Altered Specificity of DNA-Binding Proteins with Transition Metal Dimerization Domains

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The bZIP motif is characterized by a leucine zipper domain that mediates dimerization and a basic domain that contacts DNA. A series of transition metal dimerization domains were used to alter systematically the relative orientation of basic domain peptides. Both the affinity and the specificity of the peptide-DNA interaction depend on domain orientation. These results indicate that the precise configuration linking the domains is important; dimerization is not always sufficient for DNA binding. This approach to studying the effect of orientation on protein function complements mutagenesis and could be used in many systems.

Active sites of proteins are typically composed of recognition elements guided into proximity and appropriate orientation by the native protein fold. Individual recognition elements may be remote in primary structure or may be located on different polypeptide chains in multisubunit proteins. With site-directed mutagenesis, amino acids that constitute individual recognition elements can be changed without affecting the overall orientation of the recognition domain. Yet present technology does not allow predictable and routine changes in the orientation of the domains themselves.

The transcriptional activator protein GCN4 is one of a large family of DNA-binding proteins identified by a bZIP structural motif (1); this motif contains a DNA contact domain characterized by conserved basic and hydrophobic residues (b domain), and a dimerization domain identified by a helix-collared leucine repeat extended NAD (domain) (2, 3). The two domains are separated by a six-amino acid linker whose length, but not sequence, is conserved across bZIP families (1). Previous work has demonstrated that the active DNA-binding entity is generated when the ZIP domains of two protein monomers assemble (4) into a parallel coiled coil (5, 6). The scissors grip (1) and induced helical fork (7) models propose that the coiled coil, the natural dimerization
tion domain, fixes the spatial relationship between the two DNA contact domains to present the correct constellation of functional groups to the DNA (5, 8–10). Even subtle changes in basic domain orientation or organization can have dramatic effects on DNA binding (5, 11–13).

To understand better the role of dimerization domain architecture in modulating the affinity and specificity of bZIP-DNA interactions, we explored an approach to protein design (14) that exploited the well-defined geometries of transition metal ion complexes to assemble protein structural domains with defined orientations (15). Transition metal ion complexes make ideal synthetic scaffolds: the strong geometric constraint placed on the ligand by the central metal ion allows the relative orientation of two or more domains to be altered systematically by positioning them, through chemical synthesis, at various locations on a ligand of known structure. Molecules were constructed in which the GCN4 coiled coil was replaced by a series of Fe(II) complexes that systematically altered the relative orientation and spacing of the basic DNA contact domains. In our model-building studies we started with the coordinates of the scissors grip model (1) and sought a stereochemically well-defined transition metal ion complex whose geometry was complementary to the void produced on removal of the GCN4 coiled coil. These requirements were satisfied by the kinetically inert bis(terpyridyl)iron(II) complex first prepared by Morgan and Buntal in 1932 (16).

The terpyridyl substitution pattern dictated the relative orientation of G29 peptides in bis(terpyridyl)iron(II) complexes [G29T11]Fe, [G29T18]Fe, and [G29T13]Fe (17) (Fig. 1). In [G29T13]Fe, the 4′-substituted terpyridyl T13 separated the two G29 carboxamide termini by approximately 11 Å and oriented them at a 180° angle. In [G29T18]Fe, the 4-substituted terpyridyl T18 separated the carboxamide termini by approximately 8 Å and oriented them at a 90° angle. The 4′-substituted terpyridyl T13 enforced the same geometry as T18 but contained a longer and more flexible tether.

The ability of these stereochemically defined synthetic scaffolds to organize two G29 peptides into a functional DNA-binding protein was determined by monitoring the DNA-binding affinities of the resultant complexes. The disulfide dimer of G29 (G29SS) (13) and a 54-amino acid peptide containing both the DNA contact and dimerization domains of GCN4 (G35) were studied for comparison.

The binding of [G29T13]Fe complexes to duplex DNA was measured first by gel mobility shift assay (18). Incubation of CRE1, a 5′ end-labeled double-stranded DNA fragment (5′-AGTGGAGATGC-GTCTACTGGTGC-3′) containing the high affinity GCN4 binding site CRE (AT-GAGCTCAT; cAMP response element) (19), with 0.13 to 1.2 nM [G29T13]Fe resulted in the gradual appearance of a complex with lower electrophoretic mobility than CRE1 alone or a CRE1-G29 complex (Fig. 2A). Monomeric G29T13 failed to bind CRE1, but binding was restored upon addition of Fe(II). Addition of Cu(II) did not result in DNA binding. The affinity of [G29T13]Fe for CRE1 was determined by measuring the fractions of bound and free CRE1 at several [G29T13]Fe concentrations (Fig. 3). The data fit a theoretical equation that describes formation of a 1:1 complex with an equilibrium dissociation constant (Kd) of 0.13 ± 0.01 nM (18), a value comparable to the Kd measured for the G44 homodimer and the Fox-Jun heterodimer at this temperature (11).

DNA binding by [G29T13]Fe was characterized further by deoxyribonucleic acid (DNAase I) footprinting (20) and circular dichroism (CD) spectroscopy. [G29T13]Fe and G35 exhibited identical DNAase I footprints and adjacent hypersensitive sites (Fig. 2B), demonstrating that the two molecules make similar DNA contacts and cause similar structural adjustments of the flanking DNA. Previous work has shown
that the DNA contact domains of bZIP proteins undergo an increase in helical structure when bound to specific DNA targets but not to nonspecific DNA (7, 9, 10, 13). [G29Tg3]Fe had little helical structure in the absence of DNA. The intensity of the CD signal at 222 nm (a helical band) increased significantly when one molar equivalent of CRE<sub>16</sub> (5'GAGATGGAGAGTCATCTCTGTGC-3') was added (Fig. 4A). The difference spectrum (Fig. 4B) indicated that [G29Tg3]Fe was almost entirely helical when bound to CRE<sub>16</sub> (21). These results demonstrate that a metal ion complex of 612 daltons can replace the function of the leucine zipper, effectively orienting two G29 basic domains to permit high-affinity, sequence-specific recognition of a GCN4 target site.

The importance of orientation in DNA binding is illustrated by comparing the DNA affinities of [G29Tg3]Fe, [G29Tg]Fe, and [G29Tg]Fe. Although these metal complexes are structurally similar, neither [G29Tg3]Fe nor [G29Tg3]Fe bound CRE in a mobility shift assay (Fig. 2A), even at 0.2 μM. The absence of binding was not due to intractability of the relevant DNA complexes under mobility shift conditions; neither [G29Tg3]Fe nor [G29Tg3]Fe yielded a detectable DNase I footprint (Fig. 2B) at 4 μM nor reduced the extent of CRE[G29Tg3]Fe complex formation in a mobility shift competition assay. The only evidence supporting DNA binding by [G29Tg3]Fe or [G29Tg3]Fe was obtained in CD experiments performed at a concentration 10,000 times the K<sub>d</sub> of the CRE[G29Tg3]Fe complex, where a moderate increase in peptide helicity was observed (Fig. 4B). From our mobility shift data, we estimated that at 4°C the CRE[G29Tg3]Fe complex is 4 kcal mol<sup>-1</sup> more stable than either the CRE[G29Tg]Fe or the CRE[G29Tg]Fe complex. The spacing of the basic domains in [G29Tg3]Fe and [G29Tg]Fe differs by only four methylene groups, while [G29Tg3]Fe and [G29Tg]Fe differ only in the orientation with which the basic domains extend into the major groove. Thus, coordination complex architecture is important in orienting the G29 basic domain for high affinity recognition of the CRE. The transition metal ion complexes studied here provide an independent stereoelectronic variable in protein design by allowing the assembly of peptide aggregates with defined orientations.

All bZIP proteins share a common domain architecture and shared basic and hydrophobic residues, yet they differ in their ability to recognize target sites with alternative half-site spacing. The Fos-Jun subfamily prefers the AP1 site (ATGACTCAT) and the CREB/ATF subfamily prefers the CRE site (ATGACGTCA) (22). GCN4 (9, 19) and certain cysteine-linked dimers of the GCN4 basic domain (23) bind both sites with comparable affinity. Within a B-DNA context, the additional C-G base pair in the CRE displaces the two ATGA contact surfaces by an axial translation of 3.4 Å and a twist angle of 34° (24).

As the results described above demonstrate the effect of basic domain orientation on CRE affinity, we asked whether basic domain orientation would also affect discrimination between CRE and AP1. Mobility shift assays did not detect any interaction between AP1<sub>23</sub> (5'-GGTGGAGATGCCTTCATCTCTGTGC-3') and [G29Tg]Fe, even at elevated concentrations (Fig. 5A), whereas G29<sup>55</sup> bound CRE<sub>14</sub> and AP1<sub>23</sub> with comparable affinity (Fig. 5B). Neither
peptide bound AP2β (5'AGTGAGAT-GATCATCTCAGTCC-3'), an API-like DNA containing a C to T mutation that is not tolerated by GCN4 in vitro (25). DNase I footprint experiments performed with a DNA fragment containing both CRE and API sites revealed a footprint only at the CRE site in the presence of G35 Taf-Fc, whereas footprints were observed at both sites when G35 or G35-25 was added (Fig. 3B). Finally, G35 Taf-Fc displayed no increase in helical structure in the presence of API1 (5'AGATGAGAT-GATCATCTCAGTCC-3') (Fig. 4C). A mobility shift competition assay was performed to quantify the differential binding properties of G35 Taf-Fc (17, 18, 26). The preference for CRE over API1, exhibited by G35 Taf-Fc, corresponded to a differential binding free energy (ΔΔG°) of >4 kcal mol⁻¹ (17). Although G35 Taf-Fc contains the basic and linker domains of GCN4, it had a 10-fold preference for CRE over API1, mimicking the sequence-selectivity of the CREB/ATF subfamily. These results demonstrate that the orientation and the relative displacement with which the basic domains emerge from the central dimerization interface are important in controlling CRE affinity and CRE/API1 selectivity (27).

Transcriptional regulation in eukaryotes involves a finely tuned interplay of proteins with a complex set of DNA target sites. A fundamental issue in the study of gene regulation is how regulatory proteins differentiate among closely related sites with high precision. Here we demonstrate that substitution of the GCN4 coiled coil with a stereoechemically well-defined metal complex generates a molecule capable of differentiating DNA sequences that GCN4 cannot. G35 Taf-Fc contains the GCN4 basic and linker domains, yet it displays the sequence selectivity of the CREB/ATF subfamily. The bis(terpyridyl)iron(II) dimerization domain modifies the inherent selectivity of the GCN4 basic domain without altering its sequence.

REFERENCES AND NOTES

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