Dynamics of Protamine 1 Binding to Single DNA Molecules*

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Protamine molecules bind to and condense DNA in the sperm of most vertebrates, packaging the sperm genome in an inactive state until it can be reactivated following fertilization. By using methods that enable the analysis of protamine binding to individual DNA molecules, we have monitored the kinetics of DNA condensation and decondensation by protamine 1 (P1) and synthetic peptides corresponding to specific segments of the bull P1 DNA binding domain. Our results show that the number of clustered arginine residues present in the DNA binding domain is the most important factor affecting the condensation and stability of the DNA-protamine complex prior to the formation of inter-protamine disulfide cross-links. The high affinity of P1 for DNA is achieved by the coordinated binding of three anchoring domains, which together in bull P1 contain 19 Arg residues. The single DNA molecule experiments show that sequences containing two or more anchoring domains have an off-rate that is at least 3 orders of magnitude slower than those containing a single domain. The use of Arg, rather than Lys residues, and the inclusion of Tyr or Phe residues in the hinge regions between anchoring domains provide additional stability to the complex.

Several different mechanisms have been identified for packaging DNA in eucaryotic cells. The best understood is the packaging scheme used to organize DNA inside the nucleus of all somatic cells, in which the histones coil the DNA into 11-nm nucleosomal subunits that are further organized into 30-nm chromatin fibers. This type of packaging allows individual genes to be activated or repressed depending on the needs of the cell. In contrast, small basic nuclear proteins called protamines interact with DNA and inactivate the genes of the developing spermatid, we have examined the functions of specific subsets of the P1 DNA binding domain that enable protamine to bind to DNA and condense it into toroids. These experiments extend previous studies that have been conducted by others using various Arg- and Lys-containing oligopeptides (24–28) and focus on elucidating how three specific features of the P1 sequence affect the ability of P1 to bind to DNA and maintain it in a condensed state as follows: 1) the number of poly(Arg)...

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EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Bull P1 was isolated from pooled bull semen (American Breeders Service, DeForest, WI) and purified using high performance liquid chromatography. Electrospray mass spectrometry was used to confirm their sequence and purity. Peptides located in the DNA binding domain of P1 were synthesized with an acetylated amino terminus and an amide on the carboxyl terminus to minimize terminal charge effects and mimic the internal peptide sequences present in P1. The amino-terminal protamine peptide ARYRSSLTHSGSR was synthesized with a carboxyl-terminal amide group, and the carboxyterminal protamine peptide VSSRRTYVRSTQ was synthesized with an amino-terminal acetyl group.

Protein Concentration Verification—To obtain accurate concentrations of the nuclear proteins used in the condensation experiments, the purified peptides and bull P1 were dissolved in 0.1% trifluoroacetic acid, known aliquots were distributed into glass tubes, and the samples were frozen and lyophilized. Replicate samples were dissolved in 6 N hydrochloric acid and hydrolyzed at 105 °C for 16 h, and the amino acid content of each tube was determined by AAA Service Laboratory (Boring, OR). The protein content of each sample was determined using the known sequence of bull P1 and the synthetic peptides. Tube to tube variations in the aliquoted samples used for the experimental studies were found to be less than 2%.

Preparation of Stained DNA bead Sample—λ phage DNA (Sigma) was dissolved in buffer (degassed 50% sucrose (w/v), 100 mM sodium bicarbonate, pH 8, 30 mM dithiothreitol), tagged with biotin, and attached to 1-μm streptavidin-coated polystyrene spheres as described previously (21). YOYO-1 dye was then added so that the dye molecule to DNA base pair ratio was 1:4. Experiments performed with salmon and various peptides have shown that this concentration of YOYO-1 does not alter the kinetics of binding to DNA (21). The same buffer, minus the YOYO-1 dye, was used to dissolve the protein samples. In experiments involving the binding and dissociation of bull P1 to DNA, dithiothreitol was included to prevent the formation of disulfide bonds between P1 molecules following their binding to DNA.

Condensation/Decondensation Experiments—The experimental apparatus is identical to that used previously to examine the condensation of individual DNA molecules by salmon protamine (21). Separate solutions containing protein and DNA molecules attached to polystyrene beads were introduced into a dual port flow cell at a flow velocity of ~50 μm/s where they flowed side by side, in a laminar fashion, with little mixing. Beads containing individual DNA molecules were trapped using an infrared laser optical trap (λ = 1.047 μm). An argon ion laser (λ = 488 nm) was used to excite the YOYO-1-stained DNA molecules, and their fluorescence was visualized using an image-intensified CCD camera.

Working at a depth of about 20 μm below the coverslip (the flow cell depth was 40 μm), the DNA molecule was pulled across the interface separating the two flowing buffer solutions, and the shortening of individual DNA molecules was monitored as the protein or peptide bound to and induced condensation. The condensed DNA was evident as a bright fluorescent spot at the end of the DNA molecule. The length of the stained λ phage DNA molecule (48.5 kb) extended by flow was measured to be 19.2 ± 0.52 μm at a flow rate of 72 μm/s in 50% sucrose. Under these conditions, the DNA was 95% extended.1

Analyses of Protein and Peptide Binding Kinetics—The condensation rates for 5–20 individual DNA molecules were measured at several different protein or peptide concentrations, and a linear least squares fit (in conjunction with the peptide footprint) was used to determine the binding, on- and off-rates, k on and k off, of each peptide or protein to DNA. The binding of peptides and proteins to DNA was shown previously to be the rate-limiting step in condensation, and the length of the DNA molecule during condensation was shown to decrease linearly with time (21, 30) (Fig. 1, A and B). The decondensation rate was determined by pulling partially condensed DNA molecules back to the DNA side of the flow cell (where there was an absence of peptide or protein) and observing the DNA re-exist as the bound peptide left the DNA. The mean of ~10 molecules was used to determine the “off-rate,” k off, of each peptide or protein dissociating from the DNA molecule. The dissociation constant K D was determined by dividing the off-rate by the on-rate, K D = k off/k on.

Computational Analysis of Peptide Solution Conformation Using Molecular Dynamics—Molecular dynamics simulations were performed with AMBER (31) using the Cornell force field (32). Structures of the R GGR and R FGR peptides were constructed in extended conformations and solvated in boxes of TIP3P water (33) sufficient in size to have at least 15 Å of water between the protein and the solvent interface (81 × 72 × 45 Å3 initially). Twelve chloride ions were added to each system to neutralize the charge. The systems typically consisted of 21,400 atoms (~7,000 water molecules). Each system was energy-minimized using 250 steps of steepest descents and 750 steps of conjugate gradients. Constant temperature and pressure dynamics were performed on these energy-minimized systems. Periodic boundary conditions were used, and electrostatic interactions were treated by Particle Mesh Ewald methods (34) with a 9-Å cut-off in direct space, cubic interpolation, and using a 1-Å grid. SHAKE (35) was used to constrain bonds containing a hydrogen, and a time step of 2 fs was used. These systems were coupled to a heat bath at 300 K. Coupling constants of 5 and 2 ps were used for peptide and solvent, respectively. Each simulation was performed for a total of 2.5 ns, and the last 2.0 ns were used for analysis. Coordinates were saved every 200 time steps. The peptide conformers were clustered using the program NMRCORE (36). Solvent-accessible surface areas were calculated using the program surfv (37).

RESULTS

Rate of DNA Condensation by P1 and Peptides—The rates of DNA condensation induced by the binding of bull P1 and a series of peptides containing different sequences present in the bull P1 DNA binding domain (Arg15–Arg36), sequence ARYRC-
The inclusion of an aromatic amino acid between two Arg residues also appeared to increase the stability of the peptide-DNA complex. Sequences containing Tyr had substantially slower off rates than those containing only Gly. The inclusion of a single Tyr residue decreased the off-rate of the peptide by 6-fold. Peptides containing Phe exhibited a similar, but less pronounced effect. Replacing the first Gly in R6GGR_K with Phe decreased the off-rate by 2-fold.

**Computational Simulation of the Protamine Anchoring Domain by Using Molecular Dynamics**—Molecular dynamics runs were performed on the R6GGR_K and R6FGR_K peptides to examine how the presence of the aromatic Phe residue located between two anchoring domains might affect the conformation adopted by the adjacent peptide segments and ultimately impact the binding of these sequences to DNA. Structures of each peptide were constructed in extended conformation and solvated in boxes of water utilizing periodic boundaries. Molecular dynamics simulations were run for a total time of 2.5 ns using a fixed number of particles held at constant pressure and temperature.

Clustering analysis performed on 200 structures selected at 10-ps intervals during the last 2.0 ns of the simulations are shown in Fig. 2, A and B. Comparisons of these structures suggest that both peptides form a significant amount of structure along the backbone of the entire sequence. In the simulation performed with the R6GGR_K sequence, this backbone remains reasonably extended as a result of the extension and solvation of the Arg side chains. The mobility of the carboxyl-terminal region of this peptide is constrained by the formation of a salt bridge between the terminal carboxyl group and the side chain of residue Arg. The most interesting structure, however, is that developed in the R6FGR_K peptide during the last 1.1 ns of the simulation. In this peptide, the phenyl ring stacks against the peptide backbone and appears to form a stable core that is surprisingly tight (Fig. 2B). In these structures, the aromatic ring of Phe interacts with the backbone amides of Gly and Arg. One consequence of this stacking is that the backbone is bent between the two anchoring (Arg) domains.

The distances measured from the amide hydrogen to the center of mass of the Phe ring during the last nanosecond of the simulation are 3.88 Å and 3.52 Å for Gly and Arg, respectively. One effect of the interaction between the aromatic ring and backbone is that the amount of motion sampled by the ring is reduced significantly. The positional fluctuation of the Phe side chain is 4.54 Å for the first nanosecond of dynamics but reduces to 2.72 Å when stacked against the peptide backbone. The average solvent-accessible surface area of R6FGR_K (2766.5 ± 87.4 Å²) is lower than for R6GGR_K (2850.4 ± 79.4 Å²) even though R6FGR_K has the greater intrinsic surface area.
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TABLE II

| Protein         | Decondensation rate | Off-rate (k_{off}) | Kd (m) |
|-----------------|---------------------|--------------------|--------|
| Bull P1         | 1.0 ± 0.6 × 10^{-3} | 0.23 ± 0.14        | 1.1 ± 0.7 × 10^{-10} |
| Arg6            | 20.1 ± 5.0          | 16800 ± 4200       | 9.6 ± 3.0 × 10^{-5}  |
| Gly6            | 11.0 ± 3.4          | 9260 ± 2860        | 4.1 ± 1.3 × 10^{-4}  |
| R6GGR6          | 2.6 ± 0.8 × 10^{-3}  | 1.1 ± 0.33         | 7.9 ± 2.9 × 10^{-9}  |
| R6FGR6          | 7.9 ± 3.3 × 10^{-3}  | 3.3 ± 1.4          | 9.5 ± 4.3 × 10^{-9}  |
| R6GGR6GGR6      | 1.6 ± 0.7 × 10^{-2}  | 6.7 ± 3.0          | 1.8 ± 0.8 × 10^{-8}  |
| K6GGK6          | 5.9 ± 2.8 × 10^{-2}  | 24.6 ± 11.8        | 7.3 ± 3.8 × 10^{-8}  |
| R6GGR6GGRKRRK   | 1.7 ± 0.6 × 10^{-2}  | 7.2 ± 2.5          | 2.6 ± 1.0 × 10^{-8}  |
| R6GGR6GGRKRRK   | 2.2 ± 0.4 × 10^{-2}  | 6.2 ± 1.1          | 6.7 ± 1.9 × 10^{-9}  |

FIG. 2. A, the superposition of the backbone atoms of R6GGR6 from molecular dynamics calculations. Only the N, CA, and C atoms of the peptide are shown. A total of 200 structures, taken from 10-ns intervals, are shown. B, overlay of the backbone atoms for R6FGR6 from the last 1.16 ns of molecular dynamics calculations (116 structures). Only the N, CA, and C atoms of the peptide are shown for clarity. C, the last of the 116 structures shown in B. The Phe and backbone atoms of Gly6 and Arg6 are drawn as Corey-Pauling-Koltun space-filling models (49). The arrows in B and C point to the aromatic ring of Phe1 that stacks against the backbone amides of Gly6 and Arg6.

DISCUSSION

Importance of Multiple Arg6 DNA Anchoring Domains—DNA condensation and decondensation experiments were performed using a series of synthetic peptides containing one (Arg6), two (R6GGR6), and three (R6GG R6GGR6) Arg6 anchoring sequences as model subsets of the bull P1 DNA binding domain and two 14-residue peptides corresponding to the amino-terminal and carboxyl-terminal sequences flanking the bull P1 DNA binding domain. Even though the sequences of the model peptides do not correspond to the exact sequences present in bull P1 (the Cys residues were changed to Ser or Gly to prevent disulfide bond formation and model anchoring domains containing six Arg residues separated by two Gly residues were used to build up a peptide sequence corresponding to a “representative” P1 DNA binding domain), these experiments clearly showed that each of the peptides containing one or more Arg6 anchoring sequences bound to and condensed DNA. In contrast, neither the synthetic amino-terminal nor carboxyl-terminal P1 peptide sequences condensed DNA.

The number of Arg residues present in the anchoring domain peptides had a significant effect on both the concentration of the peptide required to condense DNA and the stability of the complexes. While even a single Arg6 anchoring domain condensed DNA, condensation could only be achieved at peptide concentrations that were 44-fold higher than those used for bull P1. Rates of Arg6 binding to DNA were observed to be 12-fold lower than the rate of bull P1 binding at equivalent concentrations. The ineffectiveness of Arg6 in condensing DNA was also seen in the instability of the Arg6-DNA complex. By increasing the number of Arg6 anchoring domains present in the peptide sequence from one to two (R6GGR6), the rate of dissociation of the peptide from the complex was reduced by 2500-fold.

Surprisingly, increasing the number of anchoring domains in the synthetic peptides from two to three did not increase either the effectiveness of the peptide in condensing DNA or the stability of the complex. R6GGR6 bound to and condensed DNA at rates similar to those observed for bull P1, but this rate was 2.7 times faster than that achieved with R6GGR6GGR6. In addition, the stability of the R6GGR6 and R6GGR6GGR6-DNA complexes, as reflected by the rates of dissociation of the peptides, was identical. These results show that the rate of binding, and the stability of the resulting complex, are not strictly determined by the number of Arg residues in the P1 DNA binding domain but suggest that other factors must be involved. One factor that may contribute to the rate of binding is the increase in flexibility inherent in longer peptides. Once the length of the binding domain increases beyond two anchoring domains, the increased flexibility of the sequence could adversely influence the rate of binding and condensation.

Formation and Relative Stability of Complexes Formed with Lys-containing Anchoring Domains—Previous studies (26, 39) have shown that the Arg residues in peptides and proteins bind more tightly to DNA than Lys residues because the guanidinium group in Arg forms both a hydrogen bond and salt bridge to the phosphate groups that comprise the backbone of DNA. Several different groups have examined and compared the ability of peptides containing sequences with multiple Lys or Arg residues to bind and condense DNA (26–28, 39, 40). The majority of this work focused on small (dimer to tetramer) or high molecular weight Arg or Lys polymers.

One study found that increasing the number of Lys residues in short (2–3-mers) homopolymers increases the affinity of the peptide for double-stranded DNA (24). A second study found that larger homopolymers of Arg are more effective in inducing DNA aggregation and precipitation (27). Binding affinities have not been determined or compared in previous studies for peptides above tetramers. Experiments conducted with KKK
and KXXXK peptides containing aromatic amino acids also provided evidence that aromatic amino acids located between two Lys residues did exhibit a detectable but small effect on the base sequence preference for peptides binding to DNA (24, 39, 41). These previous studies also provided evidence that the insertion of non-basic amino acids between Lys or Arg residues in short homopolymers eliminated the base sequence specificity that has been observed for poly(Lys) and poly(Arg) binding to duplex DNA (24).

In the present study, experiments conducted with one or two Lyr₆anchoring domains showed that they were remarkably ineffective in condensing DNA compared with Arg-containing peptides. At least a 10-fold higher concentration of the Lys-containing peptides was required to achieve a condensation rate similar to that of Arg-containing peptides. Once bound, however, Lyr₆was as effective as Arg in maintaining DNA in a condensed state. To within experimental error, peptides of the same length, composed of Lys and/or Arg residues, containing GG in the hinge region between domains, and corresponding to one (Arg₆ and Lys₆) or two (R₆GGR₆, K₆GGK₆, and RK-RKKKGGRKKRRK) anchoring domains, had the same off-rate. The DNA condensation and binding rate of K₆GGK₆ and RKKKGGRKKRRK were essentially identical. This suggests that the dominant factor contributing to DNA binding in sequences containing at least two anchoring domains is the number of positively charged side chains available for binding to the phosphodiester backbone of DNA.

*Contribution of Aromatic Amino Acids to DNA Binding and Complex Stability—Mammalian and other vertebrate protamines frequently contain Tyr or Phe residues located within the DNA binding domain (11, 23, 42). Bull P1, used as the model P1 sequence for these experiments, contains a single Phe residue positioned between two Arg₆anchoring domains. Condensation experiments conducted with the bull P1 sequence R₆FGR₆, and its analogs, R₆GGR₆ and R₆YGR₆ showed that the presence of an aromatic amino acid increased the stability of the DNA-peptide complex by decreasing the off-rate of the peptides. R₆YGR₆ formed the most stable complex (k₄off = 1.1 molecules/s), followed by R₆FGR₆ (k₄off = 3.3 molecules/s) and R₆GGR₆ (k₄off = 6.7 molecules/s), respectively. These results and the difference in off-rates observed for bull P1 and R₆GGR₆ indicate that these aromatic amino acids probably do contribute to the overall stability of the DNA-protamine complex.

As has been observed for other DNA-binding proteins (1, 43–46), the aromatic ring of Tyr or Phe may intercalate between base pairs or bind in the groove through hydrophobic interactions or hydrogen bonding. Numerous sites are available inside the major groove for hydrogen bonding to the Tyr hydroxyl group, including the amino groups of cytosine and adenine, the N-7 groups of guanine and adenine, and the carbonyl oxygen of thymine or guanine. Although the current experiments do not provide enough information to enable us to discriminate between these possibilities, preliminary NMR studies of a complex formed between a hairpin DNA and a slightly different but related P1 sequence containing Phe have indicated that the phenyl ring is intercalated (1). The insertion of this ring between base pairs would force the DNA molecule to bend. Bends in the DNA-protamine complex would be expected to facilitate, and may even initiate, toroid formation.

The 10–30-fold slower on-rate we observed for R₆GGR₆ sequences containing an aromatic amino acid and the molecular dynamics simulations of the conformation adopted by the peptides in solution are both consistent with binding that involves an intercalation event. The results of the molecular dynamics simulation of the conformation of the R₆FGR₆ peptide suggest that the aromatic side chain may stack against the backbone of two adjacent amino acids (Gly² and Arg²), lowering the hydration state of the peptide and kinking the backbone slightly. Aromatic ring-backbone interactions are not uncommon and have been shown to occur in other proteins (47, 48). In contrast, the equivalent peptide containing only Gly residues in the hinge region (R₆GGR₆) remains extended throughout the simulation. These results suggest that anchoring domain sequences separated by an aromatic amino acid should take longer to bind to DNA in a manner that optimizes the interactions of the Arg residues in the anchoring domains with the phosphodiester backbone of the DNA. Once the first anchoring domain binds to DNA, additional time would be required for the aromatic side chain to unstack with the protein backbone and insert into the helix before the second anchoring domain could bind properly to the phosphodiester backbone of DNA.

The stacking of the aromatic ring of Phe or Tyr against the peptide backbone might also be expected to reduce, somewhat, the flexibility of larger sequences, such as bull P1, that contain a third anchoring domain. This could explain the differences we observed for the on-rates of bull P1 and the model DNA binding domain R₆GGR₆GGR₆. Whereas the presence of an aromatic amino acid between two Arg₆anchoring domains reduced the rates of peptide binding and DNA condensation (R₆GGR₆ versus R₆FGGR₆ and R₆YGR₆) 10–30-fold, its presence is a sequence containing three anchoring domains (bull P1) increased the rate of peptide binding to DNA (P1 versus R₆GGR₆GGR₆) by 2–3-fold.

*Information About the Site of Protein/Peptide Binding Derived from Kinetics—Analyses of the binding kinetics of salmonine obtained in a previous study (21) and bull P1 and Arg₆, in this study (Fig. 1, A and B) have revealed that the length of an individual DNA molecule undergoing condensation decreases linearly with time. In each study we have observed that the binding of these proteins or peptides is the rate-limiting step in the condensation process. It follows that the rate of binding of these molecules to DNA is constant and time-independent. The first order equation describing the protein on-rate is given by Equation 1.

\[
\frac{d[P]}{dt} = k_{on} \cdot \frac{[P][FS]}{[FS]}
\]

where [FS] is the concentration of free binding sites on the DNA molecule; [P] is the protein concentration; [BS] is the concentration of bound sites on the DNA, and k₄on is the on-rate for protein binding to DNA. The rate of increase of the bound sites on the DNA molecule, d[BS]/dt, is equal in magnitude and opposite in sign to the rate of decrease in length of the DNA molecule. Clearly, the only way that this term can be time-independent is if [P] and [FS] are also time-independent. Because the binding experiments are performed in a flow stream that provides a constant concentration of protein, [P] remains constant throughout the course of the experiment. If [FS] is constant, this means that the number of binding sites available for peptide/protein binding during the condensation process is fixed. If all remaining binding sites were equally available, the binding of protein would vary exponentially with time.

One interpretation of these results is that the fixed binding site (or sites) is located at the point where the DNA bends and is condensed into the toroid, which starts in our experimental system at the free end of the extended DNA molecule. If this interpretation of the data is correct, the constant rate of condensation suggests that the protein/peptide can only bind to the DNA if the DNA is bent to a dimension on the order of its persistence length (toroids are 50 nm (3)). Whereas this hypothesis is intriguing, the best way to verify this theory is by
designing experiments that enable the direct visualization of the site of protein binding.

Relationship between DNA Binding Domain Sequence and Protamine Function—The results obtained from these single molecule DNA condensation studies have provided new insight into the features of the sequence of the P1 DNA binding domain that facilitate its function. As these experiments have demonstrated, relatively low concentrations of the protein are required to condense DNA and maintain it in a condensed state for extended periods of time if the DNA binding domain contains multiple Arg6 anchoring domains. This is particularly important in mammals because the maturation process for the developing sperm can take as long as 2–3 weeks, and the disulfide bonds that cross-link the DNA-bound protamines together (so they cannot dissociate or be displaced from DNA) are not formed until very late in the sperm maturation process (during epididymal transit).

These extremely low off-rates also suggest that large concentrations of unbonded protein are not required to maintain the entire genome in an inactive, condensed state. The low off-rate of the protamine molecule is particularly important in those non-mammalian vertebrates that use protamines to package their sperm DNA. Because the protamines in these species do not contain Cys, inter-protamine disulfide bonding cannot be used to stabilize the final structure of the mature sperm chromatin complex.

The results of these studies also appear to provide a plausible “rationale” for why protamines tend to be arginine-rich rather than lysine-rich DNA-binding proteins. Because DNA is condensed most effectively by protamines with DNA binding domains that are composed almost exclusively of Arg, selective pressures applied by the sperm during the evolution of the sequence of the protamine DNA binding domain should select against the use of Lys residues as replacements for Arg. This appears to be generally the case. Occasional Lys residues can be found in the DNA binding domains of several mammalian protamines, but they are never clustered together as the only components of an anchoring domain in P1.

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