Studies on the Mode of Ku Interaction with DNA*

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The Ku heterodimer plays a central role in non-homologous end-joining. The binding of recombinant Ku to DNA has been investigated by dynamic light scattering, double-filter binding, fluorescence spectroscopy, and band shift assays. The hydrodynamic radius of Ku in solution is 5.2 nm and does not change when a 25 bp double-strand DNA (dsDNA) fragment (D25) is added, indicating that only one Ku molecule binds to a 25-bp fragment. The dissociation constant (kd) for the binding to D25 is 3.8 ± 0.9 nm. If both ends of the substrate are closed with hairpin loops, Ku is still able to bind with little change in the kd. The kd is not affected by ATP, Mg2+, or ionic strength. However, the addition of bovine serum albumin decreases the kd by 2-fold. DNA substrates of 50 bp can bind two Ku molecules, whereas three molecules are bound to a 75-bp substrate. Data analysis with the Hill equation yields a value of the Hill coefficient (n) close to 1, and the kΔ values for the binding of Ku to both ends of these substrates are the same. Thus, we demonstrate that there is no cooperative interaction among the Ku heterodimers binding longer substrates.

DNA double-strand breaks (DSBs)1 arise in cells during physiological processes such as DNA recombination and meiosis (1, 2). Immunological diversity is generated by V(D)J rearrangement of genes encoding B cell immunoglobulins and T cell receptors (3, 4). They are also generated by both exogenously and endogenously generated DNA-damaging agents, including ionizing radiation and reactive oxygen species that arise as by-products of DNA metabolism. If left unrepair, they lead to chromosome translocation and cancer. Cells have evolved two different pathways for repairing DSBs, namely homologous recombination and non-homologous end-joining DNA (NHEJ). NHEJ is the dominant pathway in cells of multicellular eukaryotes, while homologous recombination prevails in diploid Saccharomyces cerevisiae (5). Mammalian cells utilize the same reaction to repair both radiation-induced DSBs and breaks induced during V(D)J recombination (6).

Five proteins that function in NHEJ in mammalian cells have been identified to date, namely Ku70, Ku80 DNA-PKcs, Xrcc4, and ligase IV (7). Three of these (Ku70, Ku80, and DNA-PKcs) constitute a complex termed the DNA-dependent protein kinase (DNA-PK). DNA-PKcs is a large protein of 469 kDa and a member of a sub-group of phosphatidylinositol 3-kinases, called phosphatidylinositol 3-kinase-related kinases (8). Ku70 and Ku83 are subunits of the heterodimeric protein Ku and require heterodimerization for stability and function (9). Ku has double-stranded (ds) DNA end binding activity (10) and once bound can slide along the DNA in an energy-independent manner (11). Recently, the crystal structure of the Ku heterodimer, both in the presence and absence of DNA, has been determined (12). The structure shows that Ku has the shape of a ring with a large base and a narrow "handle." When bound to DNA, the conformation of Ku does not change, and a dsDNA duplex fits precisely inside the ring. One face of the duplex DNA remains relatively accessible to the solvent, because it is only partially covered by the narrow handle of the Ku molecule. In this way, the processing enzymes may have easy access to this side of the DNA duplex to remove damaged nucleotides and fill gaps prior to ligation. Although the structural studies are very useful in providing a structure of how Ku binds to DNA, they do not provide information on the dynamics of the interaction with Ku. Here, we exploit physical studies to evaluate further the information gained from the structural studies. In addition, the structure was determined for a Ku variant that lacked the C terminus of Ku83, a region that does not seem to be involved in the binding of Ku to DNA but is required for the interaction with DNA-PKcs (13). Several laboratories have investigated the binding of Ku to DNA and have shown that Ku cannot bind any DNA substrate shorter than 14 bp (14). It has been also shown that Ku binds avidly to dsDNA ends independently of the oligonucleotide sequence and of the exact structure of the ends, whether they are blunt, with 5' or 3' overhangs, or even with hairpin loops (15). Binding studies performed by different groups with dsDNA fragments of similar length and structure have yielded values of the dissociation constants (kd) that vary from the low picomolar to the nanomolar range (10, 16, 17). Additionally, little is known about the cooperativity of Ku binding to DNA (18).

Under thermodynamic equilibrium conditions, we have examined the binding of Ku to dsDNA fragments of different

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1 The abbreviations used are: DSB, double-strand break; NHEF, non-homologous end-joining DNA; NDA-PK, DNA-dependent protein kinase; ds, double strand; rKu, recombinant Ku protein; D25, D50, D75, double-strand DNA fragments (25, 50, and 75 bp); H25, H50, H75, duplex DNA substrates with one hairpin loop (25, 50, and 75 bp); CD, circular dichroism; DLS, dynamic light scattering; ACF, autocorrelation function; BSA, bovine serum albumin.
length and structure. The stoichiometry of the complexes that Ku forms with the different substrates was determined by dynamic light scattering, which reflects the change in the value of the hydrodynamic radius of the Ku heterodimer when DNA is added (19). In addition, the value of the hydrodynamic radius of the molecule provides valuable information on the shape and the hydration shell of the protein. The affinity of Ku for DNA and the presence of cooperativity among the Ku molecules binding longer substrates was investigated by double-filter binding, an improved filter binding technique that has already been used to study protein-DNA interactions (20, 21). Fluorescence studies have also been performed to support the results obtained by double-filter binding. Our results provide an accurate estimate of the dissociation constant for binding that is consistent with the two methods employed. We show that there is no cooperativity among the Ku molecules binding to DNA substrates and that Ku binds efficiently to hairpin loops. Together our results provide novel insights into the mechanism of Ku binding to DNA.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Ku—The procedure for the expression of Ku in baculovirus was developed by Miroslav Chovanec in the laboratory of Dr. Penny Jeggo and is based on the same method already applied for the expression of the XrecligIV complex (22). The Sf9 (Spodoptera frugiperda ovary) cells were a kind gift from the laboratory of Dr. Antonino Cattaneo (Scuola Internazionale Superiore di Studi Avanzati, Trieste) and they were maintained at 27 °C in SP-900 II medium (Invitrogen) supplemented with 10 μg/ml gentamicin or stored at −80 °C in 50% conditioned media with 7.5% Me2SO. The addition of 10% fetal bovine serum did not show any improvement in the yield and the quality of the final recombinant Ku protein (rKu).

The human p70 and p83 Ku cDNAs were both subcloned into the BamHI restriction site of the pFastBac HTB vector (Invitrogen). Two different recombinant baculoviruses expressing, respectively, histidine-tagged Ku70 and Ku83 subunits were produced. The amino acid sequence preceding each subunit is the following: MSYYHHHHHHHDY-DIPTQNLKYGEMSTM and contains 6 histidines, a linker region, and an rTEV protease cleavage site. After rTEV cleavage, 7 residues remain bound to the N terminus of each subunit. Both the baculoviruses were used at the same multiplicity of infection (5–10 plaque-forming units/cell) to co-infected Sf9 cells cultured in T75 flasks (2–107 cells/flask). Seventy-two hours after infection cells were harvested, extracted by centrifugation, and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 1% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Molecular Biochemicals, Molecular Biochemicals). 5 ml of lysis buffer were used for each flask (2 × 107 cells), and the incubation was for 10 min at 4 °C. Recombinant Ku70/83 heterodimer was clarified in cell lysate by SDSPAGE and immuno blotting using specific monoclonal antibodies (data not shown). Then the lysate was cleared by centrifugation and incubated with TALON metal affinity resin (CLONTECH) (1 ml of resin/5 mg of protein) for 2 h at 4 °C. The resin was washed with lysis buffer and with buffer (20 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 12.5 mM imidazole) containing 500 mM KCl (three washes) and 100 mM KCl (two washes). The polyhistidine-tagged rKu was eluted in buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM β-mercaptoethanol, 100 mM imidazole). The purity of the preparation was verified by SDS-polyacrylamide gel stained with silver.

To remove the N-terminal polyhistidine sequence, rKu was incubated overnight at 4 °C with rTEV protease (Invitrogen) in buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol), and the digested sample was loaded again of the TALON column. The fraction not retained by the resin containing rKu was concentrated and stored in buffer (20 mM Tris-HCl, pH 8.0, 60–100 mM KCl, 5 mM β-mercaptoethanol). N-terminal sequence analysis was performed as a final step to verify the removal of the His-Tag. If protein needed to be frozen at −80 °C, 40% glycerol was added to reduce loss of dsDNA binding capability. To estimate the concentration of the rKu heterodimer by UV absorption measurements using an extinction coefficient at 280 nm of 77,660 M−1 cm−1 simply estimated from the amino acid sequence (ProtParam, available at www.expasy.ch). The extinction coefficient value increases to 87,900 M−1 cm−1 if the histidine tag is still present. Approximately 0.5 mg of rKu was obtained from 5 × 107 infected Sf9 cells. Gel shift assays showed that rKu binds a 25-mer DNA with the same affinity of the HeLa Ku used as a control.

Native HeLa Ku—The Ku heterodimer was purified from HeLa cells, as described previously (16, 23). For the last step of purification, a dsDNA-Sepharose affinity column was used. The column was equilibrated in buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol), and the elution was performed with a linear gradient of from 0.1 to 1 mM KCl (20 column volumes). The Ku heterodimer obtained after the last step of purification was judged to be at least 95% pure as determined by SDS 8% polyacrylamide gel stained both with Coomassie Brilliant Blue and silver.

DNA Substrates—All the oligonucleotides used for the binding experiments were obtained from Sigma-Genosys or Invitrogen. The DNA substrates used for all the experiments are listed in Table I. The blunt-end 25-mer duplex (D25) was made by annealing the oligonucleotide 5′-GAT CTC GCA TCA CGT GAC GAA GAT C-3′ with its complement. Complementary oligonucleotides were annealed in buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl2) first heating the samples at 95 °C for 5 min and then gradually cooling them until they reach room temperature. The 50- and 75-mer (D50) and 75-mer (D75) blunt-end DNA substrates contain the same sequence of the D25 repeated two and three times, respectively. The 25-mer duplex DNA substrate with one hairpin loop (H25) was made using a single oligonucleotide containing the D25 sequence followed by eight T and the reverse complementary sequence of the D25 (H25 = 5′-(D25) TTT TTT TGT ATG TGC GTC ACG TGA TGK GAC ATC-3′). Also the 50-mer (H50) and 75-mer (H75) substrates with one hairpin were prepared using a single oligonucleotide of 108 and 158 bp, respectively. The double-hairpin substrate (2H25) was prepared using the following oligonucleotide 5′-GTG ACG AAG ATC TTC TTT TTC TTG ATC TTC TGC GTC ACG TGA TGK GAC ATC-3′. The double-hairpin substrate (2H25) was prepared using the following oligonucleotide 5′-GTG ACG AAG ATC TTC TTT TTC TTG ATC TTC TGC GTC ACG TGA TGK GAC ATC-3′.

The double-hairpin substrate was with a nick (2H25 T). To fill the gap with the missing base and generate in this way a close double-hairpin probe (2H25), a 5′-phosphate was added, 15.9 pmol of the substrate was incubated with 16.7 pmol of [α-32P]CTP using 5 units of polymerase I Klenow fragment (New England BioLabs) and ligation was done with 400 units of T4 ligase (New England BioLabs). The efficiency of the ligation was verified comparing the 2H25 closed substrate and the 2H25’ substrate with a nick or denaturating 7 M urea 8% polyacrylamide gel (Fig. 4B).

Spectroscopic Studies—Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.). Spectra were collected at a protein concentration of about 0.1 μM in 600 μl of buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM β-mercaptoethanol) and using a quartz cuvette with a optical path of 1 cm. The parameters used for the acquisition of the fluorescence spectra were as

| Description of the substrates used in the binding experiments | D25 | D50 | D75 | H25 | H50 | H75 | 2H25 | 2H25’ |
|---|---|---|---|---|---|---|---|---|
| | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 25 | 50 | 75 | 8T | 50 | 75 | 8T | 3 | 3 |
| 25 | 25 | 25 | 50 | 50 | 50 | 75 | 3 | 3 |
| 25 | 25 | 25 | 3 | 3 | 3 | 3 | 3 | 3 |
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follows: excitation wavelength 280–282 nm, speed 300 nm/min, time constant 0.2 s, excitation and emission slits 5 nm. For titration experiments, the substrate concentration was varied between 0.01 and 100 nm. Proper corrections were applied to take in account the change in volume due to the addition of the substrates. The fluorescence data were analyzed with the Langmuir equation for the determination of the $k_d$ values like in the case of the double-filter binding studies.

Circular dichroism (CD) spectra in the far UV (200–260 nm) were collected on a JASCO J-600 dichrograph using a cylindrical cell with optical path of 0.1 cm. Proteins at a concentration of few micromolar were analyzed in 20 mM phosphate buffer, pH 7.2, containing 60 mM KCl and 1 mM MgCl$_2$. The acquisition parameters were: bandwidth 1 nm, time constant 4 s, speed of 5–10 nm/min, and step resolution 0.1 nm. The average of three to five CD spectra was baseline-corrected, expressed in terms of molar ellipticity (degcm$^2$dmol$^{-1}$) and analyzed with CDNN software (version 2.1) (24).

Dynamic Light Scattering—DLS measurements were performed using a DynaPro-801 instrument (Protein Solution, Charlottesville, VA) where the scattered light was collected at an angle of 90° through a fiber optic and converted to an electrical signal by an avalanche photo diode. The time-dependent autocorrelation function (ACF) of the photon current was monitored with a 20-channel software correlator (based on a Digital Signal Processor (DSP) unit) provided by the manufacturer. The first sampling time was 3.86 μs. The length of the subsequent channels increases in a quasi-logarithmic fashion.

The samples were gently injected into the cell through a series of Whatman filters with decreasing porosity, from 0.1 to 0.02 μm. The protein concentration was in the range of 3.5–4 μM, and the buffer used for measurements was 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol, 1 mM MgCl$_2$. The DNA substrates were added in slight excess over the protein concentration ([DNA]/[Ku] = 1.25), and the solution was incubated for 30 min at room temperature before collecting data. Autocorrelation functions (ACF) were measured every 10 s, containing $10^3$ to $10^6$ counts each. Data analysis was performed with a non-linear second order cumulant method (25, 26),

$$G(t) = \langle b(t + 1 + sn) \exp(-\Gamma_{f}t_{f} + \frac{1}{2}\Gamma_{f}^{2}t_{f}^{2})\rangle$$

(Eq. 1)

where $\Gamma_f$ and $\Gamma_{f}$ are the first and second cumulants, $b$ is a baseline in arbitrary counts and $sn$ is a parameter related to the signal-to-noise ratio (i.e. the maximum initial value of the ACF). The hydrodynamic radius $R_h$ of the molecule was derived from the first cumulant $\Gamma_f$ using the Stokes-Einstein relation:

$$R_h = \frac{k_B T}{6 \pi \eta \Gamma_f}$$

(Eq. 2)

where $k_B$ is the Boltzmann constant, $T$ is the Kelvin temperature, and $\eta$ is the solvent viscosity at the experimental Kelvin temperature $T$. The sample polydispersity ($P$) was computed as [GRAPHIC3]. Measurements were done at 20.2 ± 0.1 °C as monitored by a built in Peltier junction. The water refractive index at the laser wavelength of 843.4 nm and at a temperature of 20.2 °C was $n = 1.3282$, as interpolated from available data (27). The values of $R_h$ were corrected for the relative viscosity and refraction index of the buffer solution (20 mM Tris-HCl, 100 mM KCl). The presence of 5 mM β-mercaptoethanol was neglected.

Double-filter Binding—Double-filter binding measurements were performed with a modified 96-well dot blot apparatus that allows the addition of a DEAE membrane for the retention of unbound DNA to reduce the errors on the determination of the binding isotherm. The two membranes were subjected to autoradiography (Instant Imager, Packard Corp., Meriden, CT) to quantify the radioactivity of each dot. For the analysis of the data, we considered the ratio of DNA over the macromolecule (M) that can bind one or more ligands (Ku). Isotherms were plotted as the fraction of DNA molecules bound to Ku ($Y_f$), [DNA$_{bound}$/DNA$_{total}$], versus the total ligand (Ku) concentration ($x$). The fraction of DNA bound ($Y_f$) was calculated using the following equation:

$$Y_f = N_f/[N_f + D_f]$$

(Eq. 3)

where $N_f$ and $D_f$ are the radioactive counts retained on the nitrocellulose and DEAE membranes, respectively. All the experimental data were fitted in two different ways. First with the simple Langmuir equation,

$$Y_f = A + B e^{-x_i/(x_i + k_d)}$$

where the floating parameters are the dissociation constant ($k_d$) and two parameters, $A$ and $B$, that take in account for the free DNA retained nonspecifically by the nitrocellulose membrane (A) and for the incomplete retention of the protein-DNA complex by the nitrocellulose (B).

The baculovirus expression system allows the expression and the purification of milligram quantities of highly pure (>99% as judged from Coomassie- and Silver-stained SDS-polyacrylamide gel) recombinant Ku heterodimer (rKu) (Fig. 1A). An electrophoretic mobility shift assay showed that rKu binds the 26-bp blunt-end dsDNA substrate (D25) with the same affinity of the native Ku, purified from HeLa cells (Fig. 1B). Circular dichroism and fluorescence studies proved that the recombinant and native proteins have almost identical secondary structure (data not shown).

 Stoichiometry of Ku Binding to dsDNA—The size of the Ku molecule alone and in complex with dsDNA fragments of different length and structure was measured by Dynamic Light Scattering (DLS) (Table II). The linear trend of the autocorre-
TABLE II

| Sample | $R_g$ (nm) | Polidispersity |
|--------|-----------|----------------|
| Ku     | 5.21 ± 0.04 | 0.045 ± 0.014 |
| Ku + D25 | 5.31 ± 0.04 | 0.055 ± 0.006 |
| Ku + H25 | 5.45 ± 0.02 | 0.052 ± 0.004 |
| Ku + 2H25 | 5.33 ± 0.02 | 0.046 ± 0.006 |
| Ku + D50 | 7.06 ± 0.02 | 0.069 ± 0.008 |
| Ku + H50 | 7.55 ± 0.02 | 0.067 ± 0.005 |

Binding function (ACF) transformed according to the cumulant analysis (25)(Fig. 2B) and the low values of the polidispersity (Table II) demonstrates that the samples are monodispersed both in the absence and presence of dsDNA, showing that the Ku heterodimer does not aggregate under these conditions. In addition, no change in the average size and in the polydispersity of the protein preparation was observed under different temperatures ranging from 6 to 25 °C (data not shown). Fig. 2A shows the autocorrelation function measured for Ku alone, Ku bound to the 25-bp dsDNA (D25), and Ku bound to the 50-bp dsDNA (D50). The ACF of the solution containing Ku alone is indistinguishable from that of the solution containing the complex of Ku with D25, whereas the solution containing the Ku-D50 complex clearly shows an ACF with a longer decay time (Fig. 2A). The hydrodynamic radius of rKu alone is calculated to be 5.21 ± 0.04 nm (Table II). This value does not change significantly when Ku is complexed with the D25 substrate, demonstrating that only one Ku molecule is bound to a blunt-end DNA substrate of 25 bp. In contrast, the hydrodynamic radius increases to 7.06 ± 0.02 nm when Ku is incubated with the D50 substrate indicating that the Ku-D50 complex contains more than one Ku molecule (Table II).

The stoichiometry of Ku binding to the dsDNA fragments has been investigated also by electrophoretic mobility shift assay (Fig. 3). When Ku is incubated with the D25 substrate, a major lower band is detectable, while two distinct bands are visible in the presence of the D50 probe indicating that the 50-bp fragment can form a complex with one or two Ku molecules consistent with the conclusion based on DLS. Fig. 3 shows that Ku is also able to bind a 25-bp substrate where one (H25) or both ends (2H25) have been modified with the addition of an eight-nucleotide hairpin loop. The fainter upper bands present in the experiments done with D25, H25, and 2H25 suggest that these substrates may also be capable of accommodating two Ku molecules. Because the crystal structure shows that each Ku molecule covers about 20 bp and all these substrates are shorter than 40 bp, the upper bands may represent a less likely situation where two Ku molecules are not “fully” bound to the substrate. Experiments done with a 75-bp duplex DNA show the presence of a third upper band suggesting that, in this case, three Ku molecules are bound to DNA (see below).

Binding Affinity of Ku for dsDNA—The double-filter binding technique, which has been used previously to study protein-DNA interactions, was exploited here to measure the binding affinity of Ku for eight different DNA probes (Table III). This technique utilizes a DEAE membrane beneath the nitrocellulose membrane as an additional means to trap DNA that fails to bind to the nitrocellulose filter. This modification increases both the accuracy and precision of the measurements while reducing the time required for analysis. Fig. 4A shows the β-emission images for both the nitrocellulose and DEAE membranes where the 25-bp blunt-end dsDNA (0.8 nM) was titrated with the Ku protein. There is an inverse relationship between the radioactivity retained by the two filters and quantification of the radioactivity allowed the construction of the binding curves shown in Fig. 4B. The dissociation constant ($k_d$) for the binding of recombinant Ku to the blunt-end dsDNA substrate of 25-bp (D25) is 3.8 ± 0.9 nM. Surprisingly, the addition of one or two hairpin loops to the same substrate (H25 and 2H25) has only a small effect on the $k_d$ values (Table III) demonstrating that Ku binds to a “closed” substrate lacking free ends (2H25). As already shown by light scattering and band shift experiments, a dsDNA fragment of 50 bp (D50) can bind two Ku molecules. The dissociation constant ($k_d$) for the binding of Ku to both sites of D50 is 7.9 ± 0.6 nM, and the value of the Hill coefficient ($n$) is close to 1 indicating the absence of cooperativity (Table III). This $k_d$ value remains the same within experimental error if the data are fitted with the Langmuir equation. Thus, our experiments demonstrate that the two Ku binding sites on the D50 probe are identical and independent (Fig. 5). The 75-bp dsDNA probe (D75) can accommodate three molecules of Ku. Two are bound to the dsDNA ends, and the third must, therefore, lie internally. Again, no cooperative interaction is observed ($n$ = 1) between the molecules of Ku binding the substrate (Fig. 5). The $k_d$ values for the binding of Ku to the two ends of D75 is the same, within the experimental error, measured for the binding of the heterodimer to the D50 probe (Table III).

Similarly, for the two single-hairpin substrates of 50 and 75 bp (H50 and H75) that can bind two and three Ku molecules, respectively (Fig. 5), the experimental data indicate the absence of cooperativity (Table III).

Fluorescence Studies—The fluorescence spectrum of rKu shows a maximum of emission at 349 nm, and the signal is primarily due to the 6 Trp residues present in the rKu molecule. The 2 extra Phe and 8 extra Tyr residues that are present in the sequence of the histidine-tag significantly affect the fluorescence spectrum of the rKu molecule, shifting the emission maximum from 349 to 355 nm (Fig. 6A). The values of the dissociation constants ($k_d$) determined for the protein with and without the histidine-tag are very similar, suggesting that this extra N-terminal tail of 30 amino acids does not affect the binding of Ku to DNA. The fluorescence signal is reduced or quenched by about 10–15% when rKu is titrated with increasing amounts of dsDNA substrates (Fig. 6B). The experimental data obtained titrating Ku with a 25-bp blunt-end dsDNA fragment (D25), can be fitted very well with the simple 1:1 Langmuir equation (Fig. 6C) giving a $k_d$ value of 6.6 ± 1.5 nM that agrees with the one obtained from double-filter binding experiments. Taken together, the fluorescence studies carried out with D50 and D75 as well as with the other substrates used in this work give results consistent with those obtained by filter binding (data not shown).

Effects of Mg$^{2+}$, ATP, BSA, and Ionic Strength on the Binding Affinity—A set of double-filter binding titration experiments was collected to investigate the contribution of Mg$^{2+}$, ATP, BSA, and ionic strength on the affinity of Ku for DNA (Fig. 7, A and B). The values of the dissociation constants are not affected significantly by the presence of Mg$^{2+}$ or ATP (Fig. 7A). However, the presence of 80 μg/ml BSA improves the affinity of Ku for DNA by a factor of two (Fig. 7, top panel). The values of the dissociation constant do not change when the KCl concentration is varied from 0 to 150 mM (Fig. 7, bottom panel) in agreement with previous salt dependence studies carried out by other groups (28). This behavior of Ku raises interesting questions on the mechanism of Ku binding to DNA, because it is distinct from that observed for other DNA binding proteins where the salt concentration dramatically affects the affinity for DNA (29, 30).
Analysis of the Hydrodynamic Radius of the Ku Heterodimer—As already mentioned, the hydrodynamic radius of rKu alone in solution is calculated to be 5.21 ± 0.04 nm using the classical Stokes-Einstein relation for a sphere described under “Experimental Procedures,” where the friction coefficient $f$ is equal to $6\pi\eta R$ (Table II). Because the crystal structure shows that Ku has a shape of an ellipsoid with an axial ratio of two, the Perrin coefficient $F$ (Table II) can be used to correct the hydrodynamic radius for the ellipsoid shape (31) giving a new hydrodynamic radius of 4.93 nm. This value is similar to the one calculated for Ku by other laboratories but is greater than that predicted for a molecule of similar size (153.5 kDa) (11, 14, 32). In fact, assuming a value for the specific volume of 0.74 cm$^3$/g (31), an anhydrous molecular volume of 190 nm$^3$, and a theoretical
The hydrodynamic radius \( R_h \) of 3.57 nm would be predicted for a molecule of 153.5 kDa. Consistent with our results, the recent crystal structure of rKu indicates that the molecule has the shape of an ellipsoid with an overall dimension of 12 × 7 × 6 nm that corresponds to a molecule with an anhydrous molecular volume of 264 nm\(^3\) and an average anhydrous radius of 3.78 nm (Fig. 8). Adding the 167 amino acid residues that are missing at the C terminus of the Ku83 subunit in the crystal structure, we estimate that the value of the radius for the entire molecule would be 4.16 nm. A possible reason why this value is considerably bigger than the theoretical value measured for a globular protein of similar size \( (R_h \approx 3.57 \text{ nm}) \) is that the structure of Ku shows the presence of a 2-nm diameter hole in the center of the molecule that increases the total radius (Fig. 8). However, this value of 4.16 nm estimated from the crystal structure is still smaller than that obtained in our DLS studies. Even adding a typical 1- to 1.3-nm hydration shell \( (33) \) to the anhydrous radius estimated from the crystal structure, a value 4.45–4.52 nm is obtained. This value is 0.48–0.44 nm smaller than ours (4.93 nm) raising the possibility that Ku may be surrounded with a hydration shell thicker than 1.3 nm.
DISCUSSION

Several studies have highlighted the key role that the Ku heterodimer plays in NHEJ and V(D)J recombination (34, 35). Recently, it has been shown that this dsDNA binding protein may be involved in other fundamental physiological processes such as DNA replication (36). Notwithstanding a plethora of knowledge about Ku, the biophysical properties of this molecule in solution and the mechanism of Ku binding to DNA still demand further investigation. Because biophysical studies often require milligram quantities of material, an efficient baculovirus expression system for the Ku heterodimer was developed. With this procedure, ~0.5 mg of rKu are obtained from $5 \times 10^7$ infected Sf9 cells. Although the histidine-tag does not seem to affect the binding of Ku to dsDNA, this 30-amino acid tail was removed from both subunits.

By DLS we have demonstrated that the rKu heterodimer, with or without the histidine-tag, does not aggregate. We have also determined the stoichiometry of the complexes that Ku forms with the different DNA probes by measuring the hydrodynamic radius of the molecule in solution. The hydrodynamic radius of rKu alone in solution is 5.21 or 4.93 nm if the correction for a ellipsoid is applied. This value is greater than the one that can be predicted for a molecule of similar size (153.5 kDa) in agreement with previous results (11, 14, 32). One explanation of this difference is the presence of a 2-nm diameter hole in the center of the molecule, which may serve to increase the radius (Fig. 8). However, the value obtained from our DLS studies is also greater than the anhydrous hydrodynamic radius that can be estimated from the crystal structure (4.16 nm).

A possible explanation is that Ku may be surrounded by a thick hydration shell that increases the radius of the molecule in solution. The surface of the molecule is strongly charged, and the structure shows a disordered highly acidic N-terminal tail of 33 amino acids in the Ku70 subunit that points outside the globular domain, which is likely to be highly hydrated.

The hydrodynamic radius does not change significantly when the 25-bp duplex DNA (D25) is added to the solution containing Ku (Table II). This result indicates that only one Ku molecule is involved in the binding of a DNA probe of 25-bp in contrast to previous gel filtration studies done with DNA fragments of similar length (37) but in agreement with several studies done by other laboratories (18, 32). In contrast, a 50-bp fragment can bind two Ku molecules increasing the hydrodynamic radius from 5.31 ± 0.04 nm (D25) to 7.55 ± 0.04 nm (D50). It is worth mentioning that simply doubling the mass of a molecule with a radius of 5.21 nm would yield a new radius of 6.7 nm. The bigger value measured experimentally probably reflects the fact that the two molecules lie adjacent to one another on the 50-dp duplex DNA.

Our measurements of the dissociation constant ($k_d$) for the binding of the rKu heterodimer to the blunt-end DNA probe of 25 dp yielded a $k_d$ value of 3.8 ± 0.9 nM in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol. Surface plasmon resonance and electrophoretic mobility shift assays yielded a $k_d$ value between 0.38 and 1.4 nM for the binding of recombinant Ku to an 18-bp probe and a value of 0.16 nM for the binding to a 22-bp probe (18). On the other hand, Falzon et al. (15) measured $k_d$ values ten times lower (15-30 pM) for the binding of HeLa Ku to a blunt-end probe of 30 bp. The affinity of Ku for DNA seems to be independent of the oligonucleotide sequence of the DNA substrate and of the particular structure of the DNA ends (38), but it is affected by the length of the DNA duplex (18). Because the $k_d$ values reported above are obtained with substrates of similar length, the discrepancy in measured values may be due to the different experimental conditions and techniques used for the binding studies. Here, we show that the addition of BSA (80 μg/ml) to the reaction buffer induces a decrease in the $k_d$ value by a factor of two, and a similar effect of albumin in protein:DNA interaction has been documented (39). In addition, more than one technique should be used to determine a binding constant to prevent errors that may be coupled with the particular method chosen for the binding studies. In our case, double-filter binding studies were coupled with fluorescence measurements for a comparison of the binding constants.

Because the crystal structure shows that Ku has the shape of a ring where the hole is of the right size to fit the dsDNA duplex, our results on Ku binding to a double hairpin substrate (2H25) lacking free ends is surprising. One possibility is that Ku can enter the substrate inducing a distortion of the hairpin loop. The energy cost for this process may be reflected by the lower affinity measured for this substrate (Table III). However, a clear answer to this question will be obtained from further investigation.

One of the most important conclusions from our binding studies with DNA substrates that can accommodate two (D50) or three (D75) Ku molecules is that there is no cooperative interaction among the Ku heterodimers. The $k_d$ value for the binding of Ku to both ends of D50 and D75 is around 8 nM and the Hill coefficient ($n$) has a value close to 1 (Table III). This result is in contrast with previous studies, where it was shown that two Ku molecules bind a 45-bp substrate in a cooperative fashion (18). A possible explanation is that the 45-bp substrate, which is 5-bp shorter than the D50 probe used here, is of insufficient length to allow two molecules to bind without interaction. In agreement with this possibility, the same authors do not observe any cooperativity using longer substrates that can bind three or four Ku molecules (18). Our results suggest that, in a *in vivo* situation, when a break occurs, the first Ku molecule binds to the broken end of the DNA and moves along of the dsDNA molecule without interfering either way with the binding of additional Ku molecules to the same end.

The affinity of many DNA binding proteins for oligonucleotides and the activity of many DNA processing enzymes is controlled by ATP and Mg$^{2+}$ (40, 41). We have shown, here, that Ku binding to DNA is not affected by the addition of ATP or Mg$^{2+}$ to the reaction mixture. Our studies have also shown that the affinity of Ku for DNA is not altered when the concentration of KCl is changed from 0 to 150 mM in buffer (20 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol). This result is in agreement with previous studies that have shown that it is necessary to increase the salt concentration up to 0.3–0.4 M to decrease the affinity of Ku for DNA (28) but differs from the behavior of other DNA binding proteins whose affinity for DNA is largely dependent on the salt concentration (29, 42).

The picture that emerges from our studies and from the crystallographic data indicates that Ku binds to DNA ends with high affinity and with a “rigid body” association process, because its conformation does not change upon DNA binding. This binding event is hardly affected by any cation or ATP derivative that may be present in the solution. After binding to the DNA ends, the Ku molecules can slide along the dsDNA chain and their presence on the DNA does not favor or impair the binding of additional Ku molecules to the same dsDNA break, because there is no cooperativity of binding. This picture may become more complex if other proteins known to interact with the Ku molecule *in vivo*, such as DNA-PKcs or the Xrc64-LigIV complex, are taken into account. For this reason, further studies will be carried out to assess the effect of these other proteins in Ku binding to DNA and vice versa,
to obtain a better understanding of the mechanism of NHEJ in human cells.

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