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DNA condensation by high-affinity interaction with avidin

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Avidin, the basic biotin-binding glycoprotein from chicken egg white, is known to interact with DNA, whereas streptavidin, its neutral non-glycosylated bacterial analog, does not. In the present study we investigated the DNA-binding properties of avidin. Its affinity for DNA in the presence and absence of biotin was compared with that of other positively charged molecules, namely the protein lysozyme, the cationic polymers polylysine and polyarginine and an avidin derivative with higher isoelectric point (p*I* ≈ 11) in which most of the lysine residues were converted to homoarginines. Gel-shift assays, transmission electron microscopy and dynamic light scattering experiments demonstrated an unexpectedly strong interaction between avidin and DNA. The most pronounced gel-shift retardation occurred with the avidin–biotin complex, followed by avidin alone and then guanidylated avidin. Furthermore, ultrastructural and light-scattering studies showed that avidin assembles on the DNA molecule in an organized manner. The assembly leads to the formation of nanoparticles that are about 50–100 nm in size (DNA ≈ 5 kb) and have a rod-like or toroidal shape. In these particles the DNA is highly condensed and one avidin is bound to each 18 ± 4 DNA base pairs. The complexes are very stable even at high dilution ([DNA] = 10 pM) and are not disrupted in the presence of buffers or salt (up to 200 mM NaCl). The other positively charged molecules also condense DNA and form particles with a globular shape. However, in this case, these particles disassemble by dilution or in the presence of low salt concentration. The results indicate that the interaction of avidin with DNA may also occur under physiological conditions, further enhanced by the presence of biotin. This DNA-binding property of avidin may thus shed light on a potentially new physiological role for the protein in its natural environment. Copyright © 2004 John Wiley & Sons, Ltd.

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INTRODUCTION

Avidin, a tetrameric glycoprotein isolated from chicken egg white, is primarily known for its ability to bind biotin with high affinity ($K_d \approx 10^{-15}$ M). This property represents the basis for the exploitation of avidin as a molecular tool in many biotechnological applications (Abelson and Simon, 1990). Streptavidin, the bacterial counterpart of avidin, shares a similar affinity for biotin while differing in sequence and glycosylation. The molecular basis for the strong interaction between biotin and avidin or streptavidin is well understood. Despite the differences in sequence, the two proteins share similar tertiary and quaternary structure as well as a similar disposition of the relevant amino acids in the biotin-binding pocket. One of the most striking differences between the two proteins is in their respective p*I* values. While streptavidin is neutral, avidin is strongly basic (p*I* = 10), since it is rich in arginine (eight/subunit) and lysine (nine/subunit) residues. As a result, the protein is positively charged under physiological conditions. Nevertheless, its high p*I* does not seem to be important for biotin binding, since genetically and chemically modified avidins with neutral or acidic p*I* have been obtained without affecting this property (Nardone *et al.*, 1998; Marttila *et al.*, 2000). On the other hand, the high basicity is claimed to be the cause of avidin's interaction with DNA and cell surfaces, both undesired features in many biotechnological applications (Green, 1975; Heggeness, 1977; Duhamel and Whitehead, 1990). As a consequence, the neutral streptavidin or the chemically or genetically modified avidins with lowered p*I* are favored by far in most applications.

Despite the detailed information available concerning the molecular bases of the avidin–biotin interaction (Lindqvist and Schneider, 1996; Wilchek and Bayer, 1999), the function of the protein in nature is not well understood. Despite some evidence in early work for the existence of avidin-nucleic acid complexes in the egg white (Fraenkel-Conrat *et al.*, 1952), subsequent reports consistently described these products as being a consequence of preparation artefacts, and the avidin–DNA interaction was described as non-specific. In the present work, we investigated the DNA-binding properties of

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Abbreviations used: CT, calf thymus; ddH_2O, double-distilled water.

avidin. We characterized the interaction of avidin and other basic proteins and polymers with DNA using various techniques, with the aim of dissecting the contribution to the binding of electrostatic attraction and other recognition forces. Our results show that the interaction of avidin and the avidin–biotin complex with DNA is of high affinity, while specificity (if any) for a defined DNA sequence has not yet been identified.

METHODS

Reagents

Glycosylated chicken egg avidin was obtained from Belovo Chemicals, Belgium, polylysine and polyarginine (MW $\approx$ 20 kDa from Yeda Chemicals, Israel, lysozyme from Sigma, St Louis, IL, USA, guanyl-3,5-dimethylpyrazole from Fluka, and agarose, low EEO, from Hispanagar (Spain). Circular plasmid DNAs: pGeneGrip, 5.063 kb, were from Gene Therapy Systems; pEGF-C1, 4.7 kb, from Clontech; and GFP-T7, Luc-T7 where the Luc encoding sequence was replaced with GFP, 3.376 kb from Promega. Linear DNA: calf thymus (CT) was from Sigma and GFP-T7 linearized with Pvu II from NEB.

Avidin guanidinylation

All of the lysines in avidin were transformed into homoarginines by the use of the guanidinylating agent guanyl-3,5-dimethylpyrazole (Ariely *et al.*, 1966). Such modification induces a shift in the protein's p*I* to about 10.9. The reaction was carried out in 50 mM phosphate pH 7.4. Avidin (100 mg), dissolved in 7 ml of buffer was added to 200 mg of guanyl-3,5-dimethylpyrazole nitrate. The reaction was gently stirred for 48 h at 37 °C. The protein was purified by extensive dialysis (4 °C) against water and freeze-dried. Complete lysine modification was confirmed by amino acid analysis and ESI-TOF mass spectrometry. The modified protein maintained the same affinity for biotin as assessed by the HABA [2-(4′-hydroxyazobenzene) benzoic acid] assay (Green, 1990).

Assembly of DNA with polycations

DNA and polycations were dissolved in the desired aqueous solution [double-distilled water (ddH_2O), phosphate (10 mM, pH 7.4) or Tris (10 mM, pH 8.0) buffers, or aqueous NaCl (from 0 to 1 M)] and cooled in an ice bucket. Equal volumes of DNA and polycation solutions were then mixed while vortexing. Vortexing was maintained for 10 s after mixing and then samples were kept in an ice bucket until further analysis. As a general procedure, the DNA was added to the polycation solution.

Agarose gel shift assays

DNA and polycations were dissolved in the desired aqueous solution/buffer and mixed as described above. The DNA was added to solutions containing increasing amounts of polycation, according to the desired [+/−] charge ratio. The final DNA concentration in mixed samples was 6.25 or 12.5 μg/ml. Ten microliters of each final solution were analyzed on 0.8% agarose gels. DNA was visualized by fluorescence (ethidium bromide) using a BIS 202D transilluminator (Bio Imaging System) and image fluorescence intensity was quantified with NIH 1.61 image. The fluorescence intensity of the DNA bands in the gel was compared with that of DNA solutions of known concentration (serial dilutions from 12.5 μg/ml to 24 ng/ml) dissolved in the same buffer.

Light scattering

Light scattering measurements were performed using an in-house assembled system. Samples (300 μl) were placed in thin walled cylindrical borosilicate glass cuvettes (1 cm diameter) and placed in a vat filled with toluene as the index matching fluid. During the course of the measurements, the vat temperature was kept at 20 °C. The light source was an argon ion laser (Lexel, $\lambda = 514.5$ nm) and photons scattered by the sample were collected by a photomultiplier tube mounted on the goniometer arm at 90° to the direction of the incident radiation. The photoelectron count-time autocorrelation function was measured with a BI2030AT (Brookhaven Instruments) digital correlator and analyzed using the constrained regularization algorithm, CONTIN (Provencher, 1982). Applying the Stokes–Einstein equation to the translation diffusion coefficients provides an intensity-weighted distribution of hydrodynamic sizes (Finsy, 1994).

Electron microscopy

Transmission electron microscopy (TEM) images were obtained with a Philips Tecni-T12 microscope, operated at 120 kV. Samples were mounted on carbon grids and negatively stained with 1% uranyl acetate aqueous solution. Micrographs were taken on Megaview II (SIS, Germany) CCD camera.

Estimation of the ratio of avidin–base pairs in the complex

Equal volumes (400 μl) of avidin and DNA (circular or linearized p-GFP plasmid) solutions in water were mixed in a similar way as described for the agarose gel retardation assays. Several samples were prepared that differed by the charge ratio ([+/−] = 2, 3 and 6) and final DNA concentration (10 and 20 μg/ml). After centrifugation, 750 μl of the supernatant were recovered and particle aggregation was induced, after cooling in an ice bucket, by the addition of 7.5 μl of 1 M Tris buffer pH 8.0. After 2 h, the precipitate was centrifuged and washed three times with 10 mM Tris buffer pH 8.0. The precipitate was then quantitatively re-suspended in 0.3 M NaCl and the ratio of DNA bp to avidin was calculated from its avidin and DNA content. Avidin concentration was estimated using the Bradford reagent (Bradford, 1976). For DNA quantification, an aliquot of the solution was mixed with an equal amount of 5 M NaCl,

and the DNA concentration was measured by the fluorescence intensity of agarose gel bands as compared with that displayed by known amounts of DNA dissolved in 2.5 M NaCl.

RESULTS

Agarose gel-shift assay

Interaction of DNA with any DNA binding agent (either a specific DNA binding protein or a polycation) can be followed by gel retardation assay. This assay is based on the rationale that the electrophoretic mobility of a biomolecule in a non-denaturing gel depends on both its charge and molecular weight. If a DNA-binding agent is added to the DNA prior to its loading on the gel, the electrophoretic mobility of bound DNA will vary depending on the size and charge of the resulting complex. Both agarose and polyacrylamide gels may be used, depending on the desired sensitivity and degree of resolution. In the assay, several samples are prepared by mixing the binding agent with DNA at different molar ratios (or, in the case of polycations, charge [+/−] ratios), and the samples are then loaded in the gel. Qualitative and quantitative information on the interaction can be obtained by measuring the intensity of the band relative to the unbound (free) DNA. Quantitative information may also be obtained from the intensity and position of the band(s) relative to the bound DNA. However, quantification is only possible when stoichiometrically defined DNA/DNA-binding agent complexes are formed. When investigating the interaction of DNA with condensing agents, such as the polycations analyzed in this work, the multiplicity of the binding induces the formation of very high molecular weight complexes, which are totally retained in the application well. Therefore, quantitative information from the signal of the retained DNA cannot be obtained. Our experiments were performed on agarose gels, and the amount of free DNA in each sample was quantified by measuring the fluorescence intensity of the ethidium bromide-stained bands after normalization with a calibration curve. Binding curves were derived as a function of the charge ratio.

Effect of salts. Molecular interactions in aqueous solution are affected to a greater or lesser extent by the presence of electrolytes depending on the contribution of electrostatic forces to the binding. When salts are present in the medium, they act by shielding charges and therefore weaken such interactions. In principle, if mechanisms other than electrostatic attraction are also responsible for the binding, these should not be negatively affected by the presence of salts. This for example may occur in specific recognition which commonly results from the contribution of multiple factors such as long-range (electrostatic) and short-range (Van der Waals, hydrogen bonding and hydrophobic interaction) forces.

We evaluated the contribution of electrostatic interaction in DNA–polycation binding by monitoring the effect of the medium's ionic strength on the pattern of agarose gel-shift assays. DNA–polycation samples were prepared in either deionized water or aqueous solutions containing different amounts of electrolytes, namely NaCl and buffering salts (phosphate and Tris). Circular and linear DNAs displayed the same behavior in this assay. When DNA was mixed with the polycations in deionized water, similar retardation patterns with respect to the charge ratio were observed with all molecules investigated. However, the ability of lysozyme to retain DNA was shown to be very sensitive to the ionic strength of the medium, as expected for an interaction based purely on charge. The interaction of avidin with DNA was affected less by the presence of salt (Fig. 1), and the strength of interaction was shown to be similar to that demonstrated by high-molecular-weight ($\sim$20 kDa) polycations, polyl-L-lysine (PLK) and polyarginine (PLR) (Fig. 2). In the case of the two cationic polymers, their high charge density contributes significantly to the interaction with the DNA with a cooperative effect (Mascotti and Lohman, 1997; Liu *et al.*, 2001). Avidin and lysozyme are both basic proteins and have similar isoelectric points (lysozyme $\approx$ 9–11, avidin $\approx$ 10; Alderton *et al.*, 1945; Canfield, 1963; Green, 1975). Therefore, they are similarly charged in an aqueous environment. However, their charge density is significantly less than that of PLK or PLR, and charge-induced cooperative effect on DNA binding is not

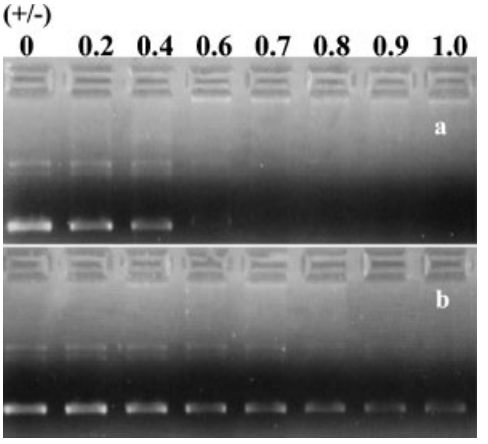

Figure 1. Agarose gel retardation pattern of DNA (pGeneGrip) mixed with either avidin (a) or lysozyme (b) at different [+/−] ratios. Samples were prepared in 0.3 M NaCl at a final DNA concentration of 6.25 μg/ml.

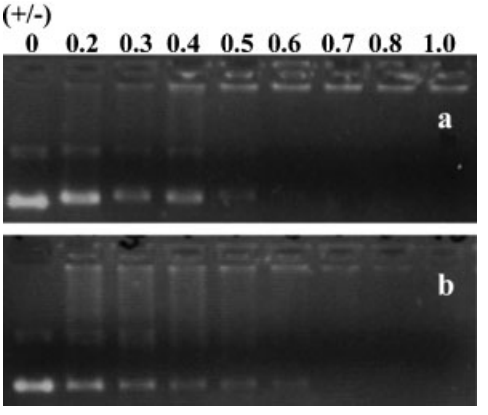

Figure 2. Comparison between DNA (pGeneGrip) agarose gel retardation patterns when mixed with either avidin (a) or polyarginine (b) at different [+/−] ratios. Samples were prepared in 0.3 M NaCl at a final DNA concentration of 25 μg/ml.

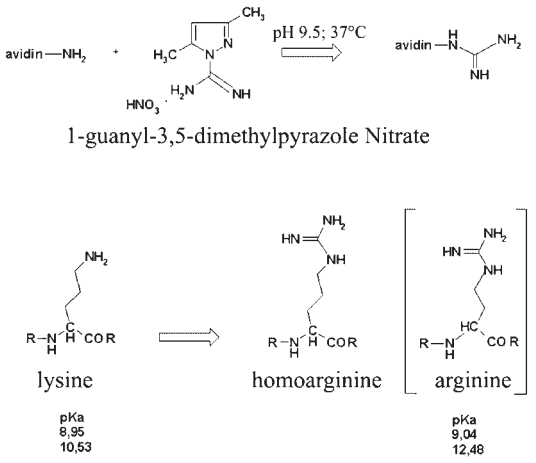

Figure 3. Reaction scheme of avidin guanidylation.

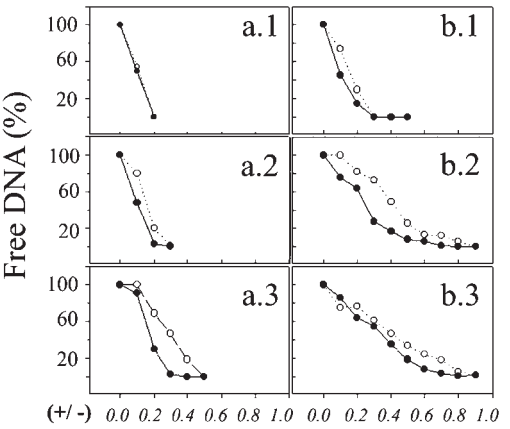

Figure 4. Agarose gel retardation patterns of DNA (pGeneGrip) mixed with avidin (a) or guanidinylated avidin (b) at different [+/−] charge ratios. Avidin(s) and DNA were mixed in solutions containing different amounts of NaCl (a.1 and b.1: [NaCl] = 0.15 M; a.2 and b.2: [NaCl] = 0.45 M; a.3 and b.3: [NaCl] = 0.6 M) and in the presence (open symbols) or absence (solid symbols) of biotin. The amount of free DNA was calculated from the band fluorescence intensity normalized according to a calibration curve. The final [DNA] in samples before loading the gel was 6.25 μg/ml.

likely to occur in this case. The differences observed between lysozyme and avidin DNA binding properties suggest that different mechanisms of interaction may exist. While in the case of lysozyme the binding is probably due primarily to electrostatic attraction, easily disrupted by the presence of salt, in the case of avidin other interacting forces may also be involved. This hypothesis is also supported by the data obtained with guanidinylated avidin. Avidin's lysine residues were transformed into homoarginines by means of the guanidinylating agent guanyl-3, 5-dimethyl-pyrazole (Fig. 3). Quantitative lysine transformation was confirmed by amino acid analysis and ESI-TOF mass spectrometry: no lysines were detected in the guanidylated product, whereas the average molecular weight of the protein subunit increased by about 420 Da, a value that corresponds to the complete transformation of the 10 primary amines (nine lysines + one alpha terminal) of the protein. The resulting protein ('avidin-R', Fig. 3) is characterized by a significantly higher isoelectric point (theoretical 10.9). Thus, in aqueous solution it is even more positively charged than native avidin. While guanidinylation did not affect the protein's biotin-binding properties (not shown), the affinity for DNA was reduced despite the significant increase of its positive charge (Fig. 4). Also, in the case of avidin and its guanidinylated derivative, the affinity for DNA was shown to depend on the medium's ionic strength. However, avidin-R was shown to be more sensitive to the presence of salts, indicating that the recognition process is affected negatively by lysine modification.

Influence of biotin. The presence of biotin increased the affinity for DNA of both unmodified and avidin-R (Fig. 4). This effect is more pronounced at NaCl concentrations above physiological level (>150 mM). It is known from the literature that biotin binding induces a conformational change in avidin where the loop connecting β-strands 3 and 4 becomes ordered and locks the biotin-binding pocket (Livnah *et al.*, 1993; Pugliese *et al.*, 1993, 1994). It is therefore possible that such a conformational change either exposes a DNA-binding motif and improves the affinity or that the increase in the rigidity of the molecule affects the binding in a positive manner.

Physico-chemical characterization

It is known that several polycations can induce DNA condensation and form small particles (polyplexes) characterized by a size of approximately 50–100 nm and different morphology (spheres, rods, toroids; Bloomfield, 1998). Condensation occurs when the large volume occupied by a DNA molecule in its extended random coil conformation decreases dramatically, leading to compact structures in which the volume fractions of solvent and DNA are comparable. Reduction of volume also occurs upon aggregation or precipitation. However, the term 'condensation' is normally used to define only situations in which the resulting aggregate is of finite size and ordered morphology (Bloomfield, 1998). Condensation by polycations is generally driven by the neutralization of the phosphodiester negative charge along the DNA backbone. The positive charge of the polycation is fundamental for both binding and condensation. However, structural features also seem to play an important role in the condensation process, since similarly charged polycations display different propensities towards promoting DNA condensation (Saminathan *et al.*, 1999; Vijayanathan *et al.*, 2001; Brewer *et al.*, 2002). We therefore evaluated the capacity of avidin to induce DNA condensation. For this purpose, we carried out dynamic light scattering and transmission electron microscopy measurements.

Light scattering. Dynamic light scattering experiments measure the relative diffusion constant of particles in solution via the time autocorrelation of the fluctuations in the intensity of the scattered light (Chu, 1992). From the diffusion constant, an average hydrodynamic particle radius may be derived. In addition, qualitative information concerning particle stability or the tendency of particles to aggregate upon modification of the environment can also be obtained by measuring changes in the time-averaged intensity of scattered light, which depends both on particle

concentration and on the molecular weight. As a consequence, changes in size, such as those derived from disassembly or aggregation, give rise to significant changes in this parameter.

We analyzed the products resulting from mixing DNA with the different cations investigated. The influence of charge ratio, sample dilution and the presence of salts in the medium on particle properties was thus evaluated.

Effect of charge ratio. DNA was mixed in distilled water with different amounts of avidin and avidin-R so that the [+/−] ratio in solution was varied between 0.5 and 7.0. All samples were analyzed by light scattering immediately after preparation and after 24 h storage at 4 °C. Provided that an excess of positive charge was present in solution ([+/−] >1), all solutions proved to contain a monomodal population of particles with an average diameter of approximately 130 nm (Fig. 5). At [+/−] = 1, partial precipitation occurred, as expected for a decrease in solubility in the absence of charge, and samples could be measured only after centrifugation. At charge ratios [+/−] <1, a population with higher dimensions was measured (diameter ≈ 170 nm). Similar data were obtained independent of sample concentration ([DNA] = 1–20 μg/ml) and the size of particles did not change upon storage. Avidin-R showed a similar behavior, although the particles demonstrated a slightly smaller size (110 nm). No significant variation in sample size could be detected upon addition of biotin, either by pre-saturating avidin before complex formation or by adding it to the assembled samples. (It should be noted that the technique might not be sensitive enough to detect small variations in size.)

Effect of sample dilution and presence of salt. These experiments were carried out in order to verify the stability of the avidin–DNA interaction as compared with those displayed by other polycations known to interact with the DNA owing to electrostatic forces enhanced by cooperativity.

Serial dilutions in water of samples obtained with each polycation were prepared and analyzed both for particle dimension and scattered light intensity. Samples were diluted down to a final [DNA] = 39 ng/ml, the lowest concentration detectable by our light scattering instrumentation.

In order to evaluate the effect of the presence of salts in solution, samples were diluted (1:100, final DNA concentration 200 ng/ml) with water containing different amounts of NaCl (0, 50, 100, 150, 200, 300 and 400 mM). Scattering intensity and particle size were measured immediately after dilution and after 5, 30 and 90 min.

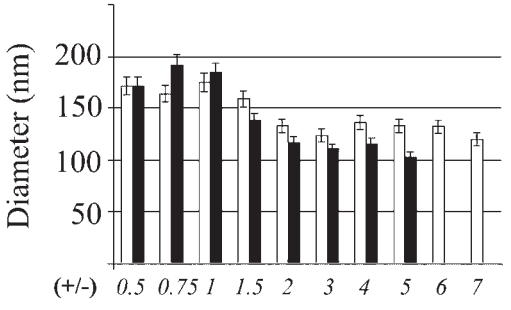

Figure 5. Avidin–DNA particle size as a function of the [+/−] charge ratio. Size was determined by light scattering measurements, assuming a spherical shape. White bars: avidin; black bars: guanidylated avidin.

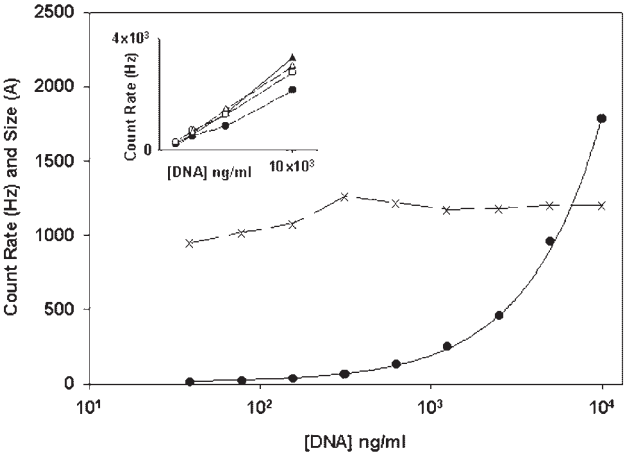

Figure 6. Scattering intensity (●) of solutions of avidin/DNA and size of the particles (×) as a function of sample DNA concentration. Avidin–DNA complexes were prepared in deionized water at charge ratio [+/−] = 3.0. Inset: scattering intensity of avidin–DNA and avidin-R–DNA solutions (with and without biotin) as a function of their concentration: (●) avidin; (▲) avidin-R; (○) avidin + biotin; (△) avidin-R + biotin.

In the case of both avidin and avidin-R a linear correlation between dilution (in water) and light intensity was obtained up to [DNA] = 39 ng/ml (Fig. 6), demonstrating that particles are very stable even at low concentration ([DNA] ≈ 10 pM). The particle size remained constant with an average hydrodynamic diameter of 130 and 110 nm for avidin and avidin-R, respectively. In contrast, the intensity of scattered light from polylysine–DNA samples decreased below the detection limit of the system after dilution to 200 ng DNA/ml with water.

Moreover, both avidin–DNA and avidin-R–DNA particles are stable with respect to disassembly at physiological salt concentrations. In fact, following 100-fold dilution of either avidin or avidin-R/DNA samples in solutions containing relatively low concentrations of NaCl (up to [NaCl] = 200 mM), the intensity of scattered light was similar to that measured for samples diluted in water. No decrease was observed, as would have occurred if the particles had disassembled. On the contrary, the intensity of scattered light slowly increased with time, probably as a result of an aggregation process. Indeed, particle size tended to increase with time. Aggregation is indeed likely to occur as a consequence of the reduction of particle surface ζ-potential, which would be induced by the presence of counterions in the medium. Dilution of avidin–DNA samples with solutions having higher salt concentration (>200 mM) caused an immediate drop in the scattering intensity, indicating rapid particle disassembly. It is likely that the binding of avidin with DNA results from a synergy of interacting forces and electrostatics is only one of the key elements involved (Luisi, 1995). Above a critical NaCl concentration, the contribution of electrostatic binding is partially weakened (it can still be observed by gel-shift assays) and the interaction is thus unable to lead to the assembly of particles that are measurable by light scattering.

Polylysine, polyarginine and lysozyme also form homogeneous populations of particles, with diameters of about 80, 65 and 75 nm respectively, when mixed with DNA in water ($[+/-]=3$). However, in all cases, the particles obtained with these polycations proved to be less stable than those obtained with avidin or avidin-R. Instability was observed with respect to either dilution (in water) or to the presence of small amounts of salt. More precisely, in the case of lysozyme, particles were stable to dilution in water (measured down to [DNA] = 200 ng/ml), but disassembled in the presence of very low amounts of salts (50 mM NaCl), thus confirming the data obtained by the gel-shift assays (Fig. 1). In the case of polylysine and polyarginine complexes, disassembly (detectable by a rapid drop in scattered light intensity) occurred rapidly both upon dilution in water and in the presence of low salt concentration. Instability in dilution for complexes obtained with low-, but not with high-molecular-weight polylysine (4 kDa) has recently been reported in the literature (Parker *et al.*, 2002). In our case, instability was observed also with the high-molecular-weight polylysine (20 kDa). It is important to notice that the range of concentration that was chosen in our experiments is significantly lower than that investigated by Parker *et al.* (2002). In fact, the sensitivity of the *in-house* assembled detection system used for these experiments is sufficient for detection of particle diffusion at concentrations lower than those reported in the literature.

Electron microscopy and avidin–DNA base pairs. Transmission electron microscopy revealed that avidin (and avidin-R)–DNA complexes prepared in water at $[+/-]>1$ preferentially assume a toroidal or rod-like conformation with an average size of 50–80 nm (Fig. 7). The same conformation is maintained even at higher $[+/-]$ ratios, although more compact structures tend to appear, together with a small percentage of globular-shaped particles. At $[+/-]=0.5$, only partial assembly into large aggregates without a defined structure could be observed [Fig. 7(a)]. In these samples, the avidin molecules appear to be concentrated in one region of the condensate, as if the assembly undergoes local nucleation.

At $[+/-]>1$, toroidal conformation appears to be favored when circular supercoiled plasmid DNA is used in the assembly. When particles are assembled using linear plasmid DNA (linearized with a restriction enzyme), rod-like structures appear to be favored. These rods show a tendency to bend or twist, and structures resembling open toroids can be observed in these preparations [Fig. 7(f)]. The size and shape of the individual condensates are consistent with the hypothesis that the particles comprise a single DNA molecule per particle. Under the same conditions of condensation with supercoiled circular DNA, polylysine, polyarginine and lysozyme form particles with a globular shape (Fig. 8). The size of particles observed by TEM is in agreement with the data obtained in light-scattering experiments. Moreover, as anticipated by the light scattering experiments, aggregates of individual condensed particles are visible in samples of avidin–DNA prepared in 10 mM Tris buffer (pH 8.0), thus confirming the that the interaction is not disrupted by the presence of salts in the medium. Even though the final complex geometry (toroids or rods) may not be relevant in the real physiological environment, the data obtained by

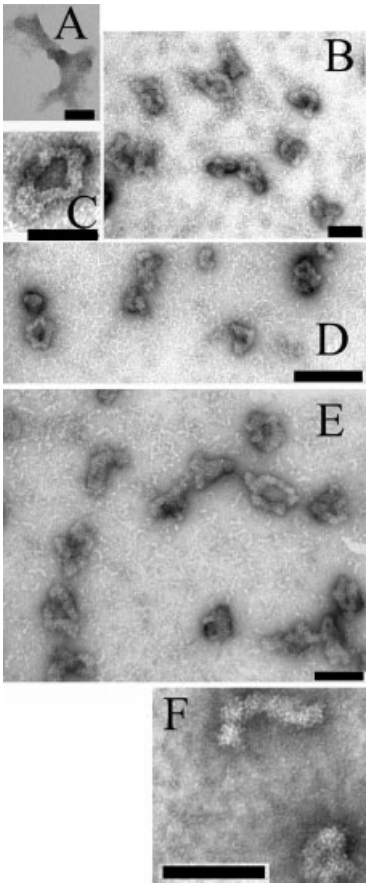

Figure 7. Transmission electron microscopy images of complexes of avidin or guanidylated avidin with circular (pGeneGrip) or linearized (GFP-T7) plasmid DNA. (A–D) Avidin–circular DNA at different $[+/-]$ charge ratios: (A) 0.5; (B, C) 3; (D) 7; (E) avidin-R–circular DNA $[+/-]=3$; (F) avidin–linear DNA $[+/-]=3.0$. Size bars: (A) 50 nm; (B, C, E, F) 100 nm; (D) 200 nm.

avidin and DNA titration indicate a high degree of organization at the molecular level. Indeed, titration carried out on complexes obtained from different DNA samples and using different component concentrations ([DNA] from 5 to 20 μg/ml) and ratios ($[+/-]=2$, 3 and 6) indicated that individual avidin molecules occupy an average of 18 ± 4 DNA base pairs. Considering the distance between two base pairs in the DNA double helix (3.4 Å), this value suggests that the avidin molecules ($\sim 7\times8\times8$ nm; Livnah *et al.*, 1993) are closely packed, probably in an organized structure. Indeed, complexes obtained with a linearized plasmid DNA preparation (GFP-T7) and the non-glycosylated (less soluble) form of avidin, when analyzed by TEM after precipitation and removal of the excess of protein in solution, were shown to contain structures that might represent intermediates of the condensation process (Fig. 9). In some of these complexes, it is possible to see the entire linear plasmid DNA [Fig. 9(A)] inundated by two rows of avidin molecules. In Fig. 9(B) the same structure appears folded upon itself (two- or three-fold), thus leading to a shorter and thicker rod (half or one-third its original length). Similar features can be observed in the toroids obtained with the circular DNA [Fig. 9(C)], thus supporting the premise of a common and organized mechanism of assembly.

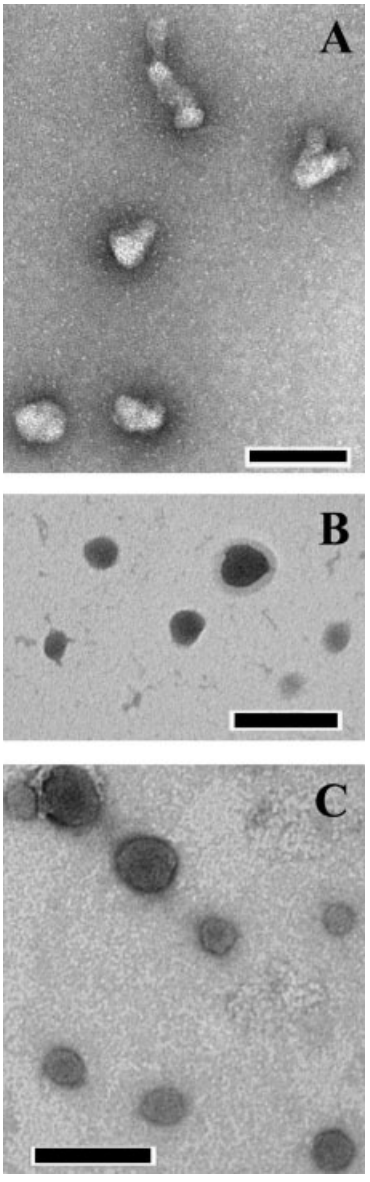

Figure 8. Transmission electron microscopy images of circular DNA (pGeneGrip) condensed with different polycations at [+/−] = 3. (A) Lysozyme; (B) polylysine; (C) polyarginine. Size bars: 100 nm.

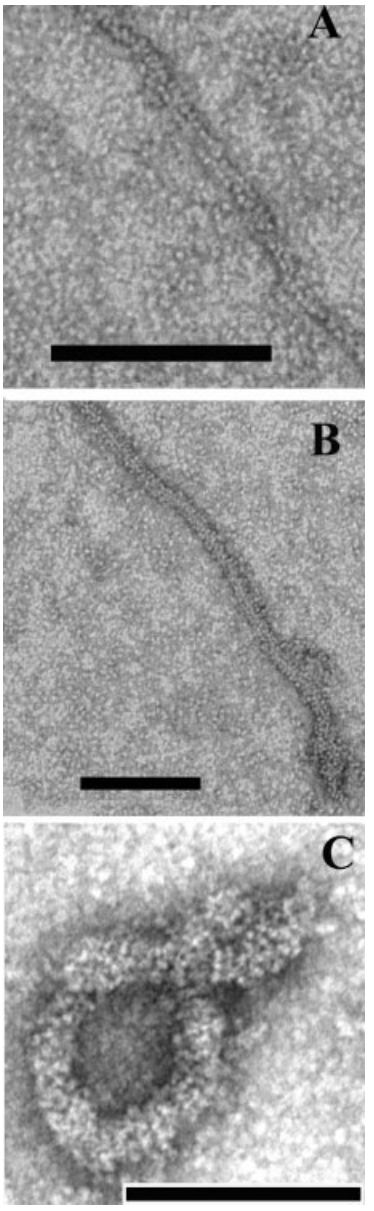

Figure 9. Transmission electron microscopy images of putative intermediates of condensation. (A, B) linear plasmid DNA; (C) circular plasmid DNA. Size bars: 100 nm.

DISCUSSION

The present work indicates that the affinity of avidin for DNA is not only due to non specific electrostatic attraction but also reflects the involvement of other recognition forces that lead to a high affinity interaction where specific regions of the protein may be involved. The affinity of this interaction is stronger in the presence of biotin, suggesting the involvement of the loop between β3–β4 strands. Besides, analysis of the amino acid distribution on avidin's surface as derived from the crystallographic data (Livnah *et al.*, 1993; Pugliese *et al.*, 1993, 1994) reveals a region at the base of the β-barrel (where both C and N-termini are located) where a high number of arginines and lysines (11!) are confined. This region may represent a potential site for electrostatic docking of the DNA.

Some of these positive residues are missing from the crystallographic data and the actual surface electrostatic potential in this region cannot be derived. Nevertheless, it is possible to propose a region of electrostatic complementarity (Lee and Tidor, 2001) where an optimal potential for the binding to DNA phosphates may exist. The fact that the even more basic guanidylated avidin exhibits a weaker interaction with DNA would support this hypothesis. Electrostatic interactions are long-range forces that might be responsible for the initial docking of the DNA (Luisi, 1995), while other regions in the protein molecule, namely the several asparagines and glutamine or the sequential Arg–Thr or Thr–Arg and Arg–Ser motifs located within the C-terminal β-strand, may provide sites for further hydrogen bonding or van der Waals interactions. In any case, the exact binding site that leads to the interaction, together with the potential specificity for defined DNA sequences, will only

be identified by future foot-printing and mutagenesis experiments and/or by crystallizing the avidin–DNA complex.

The avidin–DNA interaction is not disrupted by the presence of physiological concentrations of salts. It thus follows that the interaction may also occur in the natural environment, i.e. in the egg white. In fact, avidin concentration in the egg white (50 μg/ml; Awade, 1996) is well above the concentration levels used in this investigation. Indeed, early works have reported the isolation of avidin from the egg white in association with nucleic acids or with an acidic glycoprotein (Fraenkel-Conrat *et al.*, 1952). These findings were later disregarded for the most part and considered the consequence of an artefact (Green, 1975). However, our results indeed support the hypothesis of the occurrence of the binding *in vivo* and may shed light on a hitherto unknown physiological function of the protein in nature. Avidin is found in the egg white together with other proteins (Li-Chan *et al.*, 1995; Awade, 1996) among which many display antimicrobial properties (Li-Chan *et al.*, 1995). For example, some proteins act by sequestering nutrients or chemicals that are important for bacterial growth. In this respect avidin has also been considered an antimicrobial agent because it induces biotin depletion (Kramer *et al.*, 2000). Another important antimicrobial protein from egg white is lysozyme, an enzyme that acts by hydrolyzing the bacterial cell wall. Avidin may also act synergistically with lysozyme by sequestering the bacterial DNA after it is liberated, or it may represent a defence from viral infections. Alternatively, avidin may somehow be involved in the differentiation of the chicken embryo. In this context, the increase in affinity between avidin and DNA in the presence of biotin may represent a switch for the molecule between two different binding states, each having a distinct function.

Our results show that the avidin–DNA interaction in water induces condensation of DNA with the formation of toroids or rods of well-defined size. It is difficult to determine at this point whether the toroids or the rods observed with avidin have any physiological meaning. Toroidal conformation has been reported previously for DNA condensates (Hud *et al.*, 1995; Bloomfield, 1996, 1998; Yoshikawa, 1997; Lin *et al.*, 1998; Vijayanathan *et al.*, 2001). Indeed, DNA condensation can occur in the presence of cations with charge ratios of at least +3, and several theoretical studies have described the forces and energy involved in the process of toroid formation (Bloomfield, 1996; Stevens, 2001) upon condensation. Some authors have reported that the particle shape is independent of the condensing agent used, claiming instead that it is determined by the medium used. For example, H1 and H5 histones and clupeine all form toroids in the presence of DNA but only at high salt concentration (Garcia-Ramirez and Subirana, 1994). Under appropriate conditions, toroids have been observed with polycations that have a natural DNA binding activity (histones, spermine and spermidine; Brewer *et al.*, 1999, 2002) as well as with synthetic polymers like polylysine (Kwoh *et al.*, 1999) or inorganic salts like $Co(NH_3)_6$ (Bloomfield, 1996). Nevertheless, instability to extensive dilution or to the presence of ions has been reported for condensates obtained with this second class of molecules (Widom and Baldwin, 1980; Parker *et al.*, 2002). While it is difficult to state, at present, whether such structures have, in the case of the DNA–avidin complexes, any physiological role, their stability with respect to dilution and to the presence of salt, together with the well-known biotin-binding property of avidin, may provide potential biotechnological and biomedical applications.

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