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Distinct Roles for Interfacial Hydration in Site-Specific DNA Recognition by ETS-Family Transcription Factors

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Abstract

The ETS family of transcription factors is a functionally heterogeneous group of gene regulators that share a structurally conserved, eponymous DNA-binding domain. Unlike other ETS homologues, such as Ets-1, DNA recognition by PU.1 is highly sensitive to its osmotic environment due to excess interfacial hydration in the complex. To investigate interfacial hydration in the two homologues, we mutated a conserved tyrosine residue, which is exclusively engaged in coordinating a well-defined water contact between the protein and DNA among ETS proteins, to phenylalanine. The loss of this water-mediated contact blunted the osmotic sensitivity of PU.1/DNA binding, but did not alter binding under normo-osmotic conditions, suggesting that PU.1 has evolved to maximize osmotic sensitivity. The homologous mutation in Ets-1, which was minimally sensitive to osmotic stress due to a sparsely hydrated interface, reduced DNA-binding affinity at normal osmolality but the complex became stabilized by osmotic stress. Molecular dynamics simulations of wildtype and mutant PU.1 and Ets-1 in their free and DNA-bound states, which recapitulated experimental features of the proteins, showed that abrogation of this tyrosine-mediated water contact perturbed the Ets-1/DNA complex not through disruption of interfacial hydration, but by inhibiting local dynamics induced specifically in the bound state. Thus, a configurationally identical water-mediated contact plays mechanistically distinct roles in mediating DNA recognition by structurally homologous ETS transcription factors.

Graphical Abstract

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INTRODUCTION

The ETS family of transcription factors binds site-specific DNA via eponymous, structurally conserved DNA-binding domains that share low sequence homology. To date, a plethora of cocrystals of site-specific binary ETS/DNA complexes, as well as ternary structures in combination with other protein binding partners, show a highly conserved binding mode in which a recognition helix is inserted into the major groove of target DNA harboring the core sequence 5'-GGAA/T-3', with additional interactions along the DNA backbone at flanking minor groove positions. Despite the apparent homogeneity at the macromolecular level, heterogeneous levels and patterns of hydration pervade the protein/DNA interface. For instance, in the cocrystal of the PU.1 ETS domain with site-specific DNA, the contact interface is densely hydrated with several interfacial residues engaging in exclusively water-mediated contacts with the target DNA.¹ In contrast, the cocrystal structure for Ets-1 shows a sparsely hydrated interface where most of the corresponding residues make direct contact with the DNA.² The differences in crystallographic hydration between the two ETS domains, whose backbone trajectories are superimposable in the DNA-bound complex, have been reproduced in solution studies that perturbed ETS/DNA binding by osmotic pressure.^{3,4} These hydration differences are profoundly correlated to their binding kinetics, conformational dynamics, and site discrimination.⁵ In turn, target discrimination by ETS homologues, all of which share overlapping DNA sequence preferences,⁶ continues to be a major area of interest in understanding how biological specificity is achieved *in vivo*.^{7,8}

Since the topology of the ETS/DNA contact interface is highly conserved among ETS homologues, differences in conformational dynamics are presumably important in determining the hydration of ETS/DNA complexes. While such differences have been observed globally in ensemble experiments by dynamic light scattering, structurally resolved data do not yet exist. To address the correlation between interfacial hydration and conformational dynamics of ETS/DNA complexes, we targeted conserved ETS residues involved only in water-mediated contacts and not any other direct interactions with either itself or the DNA. In PU.1, the side chain -OH of Tyr²⁵² coordinates a bridging water with a universally conserved arginine (Arg²³⁵) and a backbone phosphate of the target DNA. This is a universally conserved configuration of interfacial hydration in all ETS homologues, including Ets-1, that harbor this equivalent Tyr. We mutated this Tyr in both PU.1 and Ets-1 and compared their DNA site recognition under osmotic pressure as well as *in silico* by molecular dynamics simulations. The two orthogonal approaches provide a deeper insight

into the mechanism of DNA recognition with respect to the absence of extensive interfacial hydration of Ets-1 (and sequence-similar homologues such as Ets-2, Fli-1, and GABP α) versus PU.1 (and its phylogenetic relatives such as ETV6).

MATERIALS AND METHODS

Nucleic Acids

Synthetic DNA oligos were purchased from Integrated DNA Technologies (Coralville, IA) and annealed to form duplex binding sites. DNA sites were the optimal binding sequences for PU.1 (5'-AGCGGAAGTG-3')⁹ and Ets-1 (5'-GCCGGAAGTG-3', termed SC1);¹⁰ the ETS-specific core consensus is in bold. Fluorescent DNA probes were constructed by annealing a TYE 563-labeled oligo with excess unlabeled complementary strand as described.¹¹ Concentrations of unmodified oligos were determined spectrophotometrically using nearest-neighbor extinction coefficients.¹²

Molecular Cloning

The cloning of the ETS domains of murine PU.1 (residues 167 to 272, designated PU.1 N167) and Ets-1 (residues 331 to 440, designated Ets-1 N331) have been described previously.^{3,4} The point mutants Y252F for PU.1 and Y421F for Ets-1 were constructed by standard PCR mutagenesis. Briefly, for each construct separate PCR reactions were performed using mutagenic primers to produce two DNA fragments with overlapping sequences that harbored the intended Tyr-to-Phe mutation. A second PCR amplification of the first-round products with external primers generated the final construct that was cloned into pET28b vector and propagated in DH5 α *Escherichia coli* cells under kanamycin selection (50 μ g/mL). Clones were verified by Sanger sequencing and transformed into BL21*(DE3) *E. coli* for expression.

Protein Expression and Purification

Wildtype or mutant ETS constructs were overexpressed in *Escherichia coli* as fusions with a thrombin-cleavable C-terminal 6 $\times$ His tag and purified as previously described.⁴ In brief, cleared lysate from sonicated cell pellets were first purified on Co-NTA by immobilized metal affinity chromatography, cleaved with thrombin, dialyzed against 10 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4) containing 0.5 M (for PU.1) or 0.15 M (for Ets-1) NaCl, and polished on Sepharose SP (GE). Buffers used with Ets-1 constructs, which harbored reduced cysteines, additionally contained 0.5 mM TCEP. Purified constructs were homogeneous as judged by Coomassie-stained SDS-PAGE. Protein concentrations were determined by UV absorption at 280 nm using the following extinction coefficients (in M⁻¹ cm⁻¹): 22 460 (wildtype PU.1 N167), 20 970 (PU.1 N167^{Y252F}), 32 430 (wildtype Ets-1 N331), and 30 940 (Ets-1 N331^{Y412F}).

Fluorescence Polarization Titrations

ETS protein binding to fluorescently labeled DNA sites was measured in solution using a Molecular Devices Paradigm plate reader. TYE-labeled DNA probe (0.5 nM) was incubated to equilibrium with purified wildtype (PU.1 N167 or Ets-1 N331) or mutant protein (PU.1 N167^{Y252F} or Ets-1 N331^{Y412F}) and graded concentrations of unlabeled high-affinity

site in 30 μL of total volume. The solution was 10 mM TrisHCl (pH 7.4) containing 150 mM total Na^+ , 5 mM DTT, 0.1 mg/mL acetylated bovine serum albumin (Promega), and betaine as indicated. Solution osmolality was measured using a freezing point depression osmometer (Osmomat 3000, GonoTec) calibrated with commercial NaCl standards. Immediately before fluorescence measurement, samples were transferred to black 384-well plates (Corning) and excited at 535/25 nm. Steady-state fluorescence parallel and perpendicular to the incident polarized light was acquired at 595/35 nm. Dark counts of a buffer-only control were subtracted from each emission count before conversion to the anisotropy using a grating factor that was independently determined with sulforhodamine B ($\langle r \rangle = 0.025$ at 25 $^{\circ}\text{C}$). Anisotropy data from multiple experiments were fitted simultaneously with a mechanistic binding model to directly estimate the dissociation constants $\pm$ SE of each protein complex with *unmodified* DNA.¹¹

Circular Dichroism Spectroscopy

Purified proteins were extensively dialyzed against 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4) containing 150 mM NaCl before scanning from 300 to 190 nm at 10 μM in a 1 mm-path length quartz cuvette at 25 $^{\circ}\text{C}$ using a Jasco J-810 instrument. Per-residue normalized spectra were decomposed using BeStSel,¹³ without scaling, to estimate their secondary structure contents. Melting experiments were carried out with 25 μM of each construct, detected at 222 nm while heated at 45 $^{\circ}\text{C}/\text{h}$ to 70 $^{\circ}\text{C}$, followed by cooling at the same rate. Melting and refolding data were simultaneously fitted to a unimolecular two-state transition with independent linear baselines.

Size Exclusion Chromatography

Purified proteins (3 nmol) were injected and eluted at 1.3 mL/min with 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4) containing 150 mM NaCl and 0.5 mM TCEP in a Superdex 75 10/300 GL column (GE) under the control of a Bio-Rad NGC instrument. The column was calibrated with bovine serum albumin (66 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa). Eluate was detected by UV absorption at 280 and 409 nm, the latter specific for the Soret band of myoglobin. The void volume of the column was 8 mL.

2D ^1H - ^{15}N HSQC NMR

Uniformly ^{15}N -labeled wildtype Ets-1 N331 and Ets-1 N331^{Y412F} were expressed in M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ and purified identically as unlabeled proteins. Samples (~0.3 mM) were extensively dialyzed against 11 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, 167 mM NaCl, and 0.1% NaN_3 and adjusted to 10% D_2O . ^1H - ^{15}N correlated measurements were made using a phase sensitive, double inept transfer with a garp decoupling sequence and solvent suppression (hsqc3gppl9). Spectra were acquired with $1\text{k} \times 144$ data points and zero-filled to $4\text{k} \times 4\text{k}$.

Molecular Dynamics Simulations

Free and site-specifically bound wildtype and mutant ETS domains of PU.1 and Ets-1 were simulated using GROMACS 2016.1 with the CHARMM36 force field. Starting coordinates for the wildtype ETS domains of PU.1 and Ets-1 were taken from available (co)-crystal

structures and trimmed to the same length after alignment of their protein sequences. The DNA was mutated to the experimental sequences without altering any existing backbone or sugar coordinate using 3DNA.¹⁴ The target Tyr residues were mutated to Phe using CHARMM-GUI.¹⁵ All crystal waters were retained as TIP3P waters. Each system was placed in a dodecahedral box of TIP3P water with a minimum of 1.0 nm from its periodic boundary and neutralized with 0.15 M NaCl. The systems were then minimized by steepest descent. The energy-minimized systems were further relaxed, in order, as *NVT* and *NPT* ensembles for 100 ps at 2 fs time steps at 298 K with restraints on the macromolecules. Afterward, restraints were removed from the equilibrated systems and run for 100 ns for unbound proteins and 200 ns for DNA-bound complexes at 2 fs time steps at 298 K. Bond constraints were introduced using the LINCS algorithm at order 4. Coordinates, velocities, and energies were saved at 10 ps intervals. Trajectories were then imaged and analyzed using GROMACS tools. In cases where the lengths of the bound DNA were different, RMS measurements were limited to corresponding protein in the structures. Hydrogen-bond enumerations were carried out using geometric cut-offs of 0.35 nm for distance and 150° for angle (between donor-H and donor-acceptor heavy atoms).

RESULTS

Despite a well-defined structural homology in terms of backbone trajectories, and overlapping DNA sequence tolerance, the DNA-binding domains of ETS transcription factors are divergent in amino acid sequence. Of the limited set of residues that are strictly or highly conserved, most are engaged in direct protein-to-DNA contacts, and alanine scans typically abolish DNA binding. The role of other conserved residues, such as an interfacial tyrosine near the C-terminus in the primary sequence of ETS domains [Figure 1A], has been less clear. This tyrosine is found in 26 of the 28 known murine (and human) ETS homologues, and all cocrystals of these homologues show a water-mediated contact coordinating the side chain –OH of the tyrosine, a universally conserved arginine in the recognition helix, and the backbone phosphate of the base upstream from the 5'-GGAA-3' core consensus. Moreover, this well-defined water-mediated “motif” is conserved whether the interface is otherwise extensively hydrated (such as in PU.1)¹ or not (such as in Ets-1)² [Figures 1B–G].

The strong conservation of this exclusively water-coordinated tyrosine among ETS homologues suggests that it plays an important role in mediating ETS/DNA binding. In the cocrystal structure of PU.1, this tyrosine anchors one end of an extensive interfacial water network, and its mutation to phenylalanine (loss of the side chain –OH) represents an attractive approach to probing this water network. We therefore mutated this Tyr to Phe in the ETS domain of PU.1 (PU.1 N167) and Ets-1 (Ets-1 N331) [Figure 2A], and compared their recognition of optimal site-specific DNA under normo-osmotic and osmotically stressed solution conditions relative to their wildtype counterparts.

In previous studies,^{3,4} we have extensively characterized the perturbative effects of compatible cosolutes on DNA binding by the ETS domains of both PU.1 and Ets-1. Using a palette of cosolutes spanning a broad range in physical chemistry, such as betaine, disaccharides (sucrose, maltose), and glycols (triethylene glycol), we observed that their

effect on site-specific binding affinity correlated only with their osmolality over an extended range of cosolute concentrations. The key conclusion from this colligative behavior is that the cosolutes did not exhibit cosolute-specific preferential interactions with the macromolecules and that their perturbations on binding could be interpreted in terms of hydration changes upon binding.^{16–19} For our studies here we used betaine, a physiologically compatible osmolyte, out of convenience given its relatively neutral effect on bulk viscosity and dielectric constant.

We carried out competitive fluorescence polarization titrations of ETS-bound, TYE 563-labeled DNA probe with an unlabeled competitor (harboring the optimal sequence for each respective protein) with or without graded concentrations of betaine [Figure 2B]. The data was analyzed with a mechanistic model¹¹ to estimate the absolute binding constant (not IC_{50} 's) for the unlabeled complex of interest. The in-solution affinities for wildtype PU.1 and Ets-1 show quantitative agreement with previous values obtained by electrophoretic mobility shift^{3,4} and surface plasmon resonance.^{20,21}

Mutation of a Conserved Interfacial Tyrosine Inducing Markedly Different Effects on DNA Recognition by the Structurally Homologous ETS Domains of PU.1 and Ets-1

Measurement of the apparent dissociation constants for the wildtype and mutant PU.1 N167 and Ets-1 N331 showed that both mutants retained high-affinity binding to their wildtypes' optimal DNA targets ($K_D < 10^{-8}$ M) under normo-osmotic conditions (150 mM Na^+ , 0.29 osmolal). However, osmotic stress to 2.5 osmolal showed distinct mutational effects on the affinity and osmotic sensitivity of DNA recognition by the ETS homologues. In the case of PU.1, both wildtype and mutant protein were negligibly different in their affinities for high-affinity site-specific DNA, but the mutant bound its target more strongly than wildtype with increasing osmotic pressure. Over the measured range of osmotic pressure, the osmotic dependence (slope) of the binding affinity was ~25% lower in magnitude for the PU.1 N167^{Y252F} mutant than wildtype PU.1 N167 [Figure 2C], consistent with a deficit of interfacial hydration in the mutant/DNA complex relative to wildtype. Quantitatively, the effect was more pronounced than if the nine water-mediated contacts seen in the cocrystal structure contributed equally to the binding free energy.

In stark contrast to PU.1, the ETS domain of Ets-1 exhibited altogether different behavior when probed by osmotic pressure. At normo-osmotic pressure, binding of Ets-1 N331^{Y412F} to high-affinity site-specific DNA was ~60-fold weaker relative to wildtype, although still >10-fold stronger than nonspecific binding.²² As observed previously,⁴ unlike PU.1, the site-specific complex of wildtype Ets-1 N331 was slightly stabilized by osmotic pressure. In comparison, the osmotic dependence of the affinity (slope) for Ets-1 N331^{Y412F} was ~35% more *positive* than wildtype, inferring relative dehydration of the mutant Ets-1/DNA complex relative to wildtype.

As binding affinity reflects the free energy change of the components from the unbound to bound state, differential perturbations of the mutations on the unbound proteins may also contribute to heterogeneity in DNA binding. Therefore, we probed wildtype and mutant PU.1 N167 and Ets-1 N331 in the absence of DNA by circular dichroism spectroscopy to determine the secondary structure contents of each construct at 25 °C under normo-osmotic

conditions [Figure 3A], and measured their stability by thermal melting [Figure 3B]. Decomposition of their CD spectra showed an apparent loss of 15 to 20% β -sheet in both mutants relative to wildtype [Table 1], which was the secondary structure at the mutated positions. Despite this difference, wildtype and mutant PU.1 N167 unfolded reversibly with experimentally indistinguishable melting temperatures and enthalpy changes. In contrast, Ets-1 N331^{Y412F} unfolded at a higher melting temperature than wildtype by ~8 °C. Although thermal denaturation was not reversible for either Ets-1 construct, as previously noted,²³ the alignment of the signal in the cooling run with the post-transition baseline of the heating run for both Ets-1 constructs indicated that irreversible change occurred after denaturation. Since Ets-1 harbored free cysteines (but not PU.1), we confirmed by size-exclusion chromatography that the mutations did not cause protein oligomerization under the solution conditions of the CD experiments [Figure 3C]. All wildtype and mutant constructs eluted quantitatively as a single species according to their nominal molecular weight (~13 kDa, at a *higher* elution volume than myoglobin at 17 kDa). To further probe the structural differences between wildtype and mutant Ets-1 N331, we acquired and compared their ¹H-¹⁵N HSQC NMR spectra [Figure 3D], which showed complete or partial overlap of >90% of resolved resonances. Using the solution NMR structure of Ets-1 N301,²³ about half of the peaks showing the largest shifts (partial or complete) are ± 20 residues from the mutated position (Y412). Thus, wildtype and mutant Ets-1 N331 showed substantially similar folding but exhibited some local and distal conformational changes in the unbound state.

In summary, one-atom mutation of a highly conserved interfacial Tyr to Phe produced distinct effects on the DNA-binding and conformational properties of the ETS domains of PU.1 and Ets-1. This heterogeneity was unexpected given the strong structural homology of the two ETS domains in general,²⁴ an identical configuration in coordinating a water-mediated contact in their cocrystal structures (cf. Figures 1F and 1G), and the similar solvent-accessible topology of the mutated Tyr.

Molecular Dynamics Simulations of Wildtype and Mutant ETS Domains of PU.1 and Ets-1 Recapitulated Experimental Data

While the free²³ and DNA-bound^{2,25} structures of the Ets-1 ETS domain are known, only the DNA-bound PU.1 structure¹ currently exists. To understand the experimental differences among wildtype and mutant PU.1 and Ets-1 in greater detail, we carried out all-atom molecular dynamic simulations of the four constructs in their free and site-specifically DNA-bound states. Unbound Ets-1 was taken from a crystallographic structure (PDB: 1GVJ) and unbound PU.1 from the cocrystal structure. The protein/DNA complexes were constructed by modifying the cocrystal structures to match the experimental sequences. All crystallographic water molecules were retained and treated as TIP3P water. Following equilibration in explicit solvent containing 0.15 M NaCl, and confirming that the crystal waters at the interface in the complexes had not exchanged with bulk solvent, each system was simulated to convergence as indicated by RMSD from the initial coordinates [Figure 4, parts A and B, for unbound proteins, 100 ns]. Enumeration of H-bonds with solvent showed identical hydration between wildtype and mutant unbound PU.1 (within 1%) in agreement with their similar melting temperatures and unfolding enthalpies [Figure 4C]. However,

wildtype Ets-1 showed >4% (average) more solvent H-bonds than the mutant, indicating less solvent exposure in the mutant [Figure 4, parts C and D] and consistent with its relative thermal stability over wildtype Ets-1 (cf. Figure 3B). In addition, the shallower slope of the mean-square displacement with respect to time (which is proportional to the self-diffusion constant) for wildtype Ets-1 ($0.2 \times 10^{-9} \text{ m}^2/\text{s}$) relative to wildtype PU.1 ($0.6 \times 10^{-9} \text{ m}^2/\text{s}$) [Figure 4, parts E and F] recapitulated published dynamic light scattering measurements showing a 14% larger hydrodynamic radius for Ets-1 N331 over PU.1 N167.⁵

In the case of the DNA-bound complexes (200 ns simulations), both wildtype and mutant complexes formed by PU.1 with its 14-bp DNA remained well-behaved through the simulation. However, the wildtype complexes formed by Ets-1 with a 14-bp site-specific target exhibited significant fraying at the DNA ends and interactions between the protein and the frayed ends. To account for this, both wildtype and mutant Ets-1 complexes were resimulated with longer DNA duplexes matching the full 23-bp experimental construct. Bases proximal to the protein remained duplex throughout the simulations with the longer DNA. Although the wildtype and mutant complexes for both ETS homologues did not differ significantly in terms of RMSD from the initial coordinates [Figure 5A], there was a substantial bias in dynamic fluctuations in favor of wildtype over mutant Ets-1 complexes. Comparison of the per-residue RMS fluctuations (RMSF; standard deviations from average positions) of the DNA-bound states indicated significant local dynamics in wildtype Ets-1 over the Y412F mutant [Figure 5B]. The average excess RMSF (wildtype over mutant) was +0.025 nm per residue for Ets-1 and lower than +0.01 nm for PU.1. To evaluate the validity of the simulated dynamics, we examined the available solution NMR structures of free²³ and DNA-bound Ets-1.²⁵ The experimental structures show excess dynamics in DNA-bound Ets-1 that are localized in elements contacting the minor groove DNA backbone flanking the 5'-GGAA-3' core consensus, as well as two loops distal from DNA-the binding interface [Figure 5C]. Although the agreement was not quantitative, the simulations correctly reproduced the presence and positions of the induced experimental dynamics in DNA-bound Ets-1. These dynamic hotspots were discontinuous in primary sequence, clustering primarily in the N-terminal half and distally from C-terminal position of Tyr⁴¹², indicating that the dynamics were transmitted through the protein's tertiary structure and probably through the DNA "substrate." In summary, the simulations' agreement with the experimental data for wildtype PU.1 and Ets-1 reported here and by others provided evidence that the simulations semiquantitatively described the solution behavior of the proteins in their free and bound states.

Hydration Dynamics in the Wildtype and Mutant Phe-to-Tyr PU.1 and Ets-1 Complexes

To probe interfacial hydration in the simulated structures, H-bonds formed by solvent to protein and nucleotide bases participating in water-mediated contacts in the contact interface were enumerated for each system based on combined distance and angle criteria from triplicate independent production runs [Figure 6]. The hydration of the tripartite water-mediated contact in the crystal structures was evaluated using the functional groups involved: the side chain -OH of Tyr²⁵²/Tyr⁴¹² (PU.1/Ets-1), the guanidinium N of Arg²³⁵/Arg³⁹⁴ (PU.1/Ets-1), and the backbone phosphate at the -1 position (relative to the 5'-GGAA-3' core consensus). To assess interfacial hydration more generally, the number of

solvent H-bond contacts made by guanidinium N of Arg²³⁵/Arg³⁹⁴ and the major groove-facing heteroatoms of the nucleobases in the 5'-GGAA-3' core consensus (i.e., O⁶ and N⁷ for G; N⁷ and N⁶ for A; O⁴ in T, and N⁴ in C) were also calculated. While these groups represent only a subset of all the interfacial residues, they are fully conserved between PU.1 and Ets-1, providing a chemically fair and equivalent basis for comparison between the two systems.

Starting from the interfacial hydration observed in the PU.1/DNA cocrystal structure, the number of solvent H-bond contacts made by the wildtype complex remained steady throughout the simulation. In particular, the 4-OH group of Tyr²⁵² was associated with a time-averaged value of ~0.9 solvent H-bond, consistent with its role in coordinating the tripartite water-mediated contact with Arg²³⁵ and a DNA backbone phosphate as observed crystallographically. Without the tyrosyl 4-OH group, hydration of Arg²³⁵, the phosphate at the -1 position, and the 5'-GGAA-3' interface all became partially depleted in the mutant complex. Nonetheless, the deficit of solvent H-bonds for interfacial residues in the mutant PU.1 complex did not significantly exceed the loss (~1 contact) already accounted for by Arg²³⁵, which was present in both sets of residues. Depletion of interfacial hydration in the PU.1/DNA complex due to the Y252F mutation therefore appeared to be limited primarily to the loss of water associated with the tyrosyl 4-OH group.

In the case of wildtype Ets-1, the time-averaged hydration at Tyr⁴¹² was significantly lower (<0.3 H-bonding contact) relative to PU.1 despite an identical configuration of water-coordinating residues, suggesting that the tripartite water-mediated contact was less persistent in Ets-1 compared to PU.1. In agreement with the cocrystal structures, hydration at the 5'-GGAA-3' interface in the wildtype Ets-1 complex was also lower, at down to half the level observed in the wildtype PU.1 complex (cf. Figure 1, parts D and E). However, abolishing the tyrosyl 4-OH contact in the Y412F mutant did not significantly perturb the hydration near the residue or in the Ets-1/DNA interface, suggesting that, like the mutant PU.1/DNA complex, any dehydration was also limited to the mutated residue.

Structural Interpretation of Interfacial Phe-to-Tyr Mutations on Affinity and Osmotic Sensitivity of DNA Binding

Osmotic stress experiments showed that loss of a conserved, interfacial H-bond donor in PU.1 and Ets-1 generated altogether different responses to osmotic pressure in their DNA complexes. In PU.1, whose wildtype high-affinity DNA complex was profoundly destabilized by osmotic stress, the Y252F mutant was less sensitive. In Ets-1, the wildtype complex was essentially insensitive to osmotic stress, but the Y412F mutant became more sensitive in the direction of increased water release. However, the experiments also showed that DNA recognition by PU.1 under normo-osmotic conditions were not affected by the partial loss of interfacial hydration in the mutant, whereas affinity for Ets-1 was reduced. The simulations provided a structural correlation in terms of induced localized dynamics in the wildtype Ets-1/DNA complex, which agreed semiquantitatively with experimental NMR data of free and DNA-bound Ets-1 (cf. Figure 5C). If induced dynamics in the bound state are specific to Ets-1, as consistent with thermodynamic observations that site-specific Ets-1/DNA binding is more entropically driven than for PU.1,⁴ the dampened fluctuations

exhibited by the Y412F mutant would reduce the favorable entropic contribution to its binding affinity. Excess dynamics in the DNA-bound state would also contribute to the skewing of osmotic sensitivity toward water release for the Y412F mutant, because hydration of dynamic elements with increased solvent exposure in the wildtype complex would be absent in the mutant complex. Thus, the analogous Tyr-to-Phe mutation in PU.1 and Ets-1 reveals different selection pressures at work (osmotically sensitivity for PU.1 vs dynamics induction for Ets-1) in the divergent evolution of ETS homologues, while maintaining a highly conserved fold characteristic of this family of transcription factors.

DISCUSSION

Mutation of a single conserved tyrosine that comprises an exclusively water-mediated interfacial contact with site-specific DNA, to phenylalanine, causes markedly heterogeneous effects on the affinities of the ETS domains of PU.1 and Ets-1. In addition to dissimilar effects on DNA recognition under normo-osmotic conditions, osmotic stress reveals disparate disposition of hydration water between the two structural homologues. In this study, molecular dynamics simulations provided a high-resolution interpretation of the experimental data. To this end, the constructs and conditions of the simulations were configured to match experimental (normo-osmotic) conditions as closely as possible using extant structural data as templates. The extensive agreement of the simulations with experimental data reported here and by others from CD spectroscopy (cf. Figure 3), dynamic light scattering,⁵ titration calorimetry,⁴ and solution NMR (cf. Figure 5C) provided confidence that the force field (CHARMM36) and the results represent an accurate semiquantitative description of ETS/DNA interactions.

PU.1 Tuned to Optimize Sensitivity to the Osmotic Environment in DNA Recognition

One of the most intriguing features of the PU.1 N167^{Y252F} mutant is its recovery from partial loss of interfacial hydration to bind site-specific DNA equally as tightly as wildtype PU.1 under normo-osmotic conditions. This unexpected observation suggests that alternative, less hydrated binding modes are accessible to PU.1. However, to what extent are interfacial hydration configurations adaptive? The very few ETS homologues that harbor a Phe at the corresponding Y252 position in PU.1 offer an insight. In the cocrystal structure of ETV6,²⁶ which binds target DNA sites equally as well as any other ETS domain (10^{-10} to 10^{-9} M under normo-osmotic conditions), Phe³⁹⁵ is the wildtype residue at the corresponding Tyr²⁵² and Tyr⁴¹² position in PU.1 and Ets-1 (cf. Figure 1A). Similar to PU.1, the ETV6/DNA interface is well hydrated, albeit in a different configurations by one fewer than the hydration network in the wildtype PU.1/DNA interface. It is also significant that ETV6 is the closest phylogenetic relative of PU.1 (but less distal from Ets-1),²⁴ suggesting that biological mechanisms may exist that discriminate specific levels of interfacial hydration in cognate ETS/DNA complexes.

Functionally, PU.1 N167^{Y252F} binds as well as wildtype to site-specific DNA in the absence of osmotic stress. On the basis of affinity alone, it would seem that the wildtype protein is at best functionally neutral with respect to the Y252F mutant under normo-osmotic conditions. What may be a selective advantage of wildtype PU.1? The evolutionary

maintenance of the wildtype as a more osmotically sensitive phenotype, in light of the hydration properties of ETV6, suggests that responsiveness to osmotic pressure is a functional advantage for PU.1. Biologically, PU.1 is a lineage-restricted transcription factor and critical regulator of hematopoiesis, the multistep process by which stem and progenitor cells in bone marrow self-renew and differentiate into terminal lineages of blood cells.^{27–31} As physiological osmotic stress has been established in lymphoid tissues *in vivo*³² due to the very high metabolic rate within rapidly dividing cells,³³ an emerging hypothesis is that strong osmotic responsiveness by PU.1 may be a favorable regulatory phenotype. We have previously discovered through a bioinformatics analysis that PU.1 targets are over-represented among known osmotically sensitive genes.⁴ The present mutagenesis data indicating that DNA binding by PU.1 is biophysically optimized for osmotic sensitivity lends further support to this hypothesis.

Role of Interfacial Hydration in DNA Recognition by Ets-1

In stark contrast with PU.1, the loss of the analogous Tyr⁴¹² water-mediated contact in Ets-1 reduces its DNA binding affinity (though >10-fold above nonspecific levels)²² under normo-osmotic conditions. Thermal denaturation of wildtype and mutant proteins in the absence of DNA showed that the Tyr-to-Phe mutation was more perturbative to the conformational stability of unbound Ets-1 than PU.1 (cf. Figure 3B). Molecular dynamics simulations suggested that the Y412F mutation in Ets-1 dampened dynamic fluctuations in the free protein, as judged by differences in their relative hydration and diffusive properties (cf. Figure 4, parts D and F), and attenuated induced fluctuations in the DNA-bound state that appeared to be crucial for Ets-1 binding. Thus, the interfacial tyrosine in Ets-1 (Y412), even though it shows the same hydration pattern as PU.1 (Y252), is not directly linked to hydration, but rather part of the structural dynamics that are specific to Ets-1. This interpretation is consistent with experimental dynamics from solution NMR of free and unbound Ets-1, titration calorimetry (Ets-1/DNA binding being more entropically driven than PU.1),⁴ and the skewing of osmotic sensitivity toward water release by the Y412F mutant (abrogation of hydration of dynamic elements in wildtype Ets-1). Strong dynamics in the N-terminal helices have also been reported in a previous molecular dynamics study of DNA-bound wildtype Ets-1,³⁴ but that study did not include a paired simulation of the unbound protein or any mutant for comparison. Taken together, the structural observation that Ets-1/DNA interface is sparsely hydrated and maintained primarily by direct contacts between protein and DNA^{2,4} should now be understood mainly in terms of induced dynamics in the DNA-bound state, rather than the lack of an adhesive role for interfacial water-mediated contacts as with PU.1 and its proximal phylogenetic ETS relatives.

CONCLUSION

By way of mutating an interfacial water-coordinating tyrosine that is highly conserved among known members of the ETS family, we showed that interfacial hydration could be modulated in a protein/DNA complex. Presumably, additional strategic point mutations could achieve further fine-grained tuning of hydration in a strongly osmotically sensitive system such as PU.1. However, interfacial hydration plays distinct roles in this family, as PU.1 N167^{Y252F} binds its target DNA site just as well as wildtype PU.1 under normo-

osmotic conditions but affinity for Ets-1 N331^{Y412F} is reduced. This disparity arises from DNA-bound Ets-1's low dependence on interfacial hydration in the first place and the coupling of a specific water-mediated contact (via Tyr⁴¹²) to induced dynamics in the DNA-bound state. The present observations add to a growing line of evidence attesting that ETS homologues, despite their structurally superimposable DNA-bound structures, recognize target DNA sites by distinct mechanisms.

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(purple ●) in panel A) are shown as cyan spheres. (D and E) Water-mediated contacts (within 3.4 Å) in the protein/DNA interface of PU.1 and Ets-1, the latter contacting DNA primarily via direct contacts. Tyr²⁵² and Tyr⁴¹² are marked by red arrows. (F and G) Expanded view of Tyr²⁵²/Tyr⁴¹² coordinating a highly conserved water-mediated contact with Arg²³⁵/Arg³⁹⁴ and a specific DNA backbone phosphate.

Figure 2.

Mutation of an interfacial tyrosine to phenylalanine perturbing DNA-binding affinity and sensitivity to osmotic pressure by the ETS domains of PU.1 and Ets-1. (A)

Electropherograms from Sanger sequencing of recombinant ETS constructs harboring the one-atom-different Tyr-to-Phe mutations in PU.1 N167 and Ets-1 N311. (B)

Representative fluorescence polarization titrations of ETS-bound, TYE-labeled DNA with unlabeled, optimal site-specific DNA under normo-osmotic (0.29 osmolal) and osmotically stressed (2.5 osmolal) conditions. DNA probe was present at 0.5 nM and protein between 10 to 100 nM, depending on construct and conditions, to establish pre- and post-titration baselines. Wildtype and mutant protein binding is denoted by solid and open *symbols*, respectively. *Curves* represent fits of the data to a 1:1 competitive model. (C) Osmotic dependence of the binding constant shows sharply different responses by the two ETS homologues and their mutants to osmotic pressure. *Lines* represent weighted linear fits to each data set.

Figure 3.

Analogous Tyr-to-Phe mutations which are differentially perturbative to the unbound PU.1 and Ets-1 ETS domains. (A) Far-UV circular dichroism scans of wildtype (*solid symbols*) and mutant (*open*) PU.1 N167 and Ets-1 N331 at 25 °C, fitted with BeStSel (*curves*) to estimate secondary structure content. Parametric values are given in Table 1. (B) Thermal melting (up triangles) and cooling curves (down triangles) of wildtype and mutant PU.1 N167 and Ets-1 N331, monitored at 222 nm. Neither Ets-1 construct unfolded reversibly (gray down triangles). Heating and, if reversible, cooling data was globally fitted with a

unimolecular two-state model (*curves*). Values are given in Table 1. (C) Size-exclusion chromatography of purified wildtype and mutant PU.1 N167 and Ets-1 N331 on a Superdex 75 column, which was calibrated with bovine serum albumin (peaks 1 and 2; a 132 kDa dimer and 66 kDa monomer), carbonic anhydrase (peak 3, 30 kDa), and myoglobin (peak 4, 17 kDa, detected via its Soret band at 409 nm). (D) Overlaid solution HSQC spectra of wildtype (gray) and Ets-1 N331^{Y412F} (black). Where possible, chemical shift assignments of *wildtype* resonances that exhibited the largest chemical shift changes (partial or complete lack of overlap) were taken from the solution NMR structure (PDB: 1R36).²³ Identity in the case of partially overlapped mutant residues was not assumed. Asterisks denote side chain resonances.

Figure 4.

Molecular dynamics simulations recapitulate experimental properties of the unbound ETS domains of PU.1 and Ets-1. Following all-atom simulations for 100 ns at 298 K, the trajectories and structures of the ETS domains of PU.1 and Ets-1 were analyzed in terms of RMS deviation from initial coordinates (RMSD, parts A and B), hydrogen bonds with solvent (parts C and D), and mean square displacement (MSD, parts E and F). The RMSD data showed rapid convergence in the dynamics of both sets of proteins. Enumeration of H-bond contacts with solvent showed a 4% bias in favor for wildtype Ets-1, consistent with its

thermal instability relative to the Y412F mutant. Finally, the lower MSD of wildtype Ets-1 relative to PU.1 reflects dynamic light scattering measurements showing a 14% larger hydrodynamic radius for Ets-1. The MSD trajectories were divergent out to 100 ns.

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Figure 5.

Molecular dynamics simulations recapitulating induced experimental dynamics in the wildtype Ets-1 complex and revealing a basis for destabilization by Y412F mutation. (A) All-atom RMS deviation from initial coordinates for wildtype and mutant PU.1 and Ets-1 in their DNA-bound states. (B) Per-residue RMS fluctuations, computed from the final 50 ns of the simulations, revealed significant dynamic bias in wildtype Ets-1 (positive values indicate excess dynamics in wildtype), but not PU.1. The red line represents the average RMSF $\pm$ SE. Dynamics of the bound DNA, not shown, exhibited identical trends. (C) Comparison with experimental NMR structures shows that the simulations reproduced the localized dynamics in the wildtype Ets-1/DNA complex. Both experimental structures were averaged from 25 conformers in the PDB ensemble; the simulated averaged structure consisted of 5000 models. Fluctuations are colored by residue based on a “B-factor” (proportional to RMSF^2) on a scale from 0 to 100 in a blue-white-red continuum, computed from the fluctuations from average positions. This scaling is intended only to facilitate comparison of the isotropic simulated and experimental fluctuations on a common scale. Corresponding sites of induced dynamics between the simulated and experimental wildtype Ets-1/DNA complexes are marked by arrows (the DNA is rendered semitransparent for clarity). The secondary

structure assignments of the simulated structures are inherited from their crystallographic templates and differ at places from the solution NMR structure. Tyr⁴¹² (wildtype) and Phe⁴¹² (mutant) are shown in green and pink, respectively.

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Figure 6.

Molecular dynamics simulations of DNA-bound PU.1 and Ets-1 ETS domain, which provide a window into the interfacial hydration of wildtype and mutant PU.1 and Ets-1. (A) Groups in the ETS/DNA interface used to probe interfacial hydration. Heteroatoms used as probes for solvent H-bonds are highlighted in red. Note that the H-bonding atoms in Group 4 (representing the 5'-GGAA-3' core consensus) specifically face the major groove toward the protein; backbone sugar and phosphates are schematized. (B) Solvent H-bond contacts made by the indicated groups given as the average of three independent simulations. The curves (dashed and solid for wildtype and mutant, respectively) represent FFT-smoothed averages based on a 2.5 ns window.

Table 1

Conformational Properties of Free Wildtype and Mutant ETS Domains of PU.1 and Ets-1^a

	secondary structure content, %				conformational stability	
	α helix	β sheet	turns	other	T_m , °C	$H(T_m) \times 10^5$, kJ/mol
PU.1 N167 ^{WT}	22.9	31.3	13.9	31.9	47.9 ± 0.2	26.6 ± 1.5
PU.1 N167Y252F	22.4	26.2	15.0	36.4	48.2 ± 0.2	29.2 ± 1.6
Ets-1 N331 ^{WT}	25.0	36.5	9.3	29.2	44.6 ± 0.1 ^b	44.2 ± 1.4 ^b
Ets-1 N331Y412F	24.2	32.0	14.2	29.6	51.9 ± 0.2 ^b	30.1 ± 1.4 ^b

^aSecondary structure content for each construct was estimated by decomposition of their CD spectra using the BeStSel algorithm.¹³ Conformational stability was measured by CD-detected thermal denaturation and renaturation, and fitted globally for both heating and cooling (where possible) by a unimolecular two-state model.

^bDetermined from heating curve only.