine on the resin as was achieved with bull P1; or clupeine Z has comparable immobilization but due to some structural hindrance, it is unable to bind more DNA fragments. The presence of two proline residues in the clupeine Z primary structure (Figure 1), possibly, can introduce obstacles in the coupling of protamine to resin. Previously, Willmitzer and Wagner (62) suggested that these two prolines assist in the proper folding of clupeine Z.

Moreover, a comparison of experiments 6-9 with experiments 12-14 shows that a four fold decrease in the concentration of bull P1 at the time of immobilization decreases the initial concentration of bound DNA at pH 9.0 and 8.6 by two fold and one half fold, respectively. These data suggest although a very high DNA binding can be achieved at both pHs and controlled by varying the protamine concentration, there could be a small effect of pH due to changes in disulfide crosslinking in bull P1 as a function of pH. Balhorn (42) demonstrated by *in vitro* studies that an increase in pH accelerates intramolecular disulfide crosslinking in bull P1. He found five different crosslinking patterns at pH 8.0 in which only one form was similar to that formed in bull P1 sperm chromatin (38).

All these observations suggest that the attachment of protamine onto the resin can be affected by varying protamine concentration at the time of immobilization. A rough estimate of 200-300 nM bound DNA indicates moderate protamine attachment onto the activated resin resulting in reversible DNA binding.

Dissociation Assay for Moderate Protamine Immobilized Resin

A sharp decrease in the % DNA binding was observed during the first 15 min when the four hour dissociation assay (experiments 8 and 9, Figure 11) was carried out with bull P1 immobilized resin at pH 9.0. The same trend was observed when bull P1 immobilized resin at pH 8.6 was used for the dissociation assay (data not shown). This observation supports the possibility of the formation of more than one type of complex. Since no data were collected during the first 15 min, it is not possible to calculate k_2 for this fast dissociation population. The fast dissociation of DNA during the initial 15 min was not observed, when experiments 19-21 were carried out with clupeine Z (data not shown). It is possible that due to intermolecular disulfide bridging of bull P1, one population of DNA might be more tightly attached to the protamine than others. The DNA molecules which are not affected by protamine disulfide crosslinks could be the fast dissociation population. Another possibility is that when the folded N-terminus (due to the formation of intramolecular crosslinks) of the bull P1 attached to the resin, it might introduce steric hindrance for proper DNA binding.

The work of Tobita (63) also supports more than one type of DNA binding populations with mammalian protamine. He studied the formation of dansylated boar and fish protamine complexes with DNA using 0.1 M NaCl and 50 mM tricine-HCl buffer at pH 7.4 as measured by fluorescence, thermal melting and sedimentation. He found

that boar protamine appears to bind to DNA with two conformations whereas fish protamine binds as a “linear molecule” with single conformation.

Dissociation Rate Constant (k_2)

There was scattering in the data points with increasing time during the four hour DNA dissociation assay (Figure 13 and 15). Therefore, the k_2 values for four hours are rough estimates (Table 4, 5 and 7). Although, the R^2 values for the total dissociation period are not good, the over all range for k_2 values are in good agreement. Since a sharp increase in dissociation of DNA from bull P1 was observed in the initial hour, the rate of dissociation for one hour was also calculated from the slope of the plot of $\ln B/B_0$ versus time (Figures 12 and 14). Note that this calculation of k_2 for the two populations is only an approximation. A better approximation of k_2 is to “strip” the data of the slow component prior to calculating the k_2 of the first component (58). The comparison of one and four hour k_2 values for fish protamine shows more uniformity as compared to the one and four hour k_2 values for mammalian protamine. The lack of disulfide bridging could be the reason for obtaining the similar k_2 values in the case of fish protamines. Table 10 shows a comparison of k_2 values for bull P1 calculated at pH 9.0 and 8.6 and for clupeine Z at pH 9.0 with literature values. The difference in k_2 values is attributed to differences in experimental conditions.

Watanabe and Schwarz (64) calculated the rate dissociation constant, referred to as k_D in their text, as 0.29 s^{-1} at 20°C and pH 7.5 for DNA-protamine complexes. Although, they studied the kinetics of similar system as used in this study, their approaches were different. They studied nonspecific binding of fluorencein labeled

Table 10: A comparison of the values of the rate of dissociation (k_2) of DNA-protamine complexes.

Protamine	k_2 (min^{-1})	Experimental Conditions	Reference
BullP1	12.78×10^{-3} (1.0 hr*) 2.38×10^{-3} (4.0 hr*)	Immobilization onto CDI resins at pH 8.6, 12 bp DNA, 0.1 M NaCl	Present study
Bull P1**	10.23×10^{-3} (1.0 hr**) 2.47×10^{-3} (4.0 hr**)	Immobilization onto CDI resins at pH 9.0, 12 bp DNA, 0.1 M NaCl	Present study
Bull P1	$5.34 \times 10^{-3} \pm 2.64 \times 10^{-3}$	Immobilization onto thiol resins at pH 7.4, 12 bp DNA, 0.1 M NaCl	58
Bull P1	$2.47 \times 10^{-2} \pm 0.5 \times 10^{-2}$	Immobilization onto CDI resins at pH 8.6, 12 bp DNA, 0.1 M NaCl	59
Clupeine Z***	2.15×10^{-3} (1.0 hr***) 1.32×10^{-3} (4.0 hr***)	Immobilization onto CDI resins at pH 9.0, 12 bp DNA, 0.1 M NaCl	Present study
Clupeine Z	17.4	Calf thymus DNA, 0.4 M NaCl	64
Clupeine Z	0.12	Phage DNA, 0.8 M NaCl	65

Notes:

*An average of k_2 values of experiments 13 and 14

** An average of k_2 values of experiments 7-9

*** An average of k_2 values of experiments 21 and 23

All experiments were carried out under same experimental conditions.

clupeine Z with high molecular weight calf thymus DNA in solution. The complexes were made by dropwise addition of concentrated protamine solution to a solution containing fixed concentrations of DNA and NaCl followed by a 4-10 minute incubation. However, in this study, complexes were made by the addition of a fixed amount of DNA to different solutions containing varied amounts of protamine and incubated for a longer time period. They employed "fluorescence titration" and "chemical relaxation" methods to measure the formation of complexes, equilibrium and kinetics of the system. However in the present study, radiolabeled DNA-protamine dissociation assay in combination with filtration and centrifugation methods were used to study kinetics of the system. The high value of the dissociation rate constant in their study can be attributed to the fact that they used high salt concentration (0.4 M) as compared to the salt concentration (0.1 M) used in this study. Since DNA-protamine binding is mainly electrostatic in nature, the high salt concentration could lead to a faster dissociation of the complexes.

Watanabe and Schwarz (64) noticed positive cooperativity in the binding process of protamine to large molecular weight DNA. According to their calculations, clupeine Z protamine occupies 20 nucleotides (10 bp) when bound to DNA. In the present study, only 12 bp long oligonucleotides were used and therefore cooperativity can not be observed.

Porschke (65) examined the binding of clupeine Z to high molecular weight phage DNA at various ionic strengths of the buffer by measuring light scattering intensities and ultracentrifugation methods. He observed that the binding of 0.1 μM clupeine Z with 2.0 μM DNA was completed at ~100 mM of buffer strength. At an ionic strength greater than 600 mM, more concentrated clupeine Z was required. Based on

his experimental results, he calculated association and dissociation rate constants of $5.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $2.0 \times 10^{-3} \text{ s}^{-1}$. Although, the association rate constant was not measured in the present study, the dissociation rate constants were measured. The variance in the k_2 values is attributed to the difference in experimental conditions employed, such as, large DNA molecules, different ionic strength of buffer and high salt concentration.

Porschke (65) also observed a unique "ligand driven" cooperativity in the DNA-protamine association process. According to him, the cooperativity was due to the "thermodynamic coupling of protamine with protamine-induced DNA association" and was not due to the "nearest neighbor interactions".

Comparison With Other Protein-DNA Interactions

Romaniuk (66) developed the nitrocellulose filter binding assay to study the kinetics of *Xenopus* transcription factor (TFIIIA) and 5S RNA complexes with an aim to examine the effect of various buffer components and incubation temperature. He observed the formation of at least three types of complexes in which 10 % of the population dissociated very rapidly and 40 % dissociated with a half life of 45 minutes. The remaining 50 % of the population was found to be very resistant to dissociation. The binding of TFIIIA to single stranded RNA is mainly nonelectrostatic, though five ionic contacts were also observed. Therefore, this interaction differs from protamine-DNA binding interactions which are predominantly electrostatic in nature.

Romaniuk (67) also studied the binding of TFIIIA with 5S RNA gene containing DNA restriction fragments. He used the nitrocellulose filter binding assay to measure equilibrium, association and dissociation rate constants. He found that the binding is

highly specific and one TFIIIA molecule binds per 5S RNA gene in contrast to protamine where one protamine molecule can bind small segments of several DNA fragments. The TFIIIA-5S DNA complex was prepared by mixing 2 nM ^{32}P -DNA with 6 nM TFIIIA at 22 °C on pH 7.5 TMK buffer containing 20 mM Tris HCl, 100 mM KCl and 5 mM MgCl_2 with other components followed by a 10 minute incubation. However, in the present study, the protamine and DNA were incubated in the low salt due to the electrostatic nature of binding, and dissociation was done by 100 fold dilution with the buffer containing 100 mM NaCl. The dissociation of TFIIIA-5S DNA complex was carried out by adding the unlabelled 5S RNA in the buffer solution containing complexes. The dissociation rate constant for TFIIIA-5S DNA was calculated as $7.53 \pm 0.9 \times 10^{-4} \text{ s}^{-1}$, having a half life 15.6 ± 1.9 minutes.

The effect of pH was also examined by Romaniuk (67) by changing buffer with different pK_a s and he found that the binding affinity of TFIIA declines with decreasing pH. In the present study, the effect of pH was not studied to determine the affinity of protamine to DNA. Here the aim was to obtain sufficient attachment of protamine onto the resin to get a measurable amount of bound DNA.

Fried and Crothers (68) did a kinetic study of binding *E. coli* cAMP receptor protein (CAP) and restriction fragments containing the lac promoter region with and without mutations. CAP binds to specific DNA sequences located in lac promoter-operator region. The complexes were formed by adding CAP to ^{32}P labeled DNA at pH 8.0 and kept for a one hour incubation at 20 °C. The complexes were separated by gel electrophoresis instead of a filter binding assay. They also observed the formation of

three types of complexes as a result of CAP binding to DNA fragments containing a wild type lac promoter region.

The dissociation of CAP to DNA fragments containing a specific sequence was carried out by replacing cAMP with cGMP. Since cAMP regulates the binding of CAP protein to its specific site, dissociation occurred. The dissociation rate constant was calculated at low DNA concentration to be $1.2 \times 10^{-4} \text{ s}^{-1}$. They observed that a "variation over a factor of 100" is not unusual in the calculated constant values in different laboratories using various experimental conditions or methods.

CONCLUSIONS

The primary aim of the present study was to optimize conditions for the immobilization of protamine onto CDI activated resins via a carbamate linkage, especially by increasing the pH of the immobilization reactions. The following conclusions were drawn on the basis of experimental results and their interpretations.

- (i) CDI activated resins can be used to immobilize bull P1 (mammalian) and clupeine Z (fish) protamines.
- (ii) The protamine attachment to the resin can be controlled by varying the protamine concentration during the immobilization reaction. The high protamine concentration at the time of binding yields highly immobilized resins which results mainly in irreversible binding. However, by decreasing the protamine concentration during the attachment reactions, a reversible DNA binding can be achieved.
- (iii) A small increase in the pH of the immobilization reaction, that is from 8.6 to 9.0, has apparently no effect on the binding of protamine onto CDI activated resin. Therefore, a pH from 8.6-9.0 can be used for protamine coupling.
- (iv) Protamine aggregation by age in solution plays an important role in the binding of bull protamine onto CDI activated resin. A smaller amount of protamine is needed to obtain the same DNA binding if protamine is less aggregated.
- (v) This method is also sensitive to other experimental conditions employed such as buffers used at the time of coupling and dissociation assay, age of the resin and specific activity of labeled DNA. Reproducibility in the data can be achieved by using constant experimental conditions.

(vi) There is a possibility of the formation of more than one type of DNA-protamine complexes in the case of bull P1 as is evident from the fast dissociation of DNA from protamine in the initial hour of the dissociation assay. The average half life of DNA protamine complexes was calculated to be approximately 68 minutes for fast dissociated complexes and 280 minutes for slowly dissociated complexes. The k_2 values used to calculate half life are an average of k_2 calculated for experiments 7-9 (Table 6 and 10). However, uniformity in one and four hour k_2 values for the dissociation of DNA-clupeine Z complexes indicates the presence of one type of complex.

FUTURE DIRECTIONS

In the present study, CDI activated resins were successfully used to couple bull P1 and clupeine Z protamines. Further studies can be carried out with protamine immobilized on CDI activated by using constant protamine concentration during the coupling reaction to get moderate substitution and then varying the DNA concentration, Na^+ ion concentration (or another monovalent ion, e.g. K^+) and ionic strength of the buffer to gain greater insight into the DNA-protamine interactions.

In the present study only the rate of dissociation of DNA-protamine complexes was calculated. The rate of association (k_1) should be calculated in further work to obtain equilibrium dissociation constant ($K_D = k_2/k_1$).

Due to the homology in structure of protamines of different species, the present study can also be extended to human protamine P1 and P2 and the systems can be compared with bull P1.

It would be of great interest if protamine immobilized on a resin can be used to make DNA-protamine complexes by gradient analysis to see the effect of slow binding on complex formation as suggested by Inoue and Ando (51).

Divalent ions play a vital role in protein-DNA binding *in vivo*. Since DNA-protamine interactions are mainly electrostatic in nature, the effect of divalent ions, such as Mg^{2+} , Zn^{2+} , Ca^{2+} , Ni^{2+} and Cu^{2+} should be examined to see how they affect the present system. Zn^{2+} is proposed to stabilize P2 protamines (69) and TFIIIA (67) by forming zinc fingers. The Ni^{2+} and Cu^{2+} complexes were also observed to bind the N-terminal sequence of human protamine P2 and facilitate oxidative DNA damage (70).

Prieto and coworkers (50) examined the interaction of DNA with a 14 amino acid long synthetic peptide which represents the proposed DNA binding site of protamine and is devoid of cysteine residues. This peptide (or another peptide) model can be immobilized on CDI activated resin and the DNA-peptide interactions can be studied. Since no information is available regarding the interaction of the imidazolyl carbamate group with an S-nucleophile, a comparative study of a model peptide with bull P1 can give valuable insight into whether a CDI resin couples with the thiol group of protamine.

Since a significant loss of cpm was observed in the present study due to a strong adsorption of DNA onto the Eppendorf tube, a coated tube should be used to increase the reproducibility of the results.

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IMAGE EVALUATION TEST TARGET (QA-3)

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