DNA non-homologous end joining (NHEJ) is the dominant pathway of DNA repair in multicellular eukaryotes (1). Six proteins involved in NHEJ in mammalian cells have been identified to date, Ku70, Ku80, DNA-PKcs, Xrcc4, DNA ligase IV, and Artemis (2–4). DNA ligase IV forms a tight complex identified to date, Ku70, Ku80, DNA-PKcs, Xrcc4, DNA ligase IV, and Artemis (2–4). DNA ligase IV forms a tight complex with two or three heterodimers were analyzed with two independent models considering the presence and absence of overlapping binding sites. This analysis demonstrated that there is no or very weak nearest-neighbor cooperativity among the Ku molecules. These models can most likely be applied to study the interaction of Ku with duplexes of any length. Furthermore, our salt dependence studies indicated that electrostatic interactions play a major role in the binding of Ku to DNA and that the $k_d$ decreases ~60-fold as the salt concentration is lowered from 300 to 200 mM NaCl. The binding isotherms for DNA duplexes with two or three heterodimers were analyzed with two independent models considering the presence and absence of overlapping binding sites. This analysis demonstrated that there is no or very weak nearest-neighbor cooperativity among the Ku molecules. These models can most likely be applied to study the interaction of Ku with duplexes of any length. Furthermore, our salt dependence studies indicated that electrostatic interactions play a major role in the binding of Ku to DNA and that the $k_d$ decreases ~60-fold as the salt concentration is lowered from 300 to 200 mM NaCl. The slope ($I_{sat}$) of the plot of log $k_d$ versus log[NaCl] is 12.4 ± 0.1. This value is among the highest reported in the literature for a protein-DNA interaction and suggests that ~12 ions are released upon formation of the Ku-DNA complex. In addition, comparison of the slope values measured upon varying the type of cation and anion indicated that approximately nine cations and three anions are released from DNA and Ku, respectively, when the complex is formed.

DNA non-homologous end joining (NHEJ) is the dominant pathway of DNA repair in multicellular eukaryotes (1). Six proteins involved in NHEJ in mammalian cells have been identified to date, Ku70, Ku80, DNA-PKcs, Xrcc4, DNA ligase IV, and Artemis (2–4). DNA ligase IV forms a tight complex with Xrcc4 (5), whereas Ku80, Ku70, and DNA-PKcs constitute a complex termed the DNA-dependent protein kinase (6).

The Ku heterodimer, formed by the Ku70 (70 kDa) and Ku80 (86 kDa) subunits, is the first molecule to bind the DNA ends generated by a double strand break (DSB) in vivo (7). The crystal structure of the Ku heterodimer shows that the two subunits are highly interdigitated and form a ring with a large base and a narrow "handle" that threads on the free DNA end like a nut on a bolt (8). The conformation of the molecule does not change upon DNA binding, and its DNA binding site consists of two full turns of the duplex corresponding to ~20–22 bp. One face of the DNA duplex remains mostly exposed to the solvent because it is only partially covered by the narrow handle of the heterodimer. In this way, processing enzymes may have easy access to this side of the duplex to remove damaged nucleotides or to fill gaps prior to ligation (8).

Crystallographic studies on individual proteins and on their DNA complexes have provided invaluable information on the molecular contacts that can occur within the complex (8). However, a complete understanding of the molecular basis of their function requires knowledge of their stability, specificity and mechanism of interaction, which can be achieved only through quantitative thermodynamic and kinetic studies (9). The only information available on the Ku heterodimer is that it avidly binds to DNA ends regardless of their structure and sequence and that, after binding, slides to internal positions of the DNA molecule in an energy-independent manner (10, 11). Binding studies performed by our group (14) and others (12, 13) reported values for the dissociation constants ($k_d$) ranging from the low picomolar to the nanomolar range. On the other hand, most of these studies were not performed under thermodynamic equilibrium conditions, and the large discrepancy in the $k_d$ values is probably a result of the different experimental conditions and techniques used for the binding experiments. Our previous study with DNA duplexes that can accommodate two or three Ku molecules suggested that there is no cooperative interaction among the heterodimers (14). Conversely, Ma and Lieber (15) have shown that Ku binds a 45-bp DNA duplex in a cooperative fashion, but that the cooperativity is lost when using longer duplexes. In this work, we labeled six DNA duplexes of various lengths with a fluorescent probe and measured the binding isotherms by following the change in the anisotropy signal upon formation of the protein-DNA complex. This approach allowed accurate determinations of the dissociation constants under equilibrium conditions. The binding isotherms obtained using DNA duplexes with a Ku/DNA stoichiometry >1 were analyzed using both a conventional non-overlapping and a more elaborate overlapping binding site model. The overlapping model takes into account the possibility that, for nonspecific DNA-interacting proteins, the binding sites on the DNA lattice might overlap (16). Analysis of our data with both models indicates that there is no cooperative interaction among the heterodimers that associate with double-
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EXPERIMENTAL PROCEDURES

Buffers—All concentrated buffers and solutions (e.g. 5 x NaCl, 1 mM Tris-HCl, etc.) were filtered through 0.22-μm Millicell filters (GP Express PLUS membrane). A small aliquot (~50–100 ml) was first filtered and then discarded to avoid any contaminants that might be leached from the filter. The solutions to be used in the fluorescence experiments were prepared by diluting the concentrated stocks in Milli-Q water and filtered again as described above. All measurements were performed in buffer A (20 mM Tris-HCl (pH 7.8 at 25 °C) and 2 mM dithiothreitol). The concentrations of NaCl, KCl, NaBr, and NaF ranged from 95 to 800 mM as indicated below.

Ku Expression and Purification—Ku protein was expressed in baculovirus and purified as previously described (14). Approximately 3–5 mg of Ku heterodimer were purified from 1.5 × 10⁹ infected Sf9 cells. The purity was assessed by SDS-polyacrylamide gels and mass spectrometry analysis. The protein concentration was measured by reading their absorbance at 280 nm as indicated below.

Ku was purified to homogeneity in buffer A (20 mM Tris-HCl (pH 7.8 at 25 °C) and 2 mM dithiothreitol). The concentrations of NaCl, KCl, NaBr, and NaF ranged from 95 to 800 mM as indicated below.

Fluorescence Studies on Ku-DNA Interaction—Fluorescence studies were performed with a FluoroMax-3 fluorometer (Jobin Yvon) equipped with a Glen-Thompson prism prism. Fluorescence was measured at a 90° angle in an L-format. Sample temperature was controlled by a Peltier cooler (Jobin Yvon F-3004). Typically, 1.5-ml samples were used in 4-mm quartz cuvettes (10 × 10 × 45 mm; Hellman). The samples were mixed by pipetting with low retention tips (Molecular Bio Products Inc.) while stirring with the magnetic bar induced protein precipitation. All binding isotherms were performed at a constant DNA concentration. Initially, a small aliquot (2–20 μl) of a highly concentrated protein solution was added to reach complete saturation of the DNA. The volume in the cuvette was maintained constant throughout all titration experiments, and the protein concentration was gradually reduced by serial dilutions with a DNA solution not containing the Ku heterodimer. At each step, a volume (vₛ) was removed from the sample cuvette and replaced with an equal volume of solution containing the same DNA concentration. This procedure allows for measurements of titration curves at a constant macromolecule concentration. The anisotropy data were collected using an excitation wavelength of 495 nm and monitoring the emission from 517 to 523 nm. The band pass was 7 nm for excitation and 9 nm for emission. The integration time was 8 ms. Typically, three to six measurements were collected and averaged for each point of the binding isotherm.

Fluorescence intensity measurements were made under magic angle conditions, setting the excitation polarizer to 0° and the emission polarizer to 55° and using an integration time rate of 0.4 s/nm.

Accuracy of the Anisotropy Measurements—In all our experiments, the fluorescence anisotropy signal originated from the dsDNA that we denoted as the macromolecule (M). The anisotropy change (ΔΔαₐₚ) is given by Equation 1,

\[
\Delta \alpha_{\text{m}} = \alpha_{\text{m}} - \alpha_{\text{d}} = \sum_i \alpha_i (1 - \alpha_i) = \sum_i \Delta A_i \alpha_i \quad (\text{Eq. 1})
\]

where \( \alpha_i \) is the anisotropy of the macromolecule in the \( i \)-state (i.e. the anisotropy of the complex formed by i Ku molecules bound to a DNA duplex), \( \alpha_{\text{d}} \) is the intrinsic anisotropy of the DNA molecule, and \( \alpha_i \) is the fraction of macromolecules in the \( i \)-state. Equation 1 is valid as long as the macromolecular binding state does not affect the quantum yield of the probe attached to the macromolecule. In all our measurements, the emission spectrum of the DNA-bound fluorescein remained constant upon Ku binding to the macromolecule.

We also investigated whether the experimentally determined increase in the anisotropy signal was directly proportional to the binding density for all duplexes listed in Table I. A series of binding isotherms were collected at constant macromolecule concentration following the procedure described previously (22, 23). The binding isotherms were collected in buffer A containing 220 mM NaCl under conditions in which their shape was dependent on the total macromolecule concentration.

Data analysis confirmed the presence of a linear relationship between the anisotropy signal and the binding density (data not shown). Furthermore, evidence for the direct proportionality also came from the stoichiometry studies showing that the anisotropy signal increased linearly with the fraction of ligand bound. The existence of this linear relationship allowed fitting of the measured binding isotherms with equations of the following general form (Equation 2),

\[
\Delta \alpha_{\text{m}} = \alpha_0 + \frac{1}{n} \chi_k \Delta A_{\text{max}} \quad (\text{Eq. 2})
\]

where the fitting parameters are the intrinsic anisotropy of the probe (\( \alpha_0 \)), the maximum change in anisotropy (\( \Delta A_{\text{max}} \)), the stoichiometry (\( n \)), and various numbers of binding constants (\( \chi_k \), \( k \), \( \chi_k \), etc.) that need to be considered depending on the binding model chosen.

Salts-back Titrations—The 25% of blunt-ended duplex DNA was initially titrated with the Ku heterodimer under stoichiometric conditions until an excess of protein was achieved. Small aliquots (3–20 μl) of buffer A containing a high concentration of salt (4.9 mM NaCl and NaBr and 3.4 μM for KCl and KBr) were added successively. In the case of NaF, the concentration of the high salt buffer was 880 mM due to the low solubility of this salt, and salt-back titrations were performed by replacing a fixed volume (50–400 μl) of the solution in the cuvette with the high salt buffer. The concentrations of Ku and DNA were kept constant throughout the experiment. The decrease in the anisotropy signal accompanying the dissociation of the Ku-DNA complex was monitored as a function of the salt concentration. For salts, the variation in the anisotropy signal due to the change in the salt concentration could be neglected since it was <10% of the total anisotropy change registered upon Ku binding. The validity of the method was confirmed by the fact that the \( \chi_k \) values obtained from salt-back titrations agreed perfectly with those determined from independent titrations performed at a constant salt concentration with various amounts of ligand.

Electrophoretic Mobility Shift Assays—Band shift assays were performed by incubating the purified Ku protein (1.74 μM) with γ-32P-end-labeled dsDNA duplexes (0.4 nm) of various lengths (25, 42, and 75 bp) in 10 μl of reaction mixture containing 20 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 80 μg/ml bovine serum albumin, and 100–275 mM KCl. After incubation for 30 min at room temperature, 2.5 μl of gel loading buffer (250 mM Tris-HCl (pH 7.5), 0.2% bromophenol blue, and 40% glycerol) were added, and the reaction products were separated on a 5% nondenaturing polyacrylamide gel run at 130 V and 4 °C in Tris borate/EDTA buffer. Labeled DNA fragments were detected on the dried gel by autoradiography (Instant Imager, Packard Instrument Co.).
RESULTS

In a previous study, we measured the binding isotherms for the interaction of Ku with nucleic acids following the change in the intrinsic fluorescence of the Ku molecule upon DNA binding (14). The maximum variation was 15%, a value that is sufficient (although not ideal) to precisely determine the equilibrium dissociation constant. In this work, accurate binding isotherms were collected on the basis that Ku binding to DNA resulted in an increase in the molecular volume, leading to a decrease in the rotational correlation time as well as an increase in fluorescence anisotropy of the labeled dsDNA molecules. The change in fluorescence anisotropy of the DNA duplex was the common theme throughout this work. It is worth noting that the fluorescence intensity of the DNA-linked fluorescein moiety did not change significantly upon Ku binding (data not shown). This observation indicated that variation in anisotropy should be exclusively related to changes in the DNA rotational correlation time induced by the binding of Ku. For this study, only one strand of all blunt-ended dsDNA molecules was labeled at the 5'-end with the fluorescein probe (Table I). The fluorescein probe induced a negligible perturbation on the Ku-DNA interaction since similar binding affinities were measured when both ends of the DNA molecule were fluorescently labeled (data not shown). This result is in agreement with the previous observation that the affinity of Ku for DNA ends is not affected by the particular structure of the ends, whether they are blunt, with 5'-or 3'-overhangs, or even with hairpin loops (10, 14).

Determination of the Active Fraction—The active fraction of the titrant affects the binding isotherms and the derived parameters. Thus, we first determined the active fraction of the recombinant Ku heterodimer purified from insect cells (Fig. 1). This was accomplished by titrating Ku into a solution of 20- or 25-bp duplexes (s20 and s25), respectively. Both DNA molecules were at 5 nM (>kd). The experiments were performed in buffer A containing 100 mM KCl, and the temperature was set at 25 °C. The solid lines are the linear fit of proper portion of the data set. The active fraction is derived from the value of the crossing point of the two straight lines (1/1.490 = 0.671 × 100 = 67.1%).

or changing the salt conditions (KCl or NaCl from 50 to 125 mM). Furthermore, the non-active protein fraction did not compromise our studies. In fact, for a given DNA fragment, we obtained the same binding energy using various protein preparations containing different active fractions.

Ku/DNA Stoichiometry—The stoichiometry of Ku for a series of blunt-ended DNA duplexes of different lengths was measured following a procedure similar to that adopted for determination of the active fraction. The DNA concentrations ranged from 5 to 20 nM, and the salt concentration was kept at 100 mM (Fig. 2a). All stoichiometry values were determined at two to three different DNA concentrations, and Table II reports the average values for the whole series of DNA templates. Blunt-ended DNA duplexes of 42 and 50 bp could accommodate two Ku heterodimers, whereas the 75-bp duplex was capable of binding three molecules. We also designed a DNA molecule of 60 bp with a gap of 20 nucleotides in the middle that could bind two heterodimers. By dividing the number of Ku molecules bound to each DNA duplex by the number of base pairs of the duplex, we estimated that Ku heterodimers cover ~22–25 bp when bound to DNA. For some of the duplexes, the stoichiometry values were also confirmed by band shift assays performed at various salt concentrations (Fig. 2b).

Ku Cooperativity and DSB Binding Affinity—Preliminary binding experiments suggested that the Ku-DNA interaction was highly salt-dependent and that the equilibrium dissociation constant (kd) was <0.5 nM at 200 mM salt. To simplify the analysis of the binding isotherms, we performed our measurements at 300 mM NaCl. Under these conditions, the total ligand (Ku) concentration was almost equal to the free ligand concentration (xfree ≈ xP), and the binding isotherms were independent of the total macromolecule concentration. The equilibrium dissociation constants were determined at this salt concentration for all duplexes listed in Table I. DNA molecules with a Ku/DNA stoichiometry of 2:1 (s42 and s50) were selected to investigate the eventual presence of cooperativity between the Ku heterodimers binding to the same DNA molecule (Fig. 3). Analysis of the binding isotherms clearly demonstrated the lack of cooperative interaction between Ku heterodimers. The data were first fitted with the Adair equation for a macromolecule with two binding sites (Equation 3),

**TABLE I**

| Duplexes | Structure and Sequence |
|----------|------------------------|
| s20      | GAACGAAACATCGGTGAGCG |
| s25      | GAACGAAACATCGGTGAGAG |
| s42      | GAACGAAACATCGGTGAGAG |
| s50      | GAACGAAACATCGGTGAGAG |
| s75      | GAACGAAACATCGGTGAGAG |
| s80      | GAACGAAACATCGGTGAGAG |

**FIG. 1. Determination of the active fraction of recombinant Ku.** Shown is a plot of the anisotropy changes versus the ratio of [Ku]total to [DNA]total. ○ and ◦, data obtained with the 20- and 25-bp duplexes (s20 and s25), respectively. Both DNA molecules were at 5 nM (>kd). The experiments were performed in buffer A containing 100 mM KCl, and the temperature was set at 25 °C. The solid lines are the linear fit of proper portion of the data set. The active fraction is derived from the value of the crossing point of the two straight lines (1/1.490 = 0.671 × 100 = 67.1%).
total DNA concentration. The duplexes used were 5 nM s50 (\(\Delta \)), 5 nM s42 (\(\bullet\)), and 10 nM s42 (\(\bullet\)). The experiments were carried out under saturating conditions, [DNA]_{total} > k_d (<0.2 nM), in buffer A containing 100 mM KCl at 25 °C. The stoichiometry was calculated from the crossing point of the two straight lines. b, band shift assays with the s20, s42, and s75 DNA molecules. The experiments were carried out with 1.74 μM Ku and 0.4 nM DNA in buffer A containing different concentrations of KCl and 80 μg/ml bovine serum albumin. The reaction mixture was incubated for 30 min at room temperature, and the reaction products were separated on a 5% nondenaturing polyacrylamide gel. Lanes 1, DNA alone; lanes 2–9, increasing concentrations of KCl from 100 to 275 mM with steps of 25 mM.

\[
v = \frac{2k_x x + 2k_x p x^2}{1 + 2k_x p + k_x p x^2}
\]  
(Eq. 3)

where \(k_1\) and \(k_2\) are the two sequential microscopic association constants that, in our case, turned out to be identical. In fact, analogous results were obtained by fitting the data with a simple equation that considers the presence of “two” independent and identical (DSB) binding sites on the macromolecule (Equation 4),

\[
v = \frac{2k_x x}{1 + k_x} \frac{2x}{k_x + x}
\]  
(Eq. 4)

where \(k_d\) is the microscopic dissociation constant for the single DSB. The \(k_d\) values for the binding of Ku to s50 and s42 were 32 ± 2 and 81 ± 3 nM, respectively. A more accurate estimate of \(k_d\) was obtained taking into account that, under our experimental conditions, the free ligand concentration (\(x\)) was still slightly dependent on the total macromolecule concentration (\(M_p\)) and could be more precisely determined using Equations 5 and 6.

\[
x = x_T - v M_T
\]  
(Eq. 5)

\[
x = -\frac{1}{2} k_d + 2 M_T - x_T + \frac{1}{2} \sqrt{(k_d + 2 M_T - x_T)^2 + 4k_d x_T}
\]  
(Eq. 6)

The new fit of the data resulted in \(k_d = 24 ± 1\) nM for the s50 duplex and \(k_d = 72 ± 3\) nM for the s42 duplex (Table III). The same analysis was performed for the ss60 molecule containing a 20-nucleotide gap in the middle, yielding \(k_d = 21 ± 1\) nM.

The short duplexes (s20 and s25) were analyzed differently from a simple 1:1 stoichiometry because of the presence of two overlapping binding sites on a single macromolecule formed by the two blunt ends of the duplex (Fig. 4). A degenerative factor was included in the fitting equation since only one of the two sites could be occupied by a Ku heterodimer (Equations 7 and 8).

\[
v = \frac{x}{k_d + x}
\]  
(Eq. 7)

\[
x = -\frac{1}{2} k_d + M_T - x_T \frac{1}{2} \sqrt{(k_d + M_T - x_T)^2 + 2k_d x_T}
\]  
(Eq. 8)

The microscopic dissociation constants for Ku binding to the 20- and 25-bp duplexes were \(77 ± 7\) and \(29 ± 2\) nM, respectively (Table III). The higher \(k_d\) value determined for s20 indicates that Ku needs >20 bp to efficiently bind DNA. Consistently, the \(k_d\) for s42 was higher than that determined for s50, suggesting that the 42-bp duplex is too short to properly accommodate two Ku molecules. The close similarity between the \(k_d\) values reported for the two shorter dsDNAs (s20 and s25) and those determined for the corresponding longer duplexes (s42 and s50) confirms that the definition of the microscopic binding site, as formed by the free DNA end plus 22–25 bp, was appropriate (Table III).

Analysis of Ku Binding to s75—The 75-bp duplex is able to accommodate three Ku heterodimers: two are bound at its blunt ends (sites 1 and 3), and one lies in the center of the DNA molecule (site 2). The experimental data were fitted using Equation 9 defined for a macromolecule with three binding sites,

\[
v = \frac{3k_x x + 2(1 + 2\omega)k_x x^2 + 3\omega k_x x^3}{1 + 3k_x x + (1 + 2\omega)k_x x^2 + \omega k_x x^3}
\]  
(Eq. 9)

where the term \((1 + 2\omega)\) takes into account that the two ligands can either touch each other (if bound to sites 1 and 2 or to sites 2 and 3) or interact with the two ends without touching each other (if bound to sites 1 and 3). In this expression, \(\omega\) corresponds to a cooperativity parameter such that if \(\omega > 1\), the contiguous ligands attract each other, and the binding is positively cooperative, whereas if \(\omega < 1\), the binding is anti-cooperative, and if \(\omega = 1\), there is no cooperative interaction. Analysis of the binding isotherms with Equation 9 yielded a \(\omega\) value that is very close to 1 (\(\omega = 1.03 ± 0.09\)) and \(k_d = 33 ± 2\) nM (Fig. 5). A \(\omega\) value close to 1 indicates that there is no cooperative interaction among the nearest-neighbor bound proteins and that the related free energy is almost zero (\(\Delta G_w = 0\)). In fact, the relationship between the cooperativity parameter and the
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free energy is given by the equation \( \omega = e^{-\Delta G/k_BT} \), where \( R \) and \( T \) are the universal gas constant and the absolute temperature, respectively (\( RT \approx 0.6 \) kcal/mol at 25 °C). Computer simulations made assuming \( \Delta G \) values greater than \((RT, 2RT, \) and \(3RT)\) or less than \((-RT, -2RT, \) and \(-3RT)\) 0 clearly showed that the best fit can be obtained for \( \omega = 1 \) (Fig. 5). This analysis indicates that the Ku heterodimer has the same affinity for the three binding sites of s75, confirming that the translocation of the Ku heterodimer along the dsDNA molecule is energy-independent. Thus, the expression for the degree of binding could be simplified into the general equation for a macromolecule with three identical and independent binding sites (Equation 10).

\[
v = \frac{3k_x}{1 + k_x}
\]  
(Eq. 10)

Also in this case, the free ligand concentration could be more precisely calculated using the expression that takes into account the total macromolecule concentration (Equation 11).

\[
x = \frac{1}{2}(k_d + 3M_T - x_T) + \frac{1}{2}\sqrt{(k_d + 3M_T - x_T)^2 + 4k_dMT}
\]  
(Eq. 11)

Remarkably, the \( k_d \) value of 22 ± 1 calculated using Equations 10 and 11 was in good agreement with those previously measured for s50 and s25 (Table III).

Analysis of Binding Isotherms Considering the Potential Overlapping Binding Sites—The binding isotherms for s42, s50, and s75 were also analyzed with a model that takes into account the overlap of ligand-binding sites (24, 25). In fact, for a nonspecific DNA-binding protein, the potential number of overlapping ligand-binding sites on a DNA lattice is \( N - m + 1 \), where \( N \) is the number of base pairs that form the duplex and \( m \) is the number of consecutive residues covered by the ligand. For example, assuming that Ku covers 25 bp when binding to DNA, the number of overlapping binding sites for the 75-bp duplex would be equal to 51. The equations describing the binding of either non-interacting or interacting ligands to a one-dimensional linear lattice of infinite length were initially developed by McGehee and von Hippel (16) and successively refined by several groups (9, 26–29). In this work, the binding isotherms were analyzed with the method derived by Di Cera and Kong (24) and Kong (25) for the interaction of ligands with a linear lattice of arbitrary length and cooperativity. This method gives the “generating function” of all partition functions of the model under study. By series expansion of the generating function, the partition functions of systems of any length can be obtained as the coefficients of the power series:

\[
G(z) = 1 + P(1)z + P(2)z^2 + P(3)z^3 + \ldots + P(N)z^N + \ldots
\]

For a system of length \( N \), its partition function \( P(N) \) is the coefficient of \( z^N \), where \( z \) is the auxiliary variable in the generating function. The general form of the generating function used for this analysis is shown in Equation 12,

\[
G(z) = \frac{1 - (\omega - 1)z^m}{1 - z - \omega z^m + (\omega - 1)z^{m+1}}
\]  
(Eq. 12)

where \( \omega \) is the unitless cooperativity parameter related to the nearest-neighbor interaction among bound ligands; \( m \) is the number of binding sites covered by a bound ligand; \( \theta = kx \), the product of the intrinsic association constant and the free ligand concentration; and \( z \) is the auxiliary variable of the generating function. The expression for the degree of binding (\( v \)) can be simply obtained from the partition function as follows (Equation 13),

\[
v = \frac{\partial}{\partial \theta} \ln P(m, \theta, \omega) dP(m, \theta, \omega) \frac{d\theta}{d\theta}
\]  
(Eq. 13)

where \( P(m, \theta, \omega) \), derived from the generating function \( G(z) \), is the partition function of a lattice of \( n \) binding sites for a protein that covers \( m \) sites. For instance, assuming \( m = 25 \), the partition function and the degree of binding for the 75-bp duplex are given by Equations 14 and 15.

\[
P(25, 75, \theta, \omega) = \omega^2 \theta^7 + 252 \omega \theta^7 + 325 \theta^7 + 51 \theta + 1
\]  
(Eq. 14)

\[
v = \frac{\partial}{\partial \theta} 3 \omega \theta^7 + 15 \omega \theta^7 + 650 \theta + 51
\]  
(Eq. 15)
Analogous expressions were derived for the s42 and s50 duplexes and for other values of \( m \). In fact, since we did not know the precise number of base pairs that were covered by one Ku heterodimer upon DNA binding, the same analysis was repeated for \( m = 20, 21, 22, 23, \) and 24 (Fig. 6). Equation 9 can be considered as a special case in this model, with \( m = 1 \) and \( n = 3 \). As expected, the results show that the \( k_d \) values depended slightly on the value \( m \), although they were all in the same range of those calculated with the previous model, after normalization of the binding sites (Table IV). The linear fit of the data showed that there was no significant difference in the salt dependence of the \( k_d \) values measured in \( \text{Na}^+ \) and \( \text{K}^+ \), indicating that an identical number of ions was released upon formation of the Ku-DNA complex (Fig. 8). The anions were varied from \( \text{Cl}^- \) to \( \text{Br}^- \) and \( \text{F}^- \) (Fig. 8). The results clearly show that the anion type and concentration affected both the magnitude and salt dependence of the \( k_d \). At 300 mM \( \text{Na}^+ \) (pH 7.8 at 25 °C), the equilibrium dissociation constants varied as follows: \( k_{d(\text{Br}^-)} = 33 \frac{k_{d(\text{Cl}^-) \text{Na}^+}}{k_{d(\text{F}^-) \text{Na}^+}} \). The slopes were \( 12.1 \pm 0.3 \) in \( \text{NaBr} \) and \( 8.8 \pm 0.2 \) in \( \text{NaF} \) compared with \( 12.4 \pm 0.1 \) in \( \text{NaCl} \) (Table V). In agreement with the observation that fluoride interacts weakly with proteins (31), the slope in \( \text{NaF} \) was significantly smaller than in the other salts, reflecting only the preferential interaction of \( \text{Na}^+ \) with nucleic acids. Therefore, the slope of 8.8 ± 0.2 measured in \( \text{NaF} \) indicates that approximately nine \( \text{Na}^+ \) ions are released from DNA upon complex formation. In addition, the difference between the slopes measured with the other anions, ~12, and the slope measured in \( \text{NaF} \), 9, suggests that about three anions are released from the Ku heterodimer upon binding to DNA when the experiments are performed in the presence of \( \text{Br}^- \) or \( \text{Cl}^- \).

**DISCUSSION**

The Ku heterodimer plays a central role in NHEJ and V(D)J recombination (32, 33). Despite the fundamental information that recently emerged from the crystal structure of the Ku-
experiments were carried out in buffer A containing 300 mM NaCl. The concentration of the DNA duplex was 5 nM, whereas the concentration of the Ku heterodimer was varied from 0 to 300 nM. The expressions of the degree of binding for \( m = 20, 21, 22, 23, 24, \) and 25 were derived from Equation 13 as described under “Results.” The lines show the fit of the binding isotherm for all different values of \( m \).

DNA complex (8), the molecular basis of this interaction demands further investigation. In this study, we have studied the interaction of Ku with nucleic acids quantitatively by following the change in fluorescence anisotropy of the DNA duplex associated with protein binding.

The binding of Ku to nucleic acids occurs at DNA ends and is independent of their structure and sequence (10, 14). After binding to the DNA termini, Ku can translocate to internal positions with a process that seems to be energy-independent (11). At first, we employed a simple binding model that takes into account these properties of the Ku heterodimer and allows a proper analysis of the binding isotherms. Thus, we were able to quantify the microscopic dissociation constants for DNA duplexes of various lengths and under different solution conditions.

Our analysis started with two short blunt-ended DNA duplexes of 20 and 25 bp that can accommodate only one Ku heterodimer. Even if these duplexes show a 1:1 stoichiometry, we consider them as macromolecules with two overlapping binding sites since they possess two DNA ends and since the binding of Ku to one end prevents the binding of a second molecule to the other end (see Equation 7). Binding studies performed at 300 mM NaCl yield \( k_d \) values of 77 ± 7 and 29 ± 2 nM for s20 and s25, respectively. The higher \( k_d \) value reported for s20 indicates that the heterodimer needs >20 bp to efficiently bind nucleic acids. In agreement with this conclusion, previous surface plasmon resonance and electrophoretic mobility shift assays yielded \( k_d \) values between 0.38 and 1.4 nM for the binding of recombinant Ku to an 18-bp blunt-ended dsDNA fragment and a value of 0.16 nM for the binding to a 22-bp blunt-ended duplex (15). Similarly, neutron scattering studies showed that a 24-bp DNA element lies almost entirely within the Ku protein envelope, and crystallographic data confirmed that Ku covers approximately two turns of DNA (8, 34). A meaningful comparison of the affinity values reported in the literature can be done only if the experiments are performed under similar solution conditions. In fact, we observed that the magnitude of the \( k_d \) for the Ku-DNA interaction is highly dependent on the experimental conditions used for the binding studies. For example, a change of ~100 mM [salt] led to an ~60-fold variation in the dissociation constant, which, in the case of the 25-bp duplex, dropped from 29 nM at 300 mM NaCl to <0.5 nM at 200 mM NaCl. In any case, our \( k_d \) values at 200 mM salt are in the same range of previous values measured with different techniques (12, 15).

In an earlier work, we demonstrated that there is no cooperative interaction among the Ku heterodimers binding to a 50-bp duplex (14). This result is in contrast with previous studies that showed that there is positive cooperativity between two Ku molecules binding a 45-bp blunt-ended dsDNA fragment (15). A possible explanation for this discrepancy is that this 45-bp dsDNA is 5 bp shorter than the 50-bp duplex that we have used. These additional 5 bp may be responsible for the fact that the two Ku molecules bound to the 50-bp dsDNA are not close enough to interact with each other. In agreement with this possibility, no cooperativity using longer duplexes that can bind three or four Ku molecules was observed (15). To verify this hypothesis, we compared the dissociation constants for the interaction of Ku with two DNA duplexes of 42 and 50 bp, respectively. Our new results clearly show that there is no cooperative interaction between the two Ku molecules binding to both DNA templates under our solution conditions, even when the duplex is short enough to allow the contact between two adjacent heterodimers. In fact, all binding isotherms are well described by the simple equation defined for macromolecules with two identical and independent binding sites.

The analysis of the Ku interaction with a 75-bp DNA duplex, capable of accommodating three heterodimers, was performed taking into account that Ku can translocate along the duplex after binding to DSBs and bind an internal site of the template. The binding curves yield a value for the cooperativity parameter of \( \omega = 1.03 \pm 0.09 \) (\( \Delta G_c = 0 \)). Thus, our results indicate that cooperative interactions are also absent when one of the Ku molecules is internally bound, indicating that Ku has the same affinity for the three binding sites of the 75-bp duplex. This finding reinforces the previous observation on the energy-independent translocation of the Ku molecule along the DNA lattice (11). Since \( \omega = 1 \), the binding equation reduces to that defined for \( n \) identical and independent binding sites, where \( n \) is the stoichiometry of the DNA molecule (see Equation 10). In the case of s75, \( n = 3 \), and the microscopic dissociation constant is \( 22 \pm 1 \) nM. This \( k_d \) value is close to those measured for s25 and s50. Thus, all our equilibrium binding data are well described by a non-interacting binding site model that considers the DNA lattice as a macromolecule with \( n = N/m \) binding sites, where \( N \) is the number of base pairs that form the duplex and \( m \) is the number of consecutive residues covered by the ligand. On the other hand, this model neglects the potential overlap of binding sites on the DNA duplexes that might need to be taken into account when studying the interaction of a nonspecific DNA-binding protein with nucleic acids. For instance, since the number of overlapping binding sites on a DNA lattice is given by \( N - m + 1 \), the 75-bp duplex would have 51 potential overlapping binding sites assuming that \( m = 25 \). Previous studies have already shown the importance of applying an overlapping model to appropriately describe the interaction of nonspecific DNA-binding proteins with linear DNA lattices (16, 27, 35, 36). On the other hand, the possibility that the binding sites might overlap was never properly considered in the case of the Ku-DNA interaction. Thus, we decided to apply this model also for the analysis of Ku binding to DNA. The binding isotherms for s42, s50, and s75 were fitted using a modified form of the McGhee and von Hippel model (16) derived for the interaction of ligands with a linear lattice of arbitrary length and cooperativity (24, 25). Since we do not know the precise number of base pairs covered by one Ku heterodimer, the same analysis was repeated for values of \( m \) ranging from 20 to 25. The results show that the values of the dissociation constants, although slightly dependent on the value of \( m \) used for the analysis, are all in the same range as

![Fluorescence Studies on Ku-DNA Interaction](image)
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The value of $k_d$ has been normalized by the number of binding sites ($N = m + 1$) of the DNA lattice.

| $m$  | $k_d$ (nM) | $m$  | $k_d$ (nM) | $m$  | $k_d$ (nM) | $m$  | $k_d$ (nM) | $m$  | $k_d$ (nM) |
|------|------------|------|------------|------|------------|------|------------|------|------------|
| 20   | $s_{42}$   | 15.9 ± 0.9 | 22.8 ± 2  | 34.0 ± 2  | 34.0 ± 2  | 34.0 ± 2  | 34.0 ± 2  |
| 21   | $s_{50}$   | 20.4 ± 0.6 | 20.4 ± 0.6 | 20.4 ± 0.6 | 20.4 ± 0.6 | 20.4 ± 0.6 | 20.4 ± 0.6 |
| 22   | $s_{75}$   | 19.3 ± 0.6 | 19.7 ± 0.6 | 20.4 ± 0.7 | 21.4 ± 0.7 | 23.2 ± 0.8 | 26.8 ± 0.8 |
| 23   | $\omega$   | 17 ± 4    | 17 ± 3    | 32 ± 3    | 44 ± 4    | 70 ± 7    | 197 ± 18  |
| 24   |           | 20 ± 2    | 25 ± 3    | 20 ± 3    | 25 ± 4    | 29 ± 6    | 16 ± 5    |
| 25   |           | 14 ± 2    | 17 ± 2    | 20 ± 3    |         |         |         |

The slope values ($\Gamma_{salt}$) as well as the dissociation constants derived from the salt-back titrations at 220 and 300 mM salt are reported.

| Salt | $\Gamma_{salt}$ | $h_d$ (nM) |
|------|-----------------|------------|
| NaBr | 12.1 ± 0.3      | 21.6 ± 0.5 | 900 ± 20  |
| KBr  | 12.6 ± 0.3      | 17.9 ± 0.4 | 890 ± 20  |
| NaCl | 12.4 ± 0.1      | 0.5 ± 0.01 | 26.9 ± 0.2 |
| KCl  | 12.1 ± 0.2      | 0.5 ± 0.01 | 20.9 ± 0.3 |
| NaF  | 8.8 ± 0.2       | 0.003 ± 0.0001 | 0.4 ± 0.01 |

Our findings indicate that the Ku molecules, after binding to the DNA ends, can slide freely along the dsDNA chain and that their presence on the duplex does not favor or impair the binding of additional heterodimers to the same dsDNA break. These results are in agreement with the emerging picture on the role of Ku in the DNA NHEJ mechanism. The binding of a single heterodimer to DSBs is thought to prevent DNA degradation and possibly to sequester other enzymes needed for the subsequent repair steps (37). In this view, the absence of positive cooperativity would prevent clustering of Ku heterodimers at the DNA ends. Instead, Ku molecules would easily slide to internal positions of the DNA molecule, allowing the binding of other factors (38).

After having described the mechanism of interaction, we moved to further dissect the energetic components of the binding free energy. Studies of the salt effect on protein-DNA equilibria can provide useful information on the number of cations and anions that are released upon formation of the complex as well as an estimate of the electrostatic component of the binding free energy (9, 39, 40). Similar studies have never been performed on any protein involved in NHEJ and, more in general, in eukaryotic DNA repair. In this work, we have demonstrated that electrostatic interactions play a major role in the binding of the Ku heterodimer to nucleic acids. A change of ~100 mM [salt] led to an almost 60-fold difference in the equilibrium dissociation constant. The slope ($\Gamma_{salt}$) of the plot of $\log h_d$ versus $\log [NaCl]$ was 12.4 ± 0.1. This value is among the highest reported for a protein-DNA interaction and indicates that ~12 ions are released upon formation of the Ku-DNA complex (Fig. 8). An estimate of the relative contributions of anions versus cations to the salt dependence of the $h_d$ can be obtained by comparing the $\Gamma_{salt}$ values from experiments performed with a common cation, e.g. Na⁺. Our experiments with Cl⁻, Br⁻, and F⁻ demonstrate that the anion type and concentration affect both the salt dependence and the magnitude of those calculated with the non-overlapping model. In addition, the values of the cooperativity parameter ($\omega$) are always ≤200. When derived from an overlapping model, $\omega$ values of this magnitude indicate that the cooperative interaction among the Ku heterodimers binding to DNA is very weak and probably negligible under our solution conditions. In fact, Lohman and co-workers have reported much higher values of $\omega$ (≥10⁵) for the cooperative interaction of the Escherichia coli single-stranded DNA-binding protein (SSB) tetramers with single-stranded DNA in the (SSB₃₅) binding mode (27) and reported values of $\omega = 420 ± 80$ for the low cooperative interaction of the same protein in the (SSB)₂₅₅ binding mode (30). Even though the overlapping binding model might be more appropriate to describe the binding of Ku to DNA duplexes able to accommodate two or more heterodimers, both the overlapping and non-overlapping models lead to similar conclusions and agree on the absence of cooperative interaction among the Ku molecules binding to DNA. Therefore, the same models can be most likely used to describe the interaction of Ku with DNA duplexes of any length.

![Fig. 7. Ku-s25 salt-back titration. The experiments were carried out in buffer A. The concentration of NaCl was gradually increased from 95 to 540 mM. The concentrations of Ku and DNA were 11.1 and 10 nM, respectively.](image1)

![Fig. 8. Effect of NaBr (●), KBr (○), NaCl (□), KCl (◆), and NaF (✦) concentrations on the $k_d$ for Ku binding to s25. The $k_d$ values were obtained from the salt-back titrations using Equation 16 as described under “Results.”](image2)
the \( k_d \). The slopes measured in NaCl and NaBr were 12.4 ± 0.1 and 12.1 ± 0.3, respectively, whereas in NaF, the \( k_d \) value decreased to 8.8 ± 0.2 (Table V). The lower \( k_d \) determined in NaF is consistent with the observation that fluoride appears to interact least with proteins compared with bromide and chloride (20, 31, 35). Hence, the salt dependence in NaF should reflect only the preferential interactions of Na\(^+\) with the negatively charged DNA molecule. In other words, the slope of 8.8 ± 0.2 in NaF indicates that approximately nine Na\(^+\) ions are released from DNA upon formation of the Ku-DNA complex. In addition, the salt dependence studies performed in NaBr and NaCl suggest that, in these two salts, there are roughly nine Na\(^+\) ions and three Br\(^-\) or Cl\(^-\) ions released when Ku binds to DNA. The anion effect should reflect the preferential interaction of Br\(^-\) or Cl\(^-\) with the Ku heterodimer versus the Ku-DNA complex. This conclusion is supported by previous studies on other DNA-binding proteins (17, 35) and by our preliminary studies on the effect of pH on the Ku-DNA interaction showing that the extent of anion binding is coupled to the number of protonation sites on the Ku protein (data not shown). The change in the anion type also induces a decrease in the magnitude of the \( k_d \) following the order Br\(^-\) > Cl\(^-\) > F\(^-\). This hierarchy follows the so-called Hofmeister series (31) and is due to the fact that it is harder to displace Br\(^-\) than Cl\(^-\) from the protein upon complex formation.

Our interpretation of the salt dependence could be further complicated by the hidden contribution of the cation uptake component. The dependence of the \( k_d \) on monovalent salt concentration can be described by Equation 19.

\[
\frac{d \log k_d}{d \log [\text{Na}^+]} = \Delta C + \Delta A \quad \text{(Eq. 19)}
\]

This equation does not include the term due to preferential hydration since it is usually negligible at salt concentrations <0.5 M. The terms \( \Delta C \) and \( \Delta A \) refer to the net release of cations and anions, respectively, upon formation of the complex. In general, any anion effect is due to preferential interaction with the protein, whereas the term \( \Delta C \) has a contribution due to the release of \( z \psi \) cations from nucleic acids as well as the uptake of b cations by the protein \( \Delta C = z \psi - b \). The symbol \( \psi \) refers to the number of counterions thermodynamically bound per phosphate, and \( z \) corresponds to the number of positively charged sites of the protein that bind to nucleic acids. In our case, \( \Delta C = z \psi - b = 8.8-9 \), meaning that there are 9 + b ions released from the nucleic acid. If \( \psi \) is unknown, we can calculate only the minimum estimate for the number of cations that are released upon complex formation. Since \( \psi = 0.88 \) for double-stranded nucleic acids, the minimum estimate for \( z \) is 9/0.88 = 10.2, meaning that at least 10 ion interactions are formed in the complex of the Ku heterodimer with DNA. On the other hand, the cation uptake component is probably negligible in the case of the Ku-DNA interaction. In fact, no significant effect on the magnitude of the \( k_d \) is observed as a consequence of the substitution of Na\(^-\) with K\(^+\). Since the affinities of Na\(^+\) and K\(^+\) for DNA are quite similar (41), this result suggests that cations are simply displaced from the nucleic acid and do not interact with Ku upon complex formation. Hence, the value of 10.2 should not need to be adjusted by the cation uptake component.

The extreme sensitivity to solution conditions observed for the Ku-DNA interaction is supported by the crystallographic data on the Ku heterodimer showing a polarization of positive electrostatic charges focused on the internal surface of the Ku ring and along the DNA-binding cradle. In addition, the lack of even a single interaction of a Ku residue with a DNA base, shown in the structure of the DNA-bound form, explains the reason for the absence of sequence specificity in DNA binding, and our salt dependence studies indicate that the mechanism of DNA recognition might be primarily driven by electrostatic forces. The same fundamental role of electrostatic interaction was also observed for many other DNA-binding proteins previously studied by other groups. For example, values \( \Gamma_{\text{salt}} \) ranging from 8 to 10 were reported for the interaction of the E. coli lac repressor with nonspecific DNA sequences (18) and of the RecA protein with single-stranded DNA (42). Rigorous salt dependence studies on the interaction of E. coli SSB with polymerized and of the A1 repressor with DNA yielded \( \Gamma_{\text{salt}} \) values between 5 and 7 depending on the type of salt and solution conditions (20, 43). Similarly, a recent work on the binding of the E. coli RecQ helicase to DNA fragments of different lengths indicated that approximately three to four ions are released upon complex formation (44). More in general, specific ion effects are likely to control most protein-DNA interactions, especially because of the polyelectrolyte nature of nucleic acids, and should not be ignored in any quantitative analysis.

In summary, the studies presented in this work provide a quantitative description of the mechanism of Ku binding to DNA and clear evidence of the dramatic effects that electrostatic forces and specific binding of ions have on the formation of this complex. Our results provide important information for the field of NHEJ and increase our knowledge about the different strategies that nonspecific DNA-binding proteins adopt to bind and translocate along the DNA lattice. Moreover, the results of the salt dependence studies can be utilized to estimate the affinity of Ku for DNA at any salt concentration and allow a proper comparison of binding data acquired under different solution conditions. The same biophysical approach will be extended to other proteins that play a key role in NHEJ and to Ku mutants that show defects in DNA repair to properly evaluate the impact that these mutations might have on the mechanism of DNA binding.

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