Mechanism of κB DNA binding by Rel/NF-κB dimers*

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The DNA binding of three different NF-κB dimers, the p50 and p65 homodimers and the p50/p65 heterodimer, has been examined using a combination of gel mobility shift and fluorescence anisotropy assays. The NF-κB p50/p65 heterodimer is shown here to bind the κB DNA target site of the immunoglobulin κ enhancer (Ig-κB) with an affinity of approximately 10 nM. The p50 and p65 homodimers bind to the same site with roughly 5- and 15-fold lower affinity, respectively. The nature of the binding isotherms indicates a cooperative mode of binding for all three dimers to the DNA targets. We have further characterized the role of pH, salt, and temperature on the formation of the p50/p65 heterodimer-Ig-κB complex. The heterodimer binds to the Ig-κB DNA target in a pH-dependent manner, with the highest affinity between pH 7.0 and 7.5. A strong salt-dependent interaction between Ig-κB and the p50/p65 heterodimer is observed, with optimum binding occurring at monovalent salt concentrations below 75 mM, with binding becoming virtually nonspecific at a salt concentration of 200 mM. Binding of the heterodimer to DNA was unchanged across a temperature range between 4 °C and 42 °C. The sensitivity to ionic environment and insensitivity to temperature indicate that NF-κB p50/p65 heterodimers form complexes with specific DNA in an entropically driven manner.

The Rel/NF-κB transcription factors constitute one of the most important families of regulatory transcription factors. Members of the Rel/NF-κB family are essential for diverse biological functions such as the regulation of innate and adaptive immunity, development, and apoptosis in a wide array of eukaryotes from Drosophila to man (1–4). Like most transcription factors, dimers of NF-κB proteins modulate transcription by directly binding to enhancer sequences located in the regulatory regions of numerous genes. These DNA sequences are collectively known as κB DNA sequences. In mammals, the Rel/NF-κB dimers arise from five polypeptides, p50, p52, p65, c-Rel, and RelB. The most abundant of these dimers are the p50/p65 heterodimer and the p50 homodimer. The existences of some, but not all, of the other possible dimers have been shown to exist in cells.

The NF-κB family can be divided into two subgroups based on the presence or absence of an activation domain. p50 and p52 do not contain a distinct activation domain and belong to class I. The other three members constitute the class II subfamily. It is generally believed that the homodimers of p50 and p52 and the p50/p52 heterodimer function as transcriptional repressors. The remaining combinations of dimeric NF-κB proteins, containing at least one monomer of p65, c-Rel, or RelB, act as activators.

Rel/NF-κB proteins share a region that shows over 45% sequence similarity across the entire family. This region, known as the Rel homology region (RHR),1 is responsible for DNA binding and subunit dimerization. High resolution x-ray crystal structures of RRHs are known for four homodimers, p50, p52, p65, and c-Rel in their DNA-bound conformations (5–8). These structures show that, as expected, Rel/NF-κB proteins also share similar structures. Most of the RHR is folded into two immunoglobulin-like domains connected by a 10-amino acid linker; the N-terminal domain confers sequence specificity in DNA binding, and the C-terminal domain is involved in dimerization as well as DNA backbone recognition. These structures show that, unlike most other transcription factors, NF-κB dimers do not use any secondary structure for contacting DNA. All the DNA-contacting residues emanate from loops connecting secondary structures. Crystal structures of these complexes suggest that in their free form the N-terminal domains should be flexible with respect to the dimerization domain.

Recently, the NMR structures of a 16-base pair duplex DNA containing the κB target from the HIV-LTR, which is identical to the κB site in the immunoglobulin light chain κ gene (Ig-κB), and a mutant form of the target site that abolishes DNA binding have been solved (9, 10). These show that the phosphodiester bonds of the sugar-phosphate backbone of the native duplex preferentially adopt a distinct conformation in the 5' and 3' regions of the κB site. The mutant site is incapable of adopting the native DNA's conformation, suggesting that κB-DNA sequence also plays a role in NF-κB-DNA complex formation. The combined flexibility of the NF-κB dimers and their target DNA allows NF-κB to adopt multiple conformations in a promoter specific manner.

Among NF-κB’s most well characterized DNA targets are the κB DNA sites of the immunoglobulin light chain κ gene and HIV-LTR (Ig-κB) and the interferon β gene (IFN-κB). A crystal structure of the NF-κB p50/p65 heterodimer bound to the Ig-κB DNA target has been completed (11). In order to understand the mechanism of DNA binding by NF-κB, thermodynamic parameters need to be determined for various NF-κB dimers and κB DNA target sites. In this study we have analyzed...
binding of Ig-κB and IFN-κB DNA targets with three different NF-κB dimers: p50 homodimer, p65 homodimer, and p50/p65 heterodimer, using both a gel mobility assay and a solution-based fluorescence anisotropy assay. The binding of NF-κB p50/p65 heterodimer to Ig-κB DNA has been further tested for its dependence on pH, salt, and temperature.

**EXPERIMENTAL PROCEDURES**

**Materials**—5'-Fluorescein-labeled oligonucleotides were purchased from the Keck Oligonucleotide Synthesis Facility at Yale University (New Haven, CT). Unlabeled oligonucleotides were synthesized using a Millipore Research Cyclone Plus DNA synthesizer. Electrophoresis and fluorescence polarization chemicals were purchased from Fisher Scientific, except for MOPS and CAPSO buffers, which were purchased from Sigma. T4-polymerase kinase was purchased from New England Biolabs. [γ-32P]ATP and poly(dI-dC) carrier DNA were purchased from Amersham Pharmacia Biotech. The Nucleotide Removal Kit was purchased from Qiagen. All proteins were purified according to Refs. 5, 6, 8, and 12.

**Site-directed Mutagenesis**—Monomeric p50 and p65 mutants were generated through a two-step polymerase chain reaction strategy using internal primers. The N- and C-terminal primers for both mutants were the same as those used for the wild type proteins (12). For the p50 Y267F/L269F mutant, the internal primers used were: N-terminal, 5'-GGGGAGGAGATGTTAATGTTGCAGAACCCCTC-3'; C-terminal, 5'-GAACCTTGTACAAATCTGTACACTCCTCCTCCC-3'. For the p62 F215D/L215D mutant, the internal primers used were: N-terminal, 5'-GGGGAGGAGATGCTAGTTGCAGAACCCCTC-3'; C-terminal, 5'-CACCTTGTCGCAATCTAGATCGATCTCATCCCC-3'.

**Electrophoretic Mobility Shift Assay (EMSA)**—The oligonucleotide used for the EMSAs was 5'-TCTGGAGACTCTCCTCCCTAGCT-3', which contains the heterodimer target site Ig-κB (underlined). This oligonucleotide was annealed to its complementary strand and end radiolabeled by the polarization value for NF-κB DNA binding. The equation is a simplified version of the equation used by Ha et al. (15).

$$\log(K_{\text{app}}) = \log(K_\text{d}) - A^*\log(\text{NaCl}) + B^*\text{NaCl}$$  \hspace{1cm} (Eq. 4)  

where $K_\text{d}$ is the extrapolated apparent $K_d$ at 1 M NaCl concentration, $Z$ is the number of cations displaced, and $\psi$ is the number of cations thermodynamically bound to each DNA backbone phosphate previously determined to be 0.88 (13).

**RESULTS**

**Binding Affinities of NF-κB p50 Homodimers for κB-DNA Targets**—We used only the RHR portions of both p50 and p65 subunits for binding experiments. The RHR of p50 and p65 homodimers and the p50/p65 heterodimer have been overexpressed and purified from overexpressing E. coli clones. We have measured the DNA binding of the p50 homodimer using a gel mobility shift assay. The DNA probe used for this assay was a 20-mer duplex DNA containing a centrally located 10-base pair Ig-κB site. Fig. 1 shows the free and bound DNA for the p50 homodimer, as well as the p65 homodimer and p50/p65 heterodimer. The data fit best to a cooperative binding model (Equations 1 and 2) describing two subunits assembling sequentially on the DNA. Fig. 2 shows the data for NF-κB p50 homodimer binding to Ig-κB DNA fit to the cooperative model. The affinity of NF-κB p50 homodimer for Ig-κB DNA was further examined using fluorescence anisotropy assays. The binding conditions were similar to those for gel mobility shift assays. This solution-based assay circumvents the problems of artificial dissociation of a protein-DNA complex as it migrates through a gel matrix. Fig. 3 shows titrations of Ig-κB DNA with the three different NF-κB dimers. The total fluorescence intensity did not change during the assay, indicating that anisotropy signals were not due to changes in fluorescence lifetime or other experimental artifacts. To determine the time required for each reaction to reach equilibrium anisotropy, a kinetic experiment was performed in which each sample was measured at different times until no change in anisotropy was observed. Accordingly, sufficient time was allowed before recording the final anisotropy value. Control experiments
showed that the presence or absence of carrier DNA poly(dI-dC) (2 μg/ml) and glycerol (5%) had no effect in binding. Additionally, we have verified the activities of each protein sample used for the assays by measuring anisotropy at various stoichiometric protein-DNA ratios (over a range from 20/1 to 1/20). We observe that approximately 85% of the NF-κB in each preparation is fully active (data not shown).

Anisotropy profiles for each binding experiment show an initial plateau indicating unbound DNA, followed by a rise in anisotropy as proteins bind to DNA, and a final plateau showing saturated binding. As mentioned previously for EMSA experiments, the binding data for anisotropy experiments fit the cooperative model. The apparent dissociation constants obtained from these anisotropy experiments are very similar to those found in EMSA experiments. Next, we measured the affinity of the p50 homodimer for the IFN-κB site. These re-
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Table I

| Ig-κB (FAA-pH 7.5) | Ig-κB (FAA-pH 8.0) | IFN-κB (FAA) |
|---------------------|---------------------|--------------|
| K_{app}             | K_{monomer}         | \( \alpha \) |
| \( \text{p50} \)    | 54.3 ± 1.1          | 210 ± 22.6   | 5.0 \times 10^{-2} ± 3.7 \times 10^{-3}   |
| \( \text{p65} \)    | 150.7 ± 29.2        | 379 ± 50.6   | 1.6 \times 10^{-1} ± 4.2 \times 10^{-2}   |
| \( \text{p50/p65} \) | 12.8 ± 2.2          | 1.7 \times 10^{-3} ± 1.6 \times 10^{-4} |

Results are presented in Table I. Our results show that the NF-κB p50 homodimer has similar affinities for both Ig-κB and IFN-κB DNA targets.

To further investigate the cooperative nature of binding, it is important to determine the affinity of a monomer for its κB half-site target. The cooperative model predicts that the monomers bind sequentially to their DNA half-sites, with the second monomer binding to its half-site with much higher affinity due to its interaction with the pre-bound first subunit. In order to test this hypothesis, we created a monomeric mutant p50 using information from crystallographic models and biochemical studies of the p50 homodimer (16, 17). The tyrosine at position 267 and leucine at position 269 are critical for subunit dimerization of p50. These residues are located away from the protein-DNA interface and are not involved in DNA contacts. We have created and purified the Tyr267L → Asp and Leu269L → Asp double mutant to homogeneity. Size exclusion experiments clearly show that the mutant p50 is monomeric even at a high protein concentration (5 mg/ml). Binding experiments have been performed with a DNA probe that bears only a single half-site (Fig. 4B). This eliminates any possible binding of two molecules of mutant p50 monomer in a non-cooperative manner. The p50 monomer binds to the target with an affinity of 210 nM (\( K_{monomer} \)). Using this value in Equation 1 yields a cooperativity factor of 0.050, suggesting that the second subunit binds to the DNA with 20 times higher affinity compared to the first monomer, 10.5 nM. The apparent equilibrium constant (\( K_{app} \)) for two monomers binding to DNA is 2.2 \times 10^{-15} M^{2}. However, in the pH, salt, and temperature studies, we focus on the overall \( K_{app} \), the concentration where half of the DNA is bound, which represents the affinity of the entire NF-κB dimer-DNA complex.

Binding Affinities of NF-κB p65 Homodimer for κB-DNA Targets—We have performed analogous binding experiments with p65 homodimer for both Ig-κB and IFN-κB DNA targets (Table I). Binding with Ig-κB DNA has been tested through both EMSA and polarization experiments at pH 8.0. EMSA experiments show that p65 homodimer binds the DNA with an affinity of 464 nM and fluorescence anisotropy gives a value of 341 nM. At pH 7.5 the p65 homodimer binds Ig-κB more tightly, with an affinity of approximately 150 nM. We also observe that the binding affinity of p65 homodimer IFN-κB DNA is similar to its affinity for Ig-κB DNA (414 nM versus 341 nM at pH 8.0).

The nature of binding isotherms also suggests a cooperative mode of binding. We therefore set out to determine the cooperativity of interactions between p65 and κB targets. We have created monomeric p65 by mutating Phe233D and Leu235D located at the subunit interface to aspartic acid. These two residues are located at the equivalent positions to that of Tyr267L and Leu269L, respectively, in p50. We overexpressed, purified, and tested the oligomeric nature by size exclusion chromatography. As expected, this double mutant was monomeric. However, the mutant tends to aggregate, preventing us from using it in binding experiments. We have overexpressed the monomeric DNA binding N-terminal domain of p65. X-ray crystal structures show that this fragment provides most of the sequence-specific binding of target DNA while lacking the phosphate backbone contacts contributed by the dimerization domain. This fragment binds a κB half-site with an affinity of approximately 1,800 nM at pH 7.5. Considering this as the absolutely upper limit, and the affinity of p50 RHR monomer, 210 nM, being the lower limit, we fit the \( K_{monomer} \) and \( \alpha \) values in Equation 1, with \( K_{monomer} \) constrained to be less than 1,800 nM, to the p65 RHR data at pH 7.5. This yielded a \( K_{monomer} \) of 379 nM and a cooperativity value (\( \alpha \)) of 0.16, suggesting that the second molecule of p65 monomer binds the second half-site of DNA with 6–7-fold higher affinity.

Binding Affinities of p50/p65 Heterodimer for κB-DNA Targets—In addition to the homodimers, we have also extensively studied the NF-κB p50/p65 heterodimer. We have determined the apparent binding affinities of the heterodimer for the Ig-κB DNA target using both gel mobility shift and fluorescence anisotropy assays. Similar to the results observed for the homodimers, we do not see any difference in binding affinities...
between these two methods. The $K_{\text{app}}$ values of the p50/p65 heterodimer for Ig-kB are approximately 20 nM at pH 8.0 in both assays. We observe that the heterodimer binds to IFN-kB with a relatively lower affinity compared with its Ig-kB targets. The apparent dissociation constants of Ig-kB and IFN-kB for the heterodimer are 19 and 27 nM, respectively, at pH 8.0. Our results show that the p50/p65 heterodimer has the highest affinity for Ig-kB DNA, p50 homodimer binds with intermediate affinity, whereas p65 shows the lowest binding affinity.

The nature of binding isotherm clearly indicates that the heterodimer binds $k$B targets with highest cooperativity of the three dimers tested here. Using the equilibrium binding constants of the p50 and p65 monomers to their DNA half-sites, we observe that the cooperativity of the heterodimer is 0.0017 (the second subunit binds 500 times tighter than the first) using Equation 2.

**Effect of pH on Complex Formation**—To test the pH sensitivity of the interactions between the heterodimer and Ig-kB DNA, we performed binding experiments at pH 7.5 and 8.0 using fluorescence anisotropy assay. These experiments showed approximately 2-fold higher affinity at pH 7.5 than at pH 8.0. To observe if both the homodimers also exhibit a similar binding trend, the homodimers were subjected to similar experiments. The homodimers did not show a large difference in affinities as was observed for the heterodimer. Nevertheless, both these dimers did show slightly higher affinities at pH 7.5 compared with pH 8.0. To further investigate the pH dependence of equilibrium binding constants of the heterodimer-DNA complex, we tested a wider pH range. The apparent binding constants were determined for the heterodimer/Ig-kB DNA complex at seven different pH values ranging from 6.0 to 9.0. At pH 6.0, no change in anisotropy was observed due to background noise, but a change of intensity was recorded with increases in protein concentration. Therefore, the binding constant was determined from the plot of increase of fluorescence intensity versus protein concentration. As shown in Fig. 5, apparent binding constants vary only roughly 2-fold between pH 6.8 and 8.0, with the highest affinity is observed at pH 7.0. Below pH 6.8 binding constants increase significantly as pH decreases. Similarly, $K_{\text{app}}$ increases as pH increases with a 5–6-fold increases of the binding constant at pH 9.0, the highest pH used in the assay.

**Effect of Salt on Complex Formation**—The dependence of the apparent $K$ for the p50/p65 heterodimer-Ig-kB DNA complex on salt concentration was determined at pH 8.0 and 37 °C using the anisotropy method. As shown in Table II, the $K_{\text{app}}$ of this complex is highly dependent on the salt concentration. $K_{\text{app}}$ remained unchanged between salt concentrations from 0 to 50 mM. Whereas $K_{\text{app}}$ is approximately 20 nM at 50 mM NaCl, it is reduced by a factor of 3–4 at 100 mM NaCl concentration. A reduction in $K_{\text{app}}$ value of 2 orders of magnitude is observed at 200 mM salt concentration. FAA experiments replacing NaCl with KCl produced no observable changes in the apparent equilibrium constants. The salt effect on the heterodimer/Ig-kB DNA complex is shown in a log-log plot of salt concentration versus $K_{\text{app}}$ in Fig. 6. The plot fits Equations 3 and 4 relating $K_{\text{app}}$ on temperature at constant salt concentration.

**Effect of Temperature on Complex Formation**—The dependence of $K_{\text{app}}$ on temperature at constant salt concentration...
(50 mM) and pH (7.5) was determined for the heterodimer/Ig-
κB DNA complex. The binding constants were measured at seven
different temperatures ranging from 4 °C to 42 °C. The results
are shown in a plot of \( \ln(K_{app}) \) versus temperature (Fig. 7).

\[ \text{DISCUSSION} \]

Over the last 5 years, three-dimensional x-ray structures of
nine different complexes of DNA-bound NF-κB dimers have
been determined. These structures have provided a wealth of
information regarding how these closely related dimers make
contacts with their DNA targets. In order to understand how
NF-κB dimers actually recognize DNA, an energetic profile of
NF-κB-DNA interactions is essential. In this study, we have
determined relative binding affinities of three NF-κB dimers,
p50 and p65 homodimers and p50/p65 heterodimer, for two
different physiological targets. We have also investigated the
effects of monovalent salt concentration, pH, and temperature
on DNA binding by the p50/p65 heterodimer.

\[ \text{Binding Affinities—We have used two different methods to} \]
\[ \text{measure binding affinities: gel mobility shift assay and solu-} \]
\[ \text{tion-based fluorescence polarization assay. Binding affinities} \]
\[ \text{obtained from both these assays are comparable for each of the} \]
\[ \text{three NF-κB-DNA complexes tested: p50/p65-DNA, p50 ho-} \]
\[ \text{modimer-DNA, and p65 homodimer-DNA complexes. The na-} \]
\[ \text{ture of the binding isotherms indicates a cooperative mode of} \]
\[ \text{binding. The source of cooperation is likely to be the stepwise} \]
\[ \text{binding of NF-κB monomers to DNA half-sites, followed by} \]
\[ \text{subunit association through the dimerization domains of each} \]
\[ \text{protein subunit. Indeed, our thorough investigation of binding} \]
\[ \text{by p50 to Ig-κB DNA clearly suggests that the dimer recognizes} \]
\[ \text{the target in a highly cooperative manner. Our results also} \]
\[ \text{show that the major source of the cooperativity is indeed the} \]
\[ \text{dimerization interactions between the two p50 subunits. Al-} \]
\[ \text{though we could not perform the similar experiment with p65} \]
\[ \text{due to the aggregation problem of monomeric p65 RHR, bind-} \]
\[ \text{ing affinity of p65 monomer was estimated to fall between the} \]
\[ \text{DNA binding affinity of the N-terminal domain p65 and affin-} \]
\[ \text{ity of monomeric p50 RHR. A binding affinity for p65 monomer} \]
\[ \text{for κB DNA of 379 nM is a good estimate for two reasons. First,} \]
\[ \text{this value fits our data best (lowest standard errors). Second,} \]
\[ \text{this value is roughly 2-fold lower than the p50 K_{monomer}, which} \]
\[ \text{is expected, because of extra DNA base contacts made by the} \]
\[ \text{p50 monomer. Using these K_{monomer} values for p50 and p65 in} \]
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a cooperative model for heterodimer binding gives a cooperativity factor of 0.0017. This suggests that the heterodimer binds the DNA much more cooperatively than either of the homodimers. Nevertheless, the apparent equilibrium binding constants provide the true affinity of the NF-κB dimer-DNA complexes. The apparent dissociation constants obtained from our experiments are somewhat higher than previous reports (19–23). Although we cannot explain the source of discrepancies, it is important to note that different binding reaction conditions may influence the relative affinity values.

Based on the three-dimensional structures of several NF-κB-DNA complexes, several important conclusions can be drawn. These complexes approximately bury 3000–3800 Å² solvent-exposed surface area; the dimers make 12–14 direct base-specific hydrogen bonds with their DNA target and 25–40 nonspecific hydrogen bonding contacts with the backbone of DNA targets (11). None of these numbers are unusual when compared with other complexes of dimeric transcription factor-DNA complexes. Whereas no direct relationship exists between the number of contacts between two complex forming macromolecules and the affinity of such a complex, it is not unusual that NF-κB binds DNA with nanomolar affinity like most other eukaryotic transcription factors. Incidentally, NFAT, a NF-κB-related transcription factor, is known to bind DNA with much lower affinity. The N-terminal specificity domain of NFAT is structurally very similar to the N-terminal domain of NF-κB and recognizes specific bases in almost identical manner to that of NF-κB (24).

pH Effect on Binding—DNA binding by the NF-κB heterodimer was determined as a function of pH. The apparent binding constants of the heterodimer/Ig-κB complex were measured at eight different pH values ranging from pH 6.0 to 9.0, using appropriate buffers. As presented in Fig. 5, the interaction of protein with DNA is optimal between pH 6.8 and 7.5. The affinity decreases below and above this pH range. However, the affinity decreases more dramatically at low pH. It is likely that partial protonation of certain residues such as Glu39 of p65 and Glu60 and His64 of p50 that are directly involved in DNA contacts are responsible for this effect. Conversely, deprotonation of DNA backbone contacting residues, Tyr36 and Cys38 of p65 and the corresponding Tyr27 and Cys29 of p50 reduce the affinity of protein for the DNA. Studies on the dimerization affinity of the p50 homodimer show no pH effects on dimer stability over the range of pH values used in these assays (16). Thus, the pH dependence of affinity is due to alterations of the amino acid residues that contribute directly to the NF-κB-DNA interface.

Salt Effect on Binding—NF-κB p50/p65 heterodimer binds Ig-κB DNA in a highly salt-dependent manner. Although no change in the binding constant is observed at NaCl concentrations between 0 and 50 mM, an increase of only 100 mM NaCl reduces the affinity by more than an order of magnitude. At 200 mM NaCl, the heterodimer binds Ig-κB practically nonspecifically. Similar strong effects of salt on p50 homodimer binding to IFN-κB DNA suggests that all NF-κB-DNA complexes are formed in a salt-dependent manner. Additionally, the formation of p50 dimers in the absence of DNA is not affected by the salt concentrations used here (16).

From the p50/p65 structure, it appears that a significant fraction of the binding affinity of NF-κB-DNA is likely to come from nonspecific salt bridges between the DNA phosphate backbone and positively charged protein side chains. There are at least 20 such contacts observed between the heterodimer and Ig-κB DNA complex (11). Additionally, from NMR and molecular modeling studies of the HIV-LTR Ig-κB DNA, it appears that the dynamics of the phosphate backbone's conformation in the 5′ and 3′ regions of the κB sequence play an active role in NF-κB recognition (25). Cooperative interactions with other transcription factors may provide the higher level of specificity at physiological salt concentrations, which is approximately 175 mM.

It is interesting to note that, during the original purification of the p50/p65 heterodimer, it was observed that the protein bound almost as tightly to nonspecific oligonucleotide columns as to specific ones. NF-κB also eluted from the oligonucleotide columns at much lower salt concentrations than other DNA-binding proteins (0.2 and 0.4 M, respectively) (26). Our data predict this weak binding at the salt concentrations used and the low protein concentration of this initial purification. At this point it is still unclear why NF-κB’s DNA binding behavior at low salt concentrations (0–50 mM) differs from that higher concentrations.

Ha et al. (14) have successfully derived an equation describing the effects of monovalent salt and water on DNA-protein complex formation (Equation 4), which has been simplified by O’Brien et al. (15). Using this ion displacement model, we calculate an A value of 6 ions (also the Z value from Equation 3) and a B value of 426 water molecules released upon complex formation. The crystal structure of the complex shows that, upon association, 3800 Å² of solvent-accessible surface area is buried (11). Considering 9 Å² as the surface area of a water molecule, theoretically 422 molecules of water would be released from this complex.

**Temperature Effect of Binding**—The dependence of the apparent binding constants on temperature at constant salt (50 mM NaCl) and pH (7.5) was determined. As shown in Fig. 7, apparent binding constants essentially remain unchanged at temperatures ranging from 4°C to 42°C. This suggests that the intrinsic enthalpy change upon complex formation is negligible. It therefore appears that the binding of Ig-κB DNA by NF-κB p50/p65 heterodimer is an entropic process driven by the release of counterion and bound waters. This is not surprising for two reasons. First, release of a large number of water molecules clearly favors entropy of binding. Second, crystallographic analysis of various NF-κB-DNA complexes reveals that several DNA contacting amino acid side chains are most likely pre-organized through interactions with each other. In fact, the structures of the dimerization domains of the p50 and p65 homodimers show that the DNA backbone contacting residues contributed by the dimerization domain adopt similar conformations in the unbound form as those found in their respective homodimer-DNA complexes (17). These observations suggest that the ordering of amino acid side chains, and the resulting loss of entropy, are minimal in the forming of NF-κB-DNA complexes.

X-ray crystallographic analyses of various NF-κB-DNA complexes have given a strong foundation upon which to initiate thermodynamic studies of these complexes. In this report we have shown qualitatively the relative binding behaviors of three NF-κB dimers, p50, p65, and p50/p65, with two different DNA targets. We have further investigated the role of pH, monovalent salt, and temperature on the ability of the p50/p65 heterodimer to recognize Ig-κB DNA. More detailed analyses are essential to determine the thermodynamic parameters of binding in more quantitative terms.

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