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Diffusion-controlled DNA recognition by an unfolded, monomeric bZIP transcription factor

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Abstract Basic leucine zipper (bZIP) transcription factors are dimers that recognize mainly palindromic DNA sites. It has been assumed that bZIP factors have to form a dimer in order to bind to their target DNA. We find that DNA binding of both monomeric and dimeric bZIP transcription factor GCN4 is diffusion-limited and that, therefore, the rate of dimerization of the bZIP domain does not affect the rate of DNA recognition and GCN4 need not dimerize in order to bind to its specific DNA site. The results have implications for the mechanism by which bZIP transcription factors find their target sites for transcriptional regulation.

Key words: Protein-DNA recognition; Kinetic mechanism; Transcription factor GCN4; Monomer-dimer equilibrium; Fluorescence-labeled DNA

1. Introduction

Transcription factors bind to promoter and enhancer regions of transcribed genes and contribute to the efficiency with which RNA polymerase II binds and initiates transcription. In basic leucine zipper (bZIP) transcription factors a C-terminal leucine zipper domain is preceded by both a basic region that binds to DNA and an activation domain that is implicated in transcriptional regulation [1–3]. Because bZIP factors dimerize in the absence of DNA and since the DNA recognition sites often have dyad symmetry, it is thought that bZIP factors have to form dimers before they bind to their target DNA sites [3,4]. However, in this compulsory dimer pathway the formation of the leucine zipper can become rate-limiting if equilibrium 1 (Fig. 1) is far on the side of the monomer. This kinetic bottleneck can be circumvented in a monomer pathway in which monomers bind sequentially and the leucine zipper dimer forms on the DNA (equilibria 3 and 4). Indeed, DNA footprinting analysis has shown monomer binding to half-sites of dyad symmetrical target sequences [5–7]. Also, DNA binding observed by band shift assays was faster than expected for a dimer pathway [8]. In this paper we present a direct kinetic comparison of monomer versus dimer binding for the yeast transcription factor GCN4. We find that both monomer and dimer bind to DNA at a diffusion-limited rate.

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Abbreviations: AP-1, activation protein-1 recognition site ATGACTCAT; CRE, cyclic AMP response element ATGACGTCAT; AP-$1_{20}{}^{F}$ and $CRE_{19}{}^{F}$, double-stranded oligonucleotides containing the AP-1 and CRE site, respectively, and the fluorescence marker NBD attached to a phosphorothioate bond preceding the recognition site; bZIP, basic leucine zipper; CD, circular dichroism; GCN4, general control of amino acid synthesis non-derepressible mutant 4, see Table 1 for abbreviations of the GCN4 derivatives used in this study; NBD, *N*,*N*′-dimethyl-*N*-acetyl-*N*′-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)ethylenediamine

2. Materials and methods

2.1. Proteins

C62GCN4 was expressed in *Escherichia coli* from plasmid pET-3AC62GCN4 [9] and purified as described [10]. Mutations (Table 1) were introduced with a QuickChange site-directed mutagenesis kit from Stratagene and expressed and purified as described [10]. Primers used were (template strands): 5′-CCGGTGATGTTATCAACAGCCACTGCCGCGTTCGCGCCAAC-3′ for $(CGCN4)_2$, and 5′-GCCACCTAATTTCTTAGCTCTGGCAGCCTCATTTTC-3′ for AAGCN4. Changes with respect to the wild-type sequence are underlined and encode the C-terminal extension GSGC in $(CGCN4)_2$ and V/A and L/A replacements in AAGCN4. Peptides GCN4br and $(GCN4br)_2$ were chemically synthesized by the Fmoc strategy as before [11]. Peptide concentrations were determined photometrically [12] and refer to single peptide chains unless indicated. The percentage of unfolded monomer was calculated as $100\times\{[-K_d+(K_d{}^2+8K_dP_0)^{1/2}]/4P_0\}$, where K_d is the dissociation constant of the leucine zipper and P_0 the concentration of peptide chains. $K_d = 1.6\times10^{-6}$ M for C62GCN4 and $>3\times10^{-5}$ M for AAGCN4 was estimated from the change with peptide concentration of the CD signal at 222 nm [13].

2.2. Oligonucleotide synthesis

$CRE_{19}{}^{F}$ and AP-$1_{20}{}^{F}$ (Table 1) were synthesized using standard phosphoramidite chemistry and were obtained from MicroSynth, Balgach, Switzerland. The fluorescent NBD group was introduced by reaction of the phosphorothioate group [14] with a 15-fold molar excess of *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)ethylenediamine (Molecular Probes, Eugene, OR) for 18 h at 50°C in 30% aqueous dimethylformamide. The oligonucleotides were purified by reversed-phase HPLC [10] and their correct mass confirmed by electrospray ionization mass spectrometry. Concentrations were determined spectrophotometrically using $\varepsilon_{254} = 180.5$ mM^{-1} cm^{-1} [15]. Concentrations refer to double-stranded $CRE_{19}{}^{F}$ or AP-$1_{20}{}^{F}$ unless indicated.

2.3. Fluorescence measurements

Fluorescence spectra were measured on a Spex Fluorolog instrument; excitation 495 nm, emission 510–600 nm. In fluorescence titrations, small aliquots of 4.8 or 60 μM peptide solutions were added to either 50 or 250 nM oligonucleotide and the fluorescence emission at 544 nm was measured 5 min after each addition. Sample dilution during titration was $<5\%$ and was not corrected for. Measurements were made at 25°C in 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$ or in 20 mM phosphate buffer with the ionic strength adjusted to either 0.1 M or 0.5 M with KCl. The same results were obtained in Tris and phosphate buffer and at low or high ionic strength. K_{app}, the apparent dissociation constant of the transcription factor-DNA complex, was obtained by fitting the observed change of fluorescence according to $\Delta F_{544} = \Delta F_{max}\{D_0+P_0+K_{app}-[(D_0+P_0+K_{app})^2-4D_0P_0]^{1/2}\}/2D_0$. The fit assumes a simple 1:1 equilibrium between free transcription factor and DNA. In the reaction with monomeric GCN4br, P_0 is the concentration of peptide chains and D_0 that of half-sites. For all other peptides, D_0 is the concentration of double-

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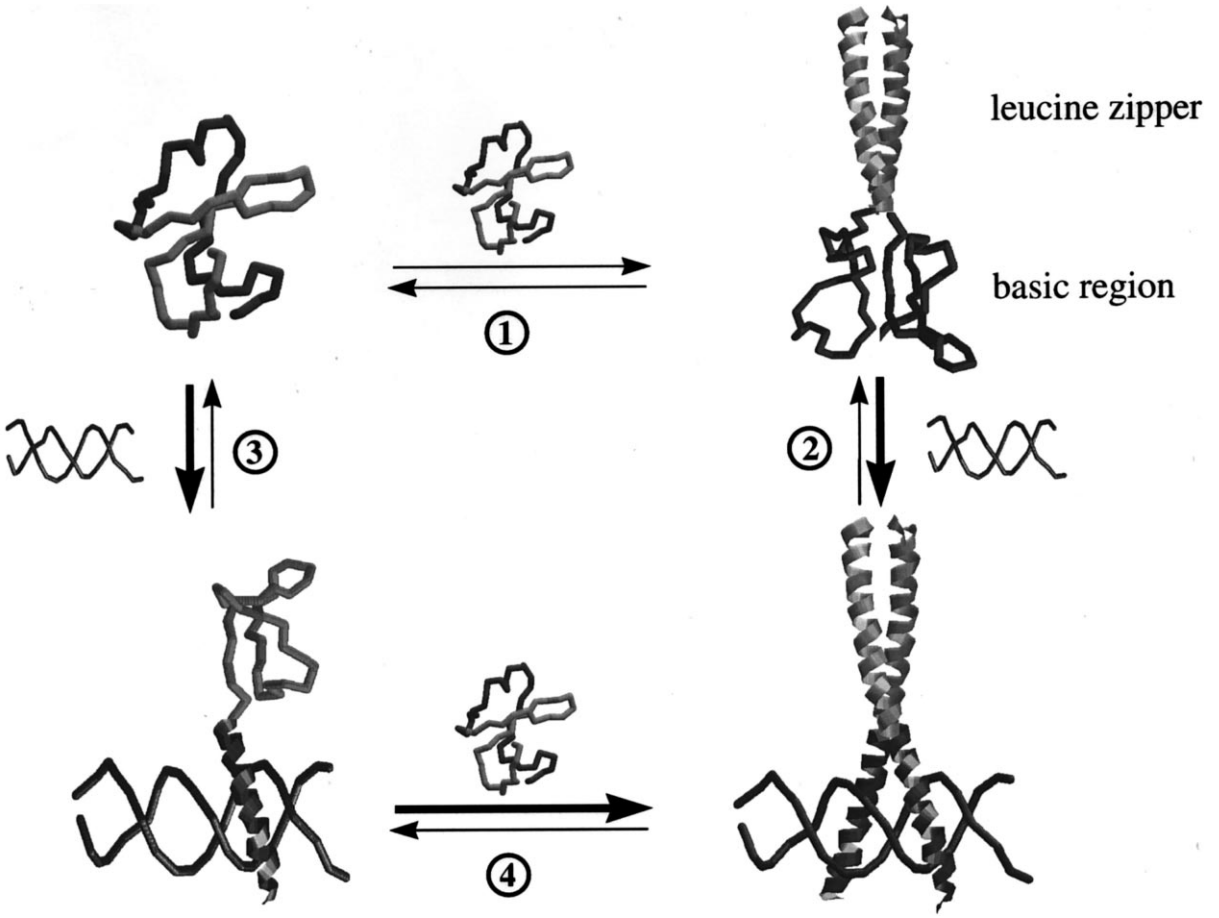

Fig. 1. Alternative pathways for DNA binding of a bZIP transcription factor. In the dimer pathway, formation of the leucine zipper (equilibrium 1) precedes DNA binding (equilibrium 2). In the monomer pathway, the two monomers bind sequentially (equilibria 3 and 4) and the leucine zipper is formed when the second monomer binds (equilibrium 4). The present work shows that DNA binding of both the monomer and the dimer is very fast (bold arrows) and that, therefore, equilibrium 1 does not affect the rate of DNA recognition.

stranded oligonucleotide, and P_0 is half the concentration of peptide chains (i.e. maximum dimer concentration). K_{app} is a true dissociation constant for GCN4br, $(CGCN4)_2$ and $(GCN4br)_2$ because these peptides exist in only two forms, free and DNA-bound. For C62GCN4 and AAGCN4, which are in a concentration-dependent monomer-dimer equilibrium, K_{app} disregards complexes with one monomer bound to one half-site (equilibrium 3 of Fig. 1). K_{app} equals $K_T^{1/2}$ where K_T=[M][M][DNA]/[M_2:DNA]; [M] and [DNA] are the molar concentrations of monomeric peptide and double-stranded nucleotide, respectively; [M_2:DNA] is the molar concentration of dimer-DNA complex.

2.4. Stopped-flow kinetic measurements

Stopped-flow experiments were made on an SF-61 instrument (High Tech Scientific, Salisbury, UK). One syringe was filled with 8 μM peptide and the other with 0.5 μM CRE_{19}^F. Equal volumes were mixed and the change of fluorescence emission above 530 nm was measured (excitation 495 nm). At least five syringe firings were averaged for each kinetic trace. In the experiment of Fig. 4, one syringe contained 36.4 μM C62GCN4 in 7.95 M urea and the other 0.92 μM CRE_{19}^F in buffer. The mixing ratio was 1:20. Measurements were made at 25°C in 20 mM potassium phosphate, pH 7.0, ionic strength adjusted to 0.1 or 0.5 M with KCl. The slow phase of the reaction was fitted to $F = F_0 + (F_\infty - F_0) \times e^{-k_{app} \times t}$; k_{app} = apparent rate constant; F, F_0 and F_∞ are the fluorescence at time t, $t = 0$, and $t = 100$ s, respectively.

3. Results

The yeast transcriptional activator GCN4 is a bZIP dimer that recognizes the CRE and AP-1 target sites as a pair of continuous α-helices [9,16]. The complex has been likened to a pair of scissors that grips the double-stranded DNA as shown schematically in Fig. 1 [17]. We used this well characterized system to distinguish between the monomer and dimer pathways of DNA binding. In the dimer pathway, but not in the monomer pathway, the observed overall rate of DNA binding depends on dimer concentration and, hence, on dimer stability, which can be manipulated by mutation. To test this prediction we directly monitored DNA binding using the fluorescence-tagged oligonucleotides CRE_{19}^F and AP-1_{20}^F (Table 1). The oligonucleotides were reacted with C62GCN4, the C-terminal fragment 220–281 of GCN4 encompassing the basic region and leucine zipper domains (Table 1). The fluorescence emission of CRE_{19}^F increased strongly on titration with C62GCN4 (Fig. 2A). A smaller fluorescence increase was observed with AP-1_{20}^F (not shown) and, therefore, CRE_{19}^F was used in the subsequent studies. $\Delta G^{\circ\prime}$ obtained by isothermal titration calorimetry was the same for the binding of C62GCN4 to CRE_{19}^F and AP-1_{20}^F, respectively ([10] and data not shown).

Under equilibrium binding conditions, the apparent dissociation constant of the protein-DNA complex was not significantly changed by mutations that either stabilized or destabilized the leucine zipper: $(CGCN4)_2$ and AAGCN4, respectively (Fig. 2B, see Table 1 for sequences of GCN4

Table 1
Sequence* of peptides derived from GCN4 and of the fluorescence-labeled double-stranded oligonucleotides CRE_{19}^{F} and $AP\text{-}1_{20}^{F}$

Abbreviation	basic region | leucine zipper	K_{app} (mol/L)
C62GCN4	MIVPESSDPAALKRARNTEAARRSRARKLQRMKQ|LEDKVEELLSKNYHLENEVARLKKLVGER	$(2.2\pm2.9)\times10^{-9}$ [b]
$(CGCN4)_2$	(MIVPESSDPAALKRARNTEAARRSRARKLQRMKQ|LEDKVEELLSKNYHLENEVARLKKLVGERGSGC)$_2$	$(1.5\pm1.0)\times10^{-9}$
AAGCN4	MIVPESSDPAALKRARNTEAARRSRARKLQRMKQ|LEDKVEELLSKNYHLENEAARAKKLVGER	$(3.9\pm7.3)\times10^{-9}$ [b,c]
GCN4br	Ac-YPESSDPAALKRARNTEAARRSRARKLQRMKQ|GGC(StBu)G-NH_2 [a]	$(1.7\pm0.1)\times10^{-6}$
$(GCN4br)_2$	(Ac-YPESSDPAALKRARNTEAARRSRARKLQRMKQ|GGCG-NH_2)$_2$ [a]	$(5.8\pm2.2)\times10^{-8}$
control peptide	EYQALKKKVAQLKAKNQALKKKVAQLKHKG-NH_2	no binding
CRE_{19}^{F} [d]	5′-TGG[NBD]AG**ATGACGTCAT**CT···CC-3′	
	3′-CC···TC**TACTGCAGTA**GA[NBD]GGT-5′	
$AP\text{-}1_{20}^{F}$ [d]	5′-TTC[NBD]CT**ATGACTCAT**CC···AGTT-3′	
	3′-AG···GA**TACTGAGTA**GG[NBD]TCAAA-5′	

Apparent dissociation constant K_{app} of complexes of GCN4 derivatives with CRE_{19}^{F} obtained by fluorescence titration.
*Sequence differences to wild-type GCN4 are underlined. The C-terminal extensions were introduced to produce stable disulfide-linked dimers. The alanine substitutions in AAGCN4 destabilize the leucine zipper by about 8 kJ/mol.
[a]Ac, N^{α}-acetyl; StBu, thio-*tert*.-butyl protection group.
[b]The large error of K_{app} shows that the experimental data are not adequately described by the simple binding model used for data analysis (<HRF>2</HRF>) because it does not account for the complex between monomeric peptide and DNA (equilibrium 3 in <FIGR>1</FIGR>).
[c]Dissociation constant for binding to $AP\text{-}1_{20}^{F}$.
[d]The CRE and AP-1 sites are in bold. NBD indicates a phosphorothioate-linked (7-nitrobenzo-2-oxa-1,3-diazol-4-yl) group.

derivatives). More important, the leucine zipper was dispensable since the basic region peptides GCN4br and $(GCN4br)_2$ alone bound to the CRE site (Fig. 2B). Titration with a basic control peptide produced no fluorescence change (Fig. 2B). Hence, the fluorescence signal was not due to non-specific electrostatic attraction between positively charged peptide and negatively charged polyphosphate backbone. K_{app} obtained by fluorescence titration was 2.2×10^{-9} M for C62GCN4 (Table 1) and was in good agreement with $K_T = 4.1\times10^{-18}$ M^2 ($K_T = K_{app}^2$, see Section 2) obtained from gel shift assays with fragment 228–281 of GCN4 and a 24 bp oligonucleotide containing the CRE site [6]. The specificity of DNA binding was further corroborated by CD spectroscopy. The basic region of GCN4 is unstructured and specific DNA binding induces a helical structure producing CD minima at 208 and 222 nm [18,19]. This spectral signature was observed when the fluorescent oligonucleotides were added to each of the peptides except the control peptide (data not shown).

Fig. 3 shows the fluorescence change observed when CRE_{19}^{F} reacted with an excess of C62GCN4 or its derivatives. The same biphasic time course was observed despite the fact that, at the start of the reaction, C62GCN4, AAGCN4 and GCN4br were 35%, >80% and 100% monomeric, respectively, whereas $(CGCN4)_2$ and $(GCN4br)_2$ were 100% dimeric. Clearly, the reaction rate did not depend on the presence of a dimeric leucine zipper. The first and major phase of the reaction was very fast, accounted for 70–80% of the total change of fluorescence, and was concentration-dependent (Fig. 3, inset) in accordance with an association reaction between peptide and DNA. Under equilibrium conditions, the complex was 10–20 times less stable in 0.5 M KCl or when the viscosity was increased by 30% (w/v) sucrose (not shown). We repeated the stopped-flow experiments under these conditions. However, the association was still too fast to be analyzed in the time frame of our stopped-flow instrument even though the increase in ionic strength and viscosity may have slowed the reaction. We can estimate a lower rate limit for the association step. The observed rate is $k_{app}\times[CRE_{19}^{F}]$, where $k_{app} = k\times[GCN4]$ and k is the association rate constant. Since the reaction was virtually over within the mixing time, $t_{1/2}$ was shorter than 2 ms and k_{app} ($=\ln2/t_{1/2}$) higher than 350 s^{-1}. With [GCN4] = 4 μM (experiments of Fig. 3) one obtains $k > 10^8$ M^{-1} s^{-1}, which is diffusion-limited [20].

The slow second phase showed the same single exponential time dependence for monomeric and dimeric peptides and, thus, was not linked to dimerization. The half-time was 13.2 ± 0.4 s for 8 measurements in the range 0.5–16 μM C62GCN4. The phase may pertain to a slow conformational rearrangement of the initial protein-DNA complex and this is supported by the small amplitude. The functional significance of such a rearrangement is unclear and could be an artifact caused by the fluorescence tag.

A further decisive experiment demonstrated that DNA binding preceded dimerization of the leucine zipper: one volume of completely unfolded monomeric C62GCN4 in 8 M urea was mixed with 20 volumes of CRE_{19}^{F} in 'benign' buffer to initiate refolding and DNA binding (Fig. 4). Once again the same diffusion-limited rate of DNA binding was observed. In comparison, the formation of the leucine zipper dimer from

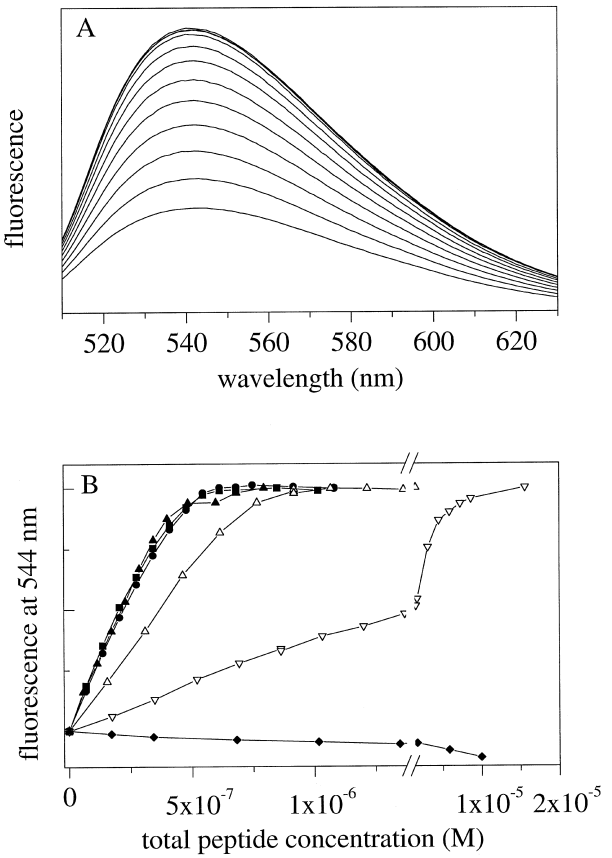

Fig. 2. DNA binding demonstrated by fluorescence titration. A: Fluorescence emission spectrum of 0.25 μM CRE_{19}^{F} in the absence (lowest spectrum) and in the presence of increasing amounts of C62GCN4. B: Fluorescence titration of 0.25 μM CRE_{19}^{F} with C62GCN4 (■), AAGCN4 (▲), $(CGCN4)_2$ (●), $(GCN4br)_2$ (△), GCN4br (▽), and control peptide (♦). The fluorescence emission at 544 nm was normalized.

two unfolded polypeptide chains is much slower and has a half-time of 0.4–4 s under the conditions of the experiment of Fig. 4 ($t_{1/2}$ calculated for a dimerization rate constant in the range 10^5–10^6 M^{-1} s^{-1} [13,21,22]). Hence, free (i.e. not DNA-bound) leucine zipper dimer was not yet formed when the rapid phase of the DNA binding reaction was already completed.

4. Discussion

A compulsory dimer pathway of DNA recognition has recently been challenged [5,7,8] and can now be definitely ruled out for GCN4 even though this transcription factor forms a relatively stable leucine zipper dimer with a K_d of approximately 10^{-6} M [23]. What are the implications for transcriptional regulation? Both monomeric and dimeric GCN4 can bind to DNA at a very rapid rate and, therefore, the monomer-dimer equilibrium of the free bZIP factor (equilibrium 1 in Fig. 1) does not affect the overall rate of DNA recognition. The function of the leucine zipper undoubtedly remains to bring two basic regions in register to bind to two DNA half-sites [3,24–26]. However, efficient DNA recognition can occur under conditions where the transcription factor is predominantly monomeric. Indeed, bZIP factors have not been selected for maximum dimer stability [3] and strands can ex-

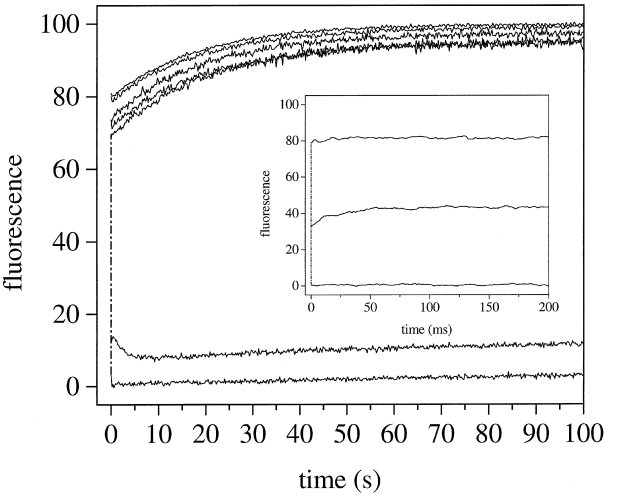

Fig. 3. Time course of DNA binding. CRE_{19}^{F} was reacted with (from bottom to top) buffer, control peptide, AAGCN4, GCN4br, $(GCN4br)_2$, C62GCN4, and $(CGCN4)_2$. Concentrations after mixing were 0.25 μM CRE_{19}^{F} and 4 μM peptide. Fluorescence emission at time zero was adjusted to the same value for each trace. The dash-dot line indicates the change of fluorescence during the dead time of mixing, which was approx. 2 ms. Inset: Initial 200 ms of the reaction of 0.25 μM CRE_{19}^{F} with (from bottom to top) buffer, 0.25 μM and 4 μM C62GCN4.

change rapidly between dimers in solution [13,27]. Once bound to DNA, the dimer is strongly stabilized by the binding of the two basic regions to the two half-sites [7,27].

The monomer and dimer pathways are thermodynamically equivalent and preference for the monomer pathway is kinetic. But why is DNA binding so fast? In this study a short piece of DNA was employed and the high binding rate can be accounted for by electrostatic attraction between properly spaced basic residues and the polyphosphate backbone. It is known that complementary charges can increase the rate of successful association to the limit of a diffusion-controlled

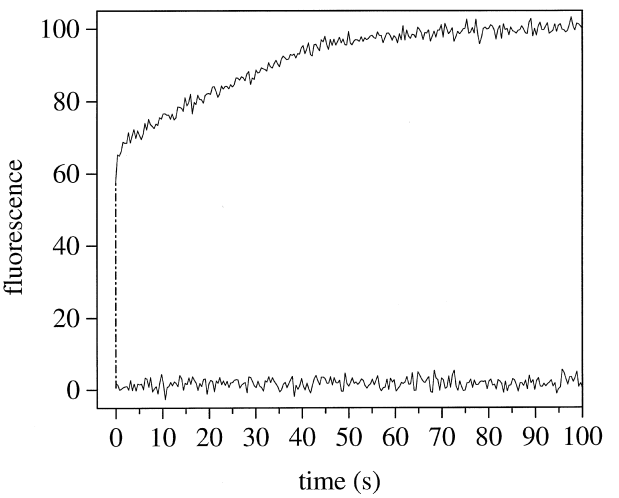

Fig. 4. Rate of DNA binding and refolding of urea-denatured, monomeric C62GCN4. Denatured peptide was diluted 21× with buffer containing CRE_{19}^{F} and the fluorescence change caused by DNA binding was followed. Conditions after mixing: 1.73 μM peptide, 0.88 μM DNA, 0.38 M urea in 20 mM phosphate buffer, pH 7.0, 0.1 M ionic strength. The bottom trace is the control without C62GCN4.

statistical encounter [28–30]. In the case of a large stretch of DNA, sliding (one-dimensional diffusion) along the DNA and 'hopping' between nearby DNA strands speeds up target finding [20,31,32]. When the bZIP factor slides along the DNA, non-specific binding should be weak. Because binding strength correlates with the number of possible interactions between peptide and DNA [20], the monomeric basic region may slide along the DNA more easily than the dimer. Less steric hindrance may also contribute to a faster diffusion rate of the monomer. (If both monomer and dimer diffuse along the DNA at the same rate, the dimer finds the target sequence faster, for statistical reasons.) Once at the target site, dimer formation prompts strong binding. We therefore suggest that an advantage of the monomer pathway could be easy and fast diffusion of the monomeric transcription factor along the DNA combined with strong 'scissors grip' binding to the dyad symmetrical target sequence.

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