Binding Studies with Mutants of Zif268

CONTRIBUTION OF INDIVIDUAL SIDE CHAINS TO BINDING AFFINITY AND SPECIFICITY IN THE ZIF268 ZINC FINGER-DNA COMPLEX

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The Zif268 zinc finger-DNA complex has served as a model system for understanding how Cys2His2 type zinc fingers recognize DNA. Structural studies of the Zif268-DNA complex revealed that residues at four positions in the α helix of each zinc finger play key roles in recognition, but there has been no information about the precise contributions of individual residues. Here we report the results of binding studies involving five mutants of Zif268 that have changes in the base-contacting residues of finger one. These studies allow us to evaluate the contributions that Arg18 (position −1 of the α helix), Asp20 (position 2), Glu21 (position 3), and Arg24 (position 6) make to the overall energy of DNA binding. Our results confirm the important role played by these arginines. By comparing the affinities of the wild type and mutant peptides for various sites, we also prove that Asp20 and Glu21 play important roles in determining binding site specificity.

The Zif268 zinc fingers of the Cys2His2 class are one of the most abundant DNA-binding motifs found in eukaryotes (1, 2). These zinc finger proteins recognize a diverse set of DNA sequences, and design and selection efforts have produced many variant fingers with modified specificities (e.g. Refs. 3–10). The first zinc finger-DNA complex to be visualized at atomic resolution involved the three fingers of Zif268 (11). This structure of the Zif268-DNA complex, since refined to 1.6 Å (12), revealed that the complex has a relatively simple, modular arrangement (Fig. 1a). Each finger contains a short, two-stranded antiparallel β sheet and an α helix. The sheet and the helix are held together by a small hydrophobic core and by a zinc ion, which is coordinated by two conserved cysteines from the sheet region and two conserved histidines from the α helix. Each of the three Zif268 fingers has a similar docking arrangement with respect to the DNA, and each uses residues from the amino-terminal portion of its α helix to contact bases in the major groove. The majority of the base contacts involve the guanine-rich strand of the binding site, and each finger makes its primary contacts with a three base pair subsite (GCG/TGG/GCG).

In the Zif268 complex, amino acid residues at four positions of the α helix make direct base contacts (positions −1, 2, 3, and 6, numbering with respect to the start of the helix). These contacts are summarized below and in Fig. 1b. (For a detailed description of the Zif268-DNA interface, the reader is referred to the original papers describing the structure (11, 12).) The residue at position −1 of the α helix is an arginine in all three fingers (Arg18, Arg46, and Arg74), and, in each case, this arginine makes a pair of hydrogen bonds with the guanine at the 3′ end of the finger’s subsite. Each of the fingers also has an aspartic acid at position 2 of the α helix (Asp20, Asp26, and Asp29), and in each finger this aspartic acid residue makes a pair of hydrogen bonds with the arginine from position −1. This interaction presumably helps orient the arginine and thus increase the specificity of the arginine-guanine interaction. The aspartic acid from position 2 may also interact with a base on the opposite strand of the DNA that is just outside the primary 3-base pair subsite of the finger. (Note that although we describe the binding site in terms of 3-base pair subsites in this paper, one could also describe recognition with respect to overlapping 4-base pair subsites.) In all three fingers, the residue at position 3 of the α helix contacts the middle base of the finger’s 3-base pair subsite, but these contacts are different in different fingers. In finger two, the residue at position 3 is a histidine. This histidine forms a hydrogen bond with guanine 6 and makes van der Waals’ contacts with thymine 5. Fingers one and three have a glutamic acid at position 3 of the helix. These glutamic acids do not make any hydrogen bonds with the bases, but the 1.6 Å structure revealed that they do make base contacts in their respective fingers; the Cy and C6 from each of these glutamic acids are in van der Waals’ contact with the C5 and C6 of the cytosine from the middle base pair of the finger’s subsite. Fingers one and three have an arginine at position 6 of the α helix. In both of these fingers, this arginine makes a pair of hydrogen bonds with the guanine at the 5′ end of the finger’s subsite. (Finger two has a threonine at the corresponding position, but this residue does not make any direct base contacts.)

Numerous modeling, design, and selection studies (3, 6, 7, 9, 13–16) confirm that the residues at positions −1, 2, 3, and 6 of the α helix in each finger play important roles in site-specific recognition. However, the precise contribution that individual residues make to binding affinity and specificity in the Zif268-DNA complex has not been known. Although a few mutants with changes in the base-contacting residues have been constructed, the subsequent binding studies used peptides expressed on the surface of phage (17, 18), and we believe that measurements with purified peptides will ultimately be more accurate. To carefully study the roles of individual residues, we have constructed five mutants with alterations in the base-contacting residues of finger one. Here we report the equilibrium dissociation constants of these peptides at a wild-type site and two mutant binding sites, and we use these data to analyze the role that each of these residues plays in site-specific recognition.


**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Mutations were introduced into pZif89 (the expression construct that encodes the three fingers of Zif268 (11)) via the four primer polymerase chain reaction method (19). The resulting polymerase chain reaction product, which included the entire zinc finger coding region, was gel purified (Qiagen), digested with NdeI and BamHI, and ligated into NdeI-BamHI-digested pET3a (R18A, D20A, R18A/D20A, and R24A) or pET21a (E21A). The sequences of all five mutant genes were verified by dideoxy sequencing (performed by the Biopolymers Laboratory, Howard Hughes Medical Institute, Massachusetts Institute of Technology). The expression constructs were transformed into E. coli BL21(DE3) cells containing either the pLysE plasmid (wild type Zif268, R18A, D20A, R18A/D20A, and R24A) or the pLysS plasmid (E21A), and expression was induced as recommended (Novagen). The peptides (Fig. 2a) were purified by reversed-phase batch extraction on Sep-Pak C18 cartridges (Waters) and reversed-phase high-performance liquid chromatography on a C4 column (Vydac) essentially as described (11). Purified peptides were folded in an anaerobic chamber (Coy Laboratory Products) by dissolving them in water and then adding ZnSO₄ to 2.75 mM and bis-tris-propane, pH 6.8, to 50 mM (3). The folded peptides were stored in aliquots at −80 °C. The peptides were about 95% pure (E21A and R24A) or 98% pure (wild type, R18A, D20A, and R18A/D20A), as estimated from examination of silver-stained SDS-polyacrylamide gels.

_Determination of Apparent Dissociation Constants_—The double-stranded oligonucleotide binding sites used in these studies are shown in Fig. 2b. The individual oligonucleotides were synthesized, gel purified, annealed, and end-labeled using [γ-³²P]ATP and T4 polynucleotide kinase (20). Binding assays were performed at room temperature in degassed binding buffer (50 mM NaCl, 5 mM MgCl₂, 10 μM ZnSO₄, 5% (v/v) glycerol, 0.1 mg/ml acetylated bovine serum albumin, 1 mg/ml Igepal-CA630, and 15 mM Hepes at pH 7.8) (3). Binding reactions were equilibrated for 3 h before being electrophoresed on 10% polyacrylamide gels in 0.5× TB. (Control experiments showed that binding reactions for these peptides reached equilibrium within 30 min.) Dried gels were stained with aniline blue.

| Protein          | K_d/K_d wild type | ΔG_mut − ΔG_wild type | kcal/mol |
|------------------|-------------------|-----------------------|----------|
| Zif268           | 1.7 (± 0.07) × 10⁻¹⁰ | 1                     | −2.7     |
| R18A             | 1.7 (± 0.08) × 10⁻⁶ | 100                   | 0.30     |
| D20A             | 1.0 (± 0.01) × 10⁻⁹ | 26                    | 1.9      |
| R18A/D20A        | 4.4 (± 0.42) × 10⁻⁹ | 26                    | 1.5      |
| E21A             | 2.5 (± 0.91) × 10⁻¹⁰ | 412                   | −3.6     |
| R24A             | 7.0 (± 1.21) × 10⁻⁸ | 412                   | −3.6     |

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**FIG. 2.** Sequences of the wild type zinc finger peptides and of the oligonucleotide binding sites. **a** sequences of the wild type Zif268 zinc finger peptide. The residues at positions 1, 2, 3, and 6 of the α-helix of finger one, which have been the focus of this study, are circled. The three fingers are aligned to highlight conserved residues and conserved secondary structure elements. The α-helix is indicated by a cylinder, and the β-strands are indicated by arrows. The cysteine and histidine residues that are ligands for the zinc ions are highlighted in bold (adapted from Ref. 12). **b** sequences of the wild type and mutant oligonucleotide binding sites used in the gel shift assays. The Zif268 binding site is highlighted in bold; the numbering scheme is the same as that used in papers describing the structure of the complex (11, 12). Boxes indicate bases that are altered in the mutant binding sites.

**FIG. 1.** *a*, overview of the Zif268 zinc finger-DNA complex. Only those side chains that make direct base contacts are shown. Finger one is red, fingers two and three are yellow; the DNA is blue, and the zinc ions are gray (adapted from Ref. 12). *b*, schematic diagram of the base contacts made by Zif268. Arrows indicate hydrogen bonds; dotted arrows represent hydrogen bonds with marginal geometry. Lines ending in filled circles represent van der Waals’ interactions. The numbering scheme is the same as that used in papers describing the structure of the complex (11, 12) (adapted from Ref. 11).
exposed to a PhosphorImager screen (Molecular Dynamics) overnight.

To derive the apparent dissociation constant for each peptide, the labeled binding site was mixed with increasing amounts of the zinc finger peptide. The binding reactions contained labeled oligonucleotide at either 2.5 μM (for wild type Zif268 and the D20A and E21A peptides) or 25 μM (for the R18A, R18A/D20A, and R24A peptides). Reactions also contained 14.7 μg/ml poly(dI-dC)-poly(dI-dC). \( K_d \) values were determined by fitting the data to the equation

\[
\theta = \frac{1}{1 + \frac{[P]}{K_d}} \quad \text{(Eq. 1)}
\]

where \( \theta \) represents the fraction of the DNA bound by the peptide. The free protein concentration, [P], was approximated by the total protein concentration (because the concentration of the binding site was considerably below the \( K_d \) in each case). The active protein concentration was determined by titrating each peptide against higher concentrations of the wild type oligonucleotide binding site; oligonucleotide concentrations were 150 nM (for wild type Zif268 and the D20A and E21A peptides), 500 nM (for the R18A/D20A peptide), or 1 μM (for the R18A and R24A peptides). Each experiment (\( K_d \) determination and measurement of the active protein concentration) was performed at least twice, using a freshly thawed aliquot of peptide each time.

RESULTS

We have constructed mutants of Zif268 in which each of the base-contacting residues in finger one, Arg\(^{18}\), Asp\(^{20}\), Glu\(^{21}\), and Arg\(^{24}\), has been individually mutated to alanine. Because the side chains of Arg\(^{18}\) and Asp\(^{20}\) interact with each other, we also have constructed an R18A/D20A double mutant. Each of these mutant peptides has been overexpressed and purified, and its affinity for a Zif268 binding site has been determined by a gel mobility shift assay. Altered binding sites also have been tested with wild type Zif268 and with some of the mutant peptides.

**Contribution of Positions 1, 2, 3, and 6 to Overall DNA Binding Energy**—We began by determining the affinity of each of the five mutants for a wild type Zif268 binding site. The apparent dissociation constants measured for the mutant peptides and for wild type Zif268 under the same conditions are listed in Table I. Wild type Zif268 binds the oligonucleotide site used in these studies (Fig. 2b) with a \( K_d \) of 0.17 nM (Fig. 3a). \( K_d \) values reported in previous studies range from 0.01 to 6.5 nM, depending on the binding conditions used (3, 7, 9–11, 16, 21). Mutating Arg\(^{18}\) (at position −1 of the helix) to alanine results in a 100-fold loss of affinity (\( K_d \) = 17 nM). Simultaneously mutating Arg\(^{18}\) and Asp\(^{20}\) (positions −1 and 2 of the helix) to alanine produces a peptide that binds with an apparent \( K_d \) of 4.4 nM (Fig. 3c). Thus, the double mutant binds 26-fold less tightly than wild type Zif268 but about four times as tightly as the R18A single mutant. At this stage, we do not know why the double mutant binds more tightly than the single mutant, but it is possible that the R18A mutant, in addition to losing contacts with the guanine, also leads to some unfavorable electrostatic interactions for the Asp\(^{20}\) residue that is now left without Waals’ contacts but no hydrogen bonds with the DNA in the wild type complex. (For example, there could be unfavorable electrostatic interactions between the acidic residues at positions 2 and 3 of the helix and the DNA.) Alternately, some rearrangement might occur in the double but not the single mutant that allows another side chain (such as Glu\(^{21}\)) to form new contacts with the DNA.

The D20A peptide binds with slightly greater affinity than does the wild type peptide (\( K_d \) = 0.10 nM; Fig. 3b). This observation also is surprising; we expected the D20A mutant to bind less tightly because this mutation eliminates the hydrogen bonds between Asp\(^{20}\) and Arg\(^{18}\) that presumably help orient the arginine side chain. The basis for the increased affinity of the D20A peptide is unclear, but it seems possible that this mutation may allow the arginine side chain to interact with Glu\(^{21}\) and contact the phosphate backbone instead of the base.
Binds the GCC site with a GCG site (Fig. 3a) mentioned previously, Asp20 (position 2 of the Zif268 and the D20A and R18A/D20A mutant peptides to a site specificity at base pair 10, we studied binding of the wild type whether this Asp-Arg interaction plays any role in determining makes a pair of hydrogen bonds with guanine 10. To determine whether this residue nonetheless plays a significant contribution to binding affinity, we examined binding of the wild type and E21A peptides to a site in which cytosine 9 was changed to adenine (the GAG site; Fig. 2b). Wild type Zif268 displays a clear preference for a wild type GCG subsite: Zif268 binds the GAG site with a $K_d$ of 2.2 nM, 13-fold less tightly than it binds the wild type binding site (Fig. 4a and Table III). However, mutating Glu21 to alanine results in a loss of specificity at base pair 9; the E21A mutant peptide displays no preference for a wild type binding site and actually binds the GAG site slightly more tightly than the wild type site ($K_d$ of 0.10 nM; Fig. 4b). It appears that Glu21 has a greater effect on specificity than it has on affinity per se.

### DISCUSSION

The data presented here allow us to evaluate the energetic contributions to binding affinity made by the base-contacting residues from finger one of Zif268 and to examine their contributions to specificity. In the cocrystal structure of the Zif268-DNA complex (11, 12), Arg18 (position −1 of the α helix) and Arg24 (position 6) each make a pair of hydrogen bonds with a guanine. Our binding studies show that each of these arginine residues makes a significant contribution to the overall binding energy (Table I). The acidic residues from positions 2 and 3 of the α helix, Asp20 and Glu21, do not appear to contribute significantly to binding affinity (Table I). However, these residues do contribute to specificity, as indicated by comparing the affinities of wild type and mutant peptides for wild type and mutant binding sites. In particular, we find that mutating Asp20 results in a reduced specificity for guanine at position 10 of the binding site (Table II). This decrease in specificity is probably due to the loss of the hydrogen bonds between Asp20 and Arg24 (and possibly also to the loss of the water-mediated hydrogen bond Asp20 normally makes to cytosine 10). Glu21 also contributes to specificity; mutating this glutamic acid to alanine results in a loss of specificity at base pair 9 (Table III). The structural basis for this observation is not entirely clear, but the Glu21 side chain, through its proximity to base 9, may help exclude bases other than cytosine from this position of the binding site. It also seems possible that favorable van der Waals’ contacts between the glutamic acid and the cytosine may be energetically balanced by the unfavorable effects of having this glutamate near the DNA. (This could explain how Glu21 contributes to specificity even though mutating this residue to alanine has very little effect on the net binding energy.) We also note that, as in all experiments of this sort, there is the possibility of compensating structural rearrangements that may complicate the interpretation, and crystal structures of the mutant complexes would be needed for a really definitive interpretation of the energetics.

In summary, our data show that Arg18 and Arg24 both make significant contributions to binding affinity (and presumably...
also to specificity). The acidic residues Asp\textsuperscript{20} and Glu\textsuperscript{21} do not make large contributions to the overall energy of DNA binding but do help determine specificity. These studies clarify several important questions about recognition and provide a better basis for understanding zinc finger-DNA interactions.

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