Solvation and the hidden thermodynamics of a zinc finger probed by nonstandard repair of a protein crevice

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Abstract

The classical Zn finger contains a phenylalanine at the crux of its three architectural elements: a β -hairpin, an α -helix, and a Zn^{2+} -binding site. Surprisingly, phenylalanine is not required for high-affinity Zn^{2+} binding, but instead contributes to the specification of a precise DNA-binding surface. Substitution of phenylalanine by leucine leads to a floppy but native-like structure whose Zn affinity is maintained by marked entropy–enthalpy compensation ($\Delta\Delta H$ -8.3 kcal/mol and $-T\Delta\Delta S$ 7.7 kcal/mol). Phenylalanine and leucine differ in shape, size, and aromaticity. To distinguish which features correlate with dynamic stability, we have investigated a nonstandard finger containing cyclohexanylanine at this site. The structure of the nonstandard finger is similar to that of the native domain. The cyclohexanyl ring assumes a chair conformation, and conformational fluctuations characteristic of the leucine variant are damped. Although the nonstandard finger exhibits a lower affinity for Zn^{2+} than does the native domain ($\Delta\Delta G$ -1.2 kcal/mol), leucine-associated perturbations in enthalpy and entropy are almost completely attenuated ($\Delta\Delta H$ -0.7 kcal/mol and $-T\Delta\Delta S$ -0.5 kcal/mol). Strikingly, global changes in entropy (as inferred from calorimetry) are in each case opposite in sign from changes in configurational entropy (as inferred from NMR). This seeming paradox suggests that enthalpy–entropy compensation is dominated by solvent reorganization rather than nominal molecular properties. Together, these results demonstrate that dynamic and thermodynamic perturbations correlate with formation or repair of a solvated packing defect rather than type of physical interaction (aromatic or aliphatic) within the core.

Keywords: NMR spectroscopy; isothermal titration calorimetry; protein design; nonstandard mutagenesis; structural water

Supplemental material: see www.proteinscience.org

The classical Zn finger (Fig. 1) defines a conserved class of nucleic acid binding proteins (Klug and Rhodes 1987). Single fingers exhibit metal-dependent folding as globular minidomains (Lee et al. 1989), which are similar in struc-

ture to corresponding elements of protein–DNA complexes (Pavletich and Pabo 1991; Jamieson et al. 2003). Despite its small size, the Zn finger exhibits general features of globular proteins and thus provides a model for investigation of

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Abbreviations: CD, circular dichroism; Cyc, cyclohexanylanine; DG, distance geometry; EEC, entropy–enthalpy compensation; ITC, isothermal

titration calorimetry; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; RMD, restrained molecular dynamics; RMS, root-mean-square; RMSD, RMS difference. Natural amino acids are designated by standard single- and three-letter codes; 2D, two-dimensional; 3D, three-dimensional.

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Figure 1. Sequence and structure of a Zn finger. (A) Sequence and schematic representation of parent (F10), variant (L10), and cyclohexanylalanine-repaired (X10) fingers. Residues are color coded according to ribbon model in *B* with Zn^{2+} highlighted in pink, ligands (two cysteate and two histidine side chains) and conserved leucine in blue, and the central aromatic side chain (F10) in red. Numbering scheme refers to synthetic peptide (30 residues). (B) Ribbon model of parent Zn finger (Protein Data Bank entry 1KLR) (Qian and Weiss 1992). Red asterisk indicates site of Leu and Cys substitution (residue 10). (C) Molecular surfaces of Phe (left) and Cyc (right). The Cyc side chain is similar in overall size and shape to Phe (including the volume of its π clouds). The nonplanarity of the cyclohexanyl ring and its additional hydrogens lead to volume increase of 32 $\text{\AA}^3$, predicted to be accommodated at the edge of the finger. Top and side views are shown.

protein stability. Of central interest is a conserved aromatic side chain at the juxtaposition of its three architectural elements: an N-terminal β -hairpin, a C-terminal α -helix, and a tetrahedral Zn^{2+} -binding site (the $\beta\beta\alpha$ motif) (Berg 1988; Gibson et al. 1988).⁴ This side chain (shown in red in Fig. 1) underlies the motif's DNA-binding surface (Pavletich and Pabo 1991); its packing against the proximal histidine leads to desolvation of the core. Nonaromatic substitutions impair DNA binding and transcriptional activation in vitro (Kehres et al. 1997; Bumbulis et al. 1998), and are associated with cancer and birth defects in vivo (Kohsaka et al. 1999). Single fingers containing a Phe→Leu substitution retain the $\beta\beta\alpha$ motif but lack dynamic stability (Mortishire-Smith et al. 1992; Lachenmann et al. 2002).⁵ The

substitution Phe→Tyr, occasionally found in nature, is in contrast well tolerated (Qian and Weiss 1992). Here, we employ nonstandard mutagenesis to investigate the contribution of the central aromatic residue to structure, stability, and dynamics. Extending the repertoire of amino acids (Wang et al. 2001) enables effects of side-chain volume and aromaticity to be distinguished.

The present study builds on a recent analysis (Lachenmann et al. 2002) of the role of the central Phe in the core of a Zn finger derived from the human Y-encoded protein ZFY (Fig. 1; Qian and Weiss 1992). Solution proton nuclear magnetic resonance (^1H -NMR) studies of a Leu analog demonstrated retention of a native-like structure in which the variant side chain occupied a similar position as the native phenylalanine. Enhanced conformational fluctuations nonetheless led to accelerated amide proton exchange and attenuation of a subset of α -helix-related and long-range nuclear Overhauser effects (NOEs). Despite its marked dynamic instability, the Leu analog exhibited native (or even somewhat enhanced) thermodynamic stability (Lachenmann et al. 2002). To investigate this seeming paradox, the coupling between metal binding and peptide folding was investigated by isothermal titration calorimetry (ITC): Apparent changes in Gibbs free energies (ΔG) were resolved into enthalpic (ΔH) and entropic ($T\Delta S$) contributions. Comparison between Phe and Leu domains demonstrated that their small difference in free-energy change ($\Delta\Delta G$) masked large and opposing changes in enthalpy ($\Delta\Delta H$) and entropy ($T\Delta\Delta S$). Further, entropy–enthalpy compensation (EEC) (Dunitz 1995) was mediated not by relative changes in configurational entropy, as would be expected surrounding a packing defect (Takano et al. 1995), but instead by changes in enthalpy. By analogy to the thermodynamics of organic complexation of metals (Danil de Namor et al. 1991; Smithrud et al. 1991; Searle and Williams 1992; Searle et al. 1995), we proposed that such “hidden” compensation arises from solvent reorganization (Lumry and Rajender 1970).⁶ Phenylalanine and leucine differ in aromaticity, size, and shape. In this article we employ nonstandard mutagenesis to investigate which of these features correlates with dynamic- and thermodynamic stability. The essential idea is to repack the core of the finger without use of an aromatic side chain. Our strategy thus exploits the distinct physical properties of aromatic and aliphatic systems (Burley and Petsko 1988). Cyclohexanylalanine (Cyc) (Morii et al. 1999) was chosen based on its approximate fit within the core. The proximal portion of Cyc (through its δ carbons) recapitulates Leu, whereas its distal ring restores native-like side-chain vol-

⁴Zn fingers exhibit alternative placements of the central aromatic residue, “consensus” (C-X₂-C-X₃-E-X₇-L-X₂-H-X₃₋₅-H) or “swapped” (C-X₂-C-X₃-E-X₉-L-X₂-H-X₃₋₅-H; Kochoyan et al. 1991). In each case the aromatic side chain contacts the conserved leucine (L18 in Fig. 1A) with either edge-to-face (consensus) or displaced-stacking (swapped) aromatic–histidine interactions.

⁵The terms “stability” and “instability” are often used in reference to either thermodynamic free energies (ΔG) or dynamic processes. To distinguish between these concepts, “stability” is used herein in the thermodynamic sense unless otherwise stated. “Dynamic stability” or “instability” is used in reference to structural fluctuations and configurational entropy.

⁶The phrase “hidden thermodynamics” is due to Karplus and coworkers (Gao et al. 1989) and meant to emphasize the complex interplay of physical processes underlying the free energy change of a reaction. “Nominal” properties refer to structural and dynamic features of a protein without consideration of solvent (Grunwald and Steel 1995).

ume (Fig. 1C). (The volume of cyclohexane [$180 \text{ \AA}^3$] is larger than that of benzene [$148 \text{ \AA}^3$] due to its nonplanarity and additional hydrogens. The additional volume [ΔV $32 \text{ \AA}^3$, presumably similar to the difference in volume between Phe and Cyc] would be expected to be accommodated by protrusion of the distal ring into solvent, accentuating a convex surface of the finger [Fig. 3, see below].) Despite the nonplanarity of cyclohexane, preliminary modeling studies suggested that the Cyc side chain in a canonical "chair" conformation could pack within the finger, draping over the proximal histidine to seal one face of the protein surface. 2D-NMR studies of the nonstandard domain are in accord with these expectations. Distance-geometry (DG) models indicate that close packing of the core occurs without formation of a cavity or crevice. Remarkably, Leu-associated conformational fluctuations are largely damped, as indicated by analysis of interresidue nuclear Overhauser enhancements (NOEs) and amide-proton exchange rates. Although the affinity of the Cyc finger for Zn^{2+} is reduced, calorimetric studies demonstrate that thermodynamic excursions in entropy and enthalpy, so extreme in the Leu analog, are almost completely attenuated. Although aromatic-aromatic interactions (Burley and Petsko 1988) in the core may contribute to thermodynamic stability in accord with past studies of mutant proteins (Serrano et al. 1991), our results demonstrate that side-chain volume, rather than aromaticity, is the key determinant of dynamic and entropic differences. Our results further suggest that the marked EEC in this system is dominated by solvent reorganization (Grunwald and Steel 1995) rather than the nominal properties of the polypeptide (Dunitz 1995). Together, these findings highlight the utility of nonstandard mutagenesis in comparative studies of mutant proteins.

Results

Consensus and variant peptides exhibit analogous Zn^{2+} -dependent folding transitions as monitored by circular dichroism (CD): Spectra in the absence of Zn^{2+} are consistent with a predominance of random coil and in the presence of Zn^{2+} with the $\beta\beta\alpha$ motif. As expected, ^1H -NMR spectra of the three apo-peptides are consistent in each case with random coil (Supplemental Material). On binding of equimolar Zn^{2+} ions, the ^1H -NMR spectrum of the Cyc10 analog is well resolved and displays a pattern of chemical shifts analogous to that of the Leu variant (Fig. 2). Sequential assignments are readily obtained by homonuclear 2D-NMR methods (Supplemental Material). Chemical shifts are similar to those of the parent finger with the exception of resonances affected by the ring current of Phe10; differences are largely consistent with ring-current simulations as described (Lachenmann et al. 2002). Of particular interest, the δ methylene protons of Cyc10 (Fig. 2B) exhibit large upfield secondary shifts similar to those of the δ -methyl resonances of

Figure 2. 1D ^1H -NMR spectra of parent Zn finger (A), Cyc10-repaired finger (B), and Leu10 finger (C) at 500 MHz and 25°C . Asterisks in C indicate upfield methyl resonances of Leu10. The upfield region of the wild-type spectrum is remarkable for the β -methylene protons of K25 (labeled in A). Downfield amide resonance of Tyr7, a characteristic feature of Zn fingers (Lee et al. 1992), is labeled in A. Asterisks in C highlight the similarity in secondary shift between the δ methylene resonances of Cyc10 and the δ -methyl resonances of Leu10, presumably due in each case to the ring current of His21 (Lachenmann et al. 2002). The numbering scheme for Cyc proton resonances is provided in the supplement.

Leu10 (asterisks in Fig. 2C), presumably due in each case to the ring current of His21. ^1H -NMR spectra and tables of chemical shifts are provided in the supplement. Large differences between the chemical shifts of vicinal methylene ring protons (for example, H_{12} and H_{22} at 0.70 ppm and -0.28 ppm, respectively; see Supplemental Material for Cyc numbering scheme) indicate the presence of steep and stably maintained magnetic-field gradients in the interior of the finger. The pattern of sequential and helix-related NOEs and the distribution of $^3J_{\alpha\text{N}}$ coupling constants in the Cyc10 domain are essentially identical to those of the Phe10 domain (Qian and Weiss 1992).

NMR features of the cyclohexanyl ring are distinctive. Whereas in the wild-type finger the central aromatic residue exhibits degenerate ring resonances due to rapid rotation around the $\text{C}_\beta\text{--C}_\gamma$ bond axis, the Cyc ring has clearly distinguishable sides. Its five pairs of methylene resonances are individually resolved; each gives rise to a distinct pattern of interresidue NOEs (Supplemental Material). The C_2 methylene resonances, for example, exhibit chemical shifts of 0.70 and -0.28 ppm, well resolved from C_6 methylene resonances at 0.20 and 1.46 ppm. These differences reflect dis-

tinct spatial environments. The C₂-C₃ side of the Cyc ring is closer to the side chains of Ser12 and Leu18 than is the C₅-C₆ side. Similarly, the side chain of His21, beneath the ring, is positioned with its H₈₂ proton closer to the C₂-C₃ side, whereas H_{ε1} is equidistant from both sides. The C₅-C₆ side is not close to other side chains and so protrudes at the surface. Preliminary stereospecific assignments of Cyc ring methylene protons, obtained by standard methods, were verified by DG models calculated in their absence. Together, these observations indicate that the conformation of the cyclohexanyl ring is well defined. NOEs otherwise involving cysteate- and histidine side chains in the Zn²⁺-binding site are indistinguishable from those in the Leu10 or Phe10 domains. Differences in chemical shifts and NOEs relative to the Leu10 domain (Supplemental Material) in part reflect altered dynamics (see below).

The structure of the Cyc10 domain, calculated on the basis of 273 interresidue NOEs, 27 dihedral-angle restraints, and 10 hydrogen bond-related distance restraints (Supplemental Material), retains a canonical ββ α structure (shown in red in Fig. 3A, left panel). The main chain closely superimposes with that of the Phe domain (shown in green in Fig. 3A, left panel). The precisions of the two ensembles are essentially identical: main-chain root-mean-square (RMS) deviations in the Cyc10 and Phe10 ensembles are 0.78 Å and 0.79 Å, respectively, whereas side-chain values are in

each case 1.33 Å (see supplement for stereo views and statistical parameters). By contrast, the Leu ensemble is less precise (main-chain and side-chain RMS difference values 0.93 Å and 1.59 Å, respectively) (Lachenmann et al. 2002). The mean pair-wise RMS deviation between main-chain atoms of the Cyc10 and Phe10 ensembles (residues 2–26) is 1.02 Å, similar to that between Leu and Phe ensembles (1.04 Å) and between the Cyc and Leu ensembles (1.12 Å). Region-specific alignment according to the main-chain atoms in the metal-binding site (residues 5–8 and 21–26) demonstrates that structures at the “base” of the fingers are essentially identical. In particular, the geometry of the Zn-binding sites are very similar (Fig. 3A, right panel), although small changes are possible in Zn-related bond angles and dihedral angles (e.g., 5-C β -S γ -Zn; see Supplemental Material); these are nonetheless within the range of parameters observed among crystal structures of Zn finger/DNA complexes (Pavletich and Pabo 1991; Fairall et al. 1993; Pavletich and Pabo 1993; Elrod-Erickson et al. 1996; Kim and Berg 1996; Nolte et al. 1998).

The Cyc10 side chain packs in a chair conformation between Leu18 and His21 to occupy a site similar to that of Phe10 (Fig. 3A, right panel). Despite this and the above structural similarities, differences are observed between the packing of Cyc10 and Phe10, which in turn, lead to differences in the relative orientation of the “fingertips” and first turn of the helix (residues 12–18). Front and side views of these structural relationships are shown in Figure 3.

Figure 3. Nonplanarity of Cyc10 is associated with its partial swiveling into solvent. (A) (Left) Front view of Zn fingers (stereo views provided in Supplemental Material) on alignment of Phe and Cyc ensembles (green and red, respectively) according to the main-chain atoms of residues 3–12 and 16–25. (Right) Stereo pair showing relative orientation of Cys₂-His₂ ligands, residue 10 (Phe or Cyc) and Leu18. (B) (Left) Side view of Phe ribbon (green) and side chains of His21 and residue 10 (Phe or Cyc; see Fig. 1A). (Right) Stereo pair showing expansion of boxed region illustrating relative orientation of nonplanar Cyc10 side chain relative to representative Phe10 side chain. The nonplanar shape of the Cyc ring is accommodated by draping over the ring of H21; the distal edge of the Cyc ring contributes to a convex protein surface (see Fig. 6B).

1. *Draping of cyclohexanyl ring.* Because the nonplanar Cyc10 side chain cannot stack evenly over the imidazole ring of His21, it is partially displaced outward. The proximal portion of the cyclohexanyl ring overlies His21, whereas the distal portion drapes over the ring into solvent (shown in red in Fig. 3B). Such packing rationalizes the extreme upfield shift of the δ methylene resonances of Cyc10 relative to the smaller H21-specific ring-current shifts of the ϵ and ζ resonances (Supplemental Material). Differences between the chemical shifts of vicinal methylene resonances, and hence local magnetic-field gradients, attenuate toward the end of the ring (Supplemental Material). Also in accord with the draping model, NOEs are not observed between Leu18 and the distal ϵ - and δ -methylene resonances of Cyc10 (attached to ring carbons C₃, C₄, and C₅). Restraints based on such unobserved NOEs were not utilized in the DG/RMD calculations.

2. *Displacement of fingertip.* Displacement of Cyc10 is communicated to the fingertip and helix via the side chain of Leu18 (Fig. 3A). The conserved leucine retains its canonical position in the core as indicated by NOEs between its δ_1 -CH₃ methyl resonance and the side chains of Cys5, His21, and Ile22; and likewise by NOEs be-

tween Leu18 δ_2 -CH₃ and the side chains of Tyr3, Cys5, and a ring resonance of Cyc10 itself (H₂I) (see Supplemental Material). Unlike the fingertip of the Leu10 ensemble, which is less well ordered, the Cyc10 ensemble exhibits an ≈ 1 Å relative shift in fingertip trajectory. Despite these differences, Cyc and Phe each enable close packing of the core to yield a convex protein surface. Despite its nonplanarity, the larger volume of the Cyc side chain is able to compensate for the smaller Leu10 side chain.

Dynamic instability of the Leu10 domain was previously demonstrated by attenuation of selected interresidue NOEs and accelerated amide proton exchange via subglobal mechanisms (Lachenmann et al. 2002). These perturbations are partially repaired in the Cyc10 domain as described in turn below. Because the Leu10 and Cyc10 domains both lack the native Phe10 ring current, physical differences between ensembles are demonstrated by differences in chemical shifts, such as observed among the H _{α} resonances of neighboring residues 11, 13, and 14 ($|\Delta\delta| \sim 0.2$ ppm) and amide resonances in the C-terminal helix ($|\Delta\delta| \sim 0.1$ – 0.5 ppm; Supplemental Material). Such changes presumably reflect perturbations associated with the Leu10-associated packing defect, including transmitted effects on the dynamic stability of the helix. Evidence for partial repair by Cyc10 is as follows:

1. *Pattern of NOE intensities.* The majority of helix-related NOEs that are attenuated in the spectrum of the Leu10 domain (relative to the Phe10 domain) regain native-like intensity in the spectrum of the Cyc10 domain. For example, four helix-related NOEs are observed in the Phe10 and Cyc10 domains but not in the Leu10 domain: two $d_{\alpha\beta(i,i+3)}$ contacts (15–18 and 17–20) and two $d_{\alpha N(i,i+4)}$ contacts (17–21 and 19–23). (Instead, the Leu domain contains anomalous 24–25 $d_{\alpha N(i,i+1)}$ and 24–26 $d_{\alpha N(i,i+2)}$ contacts that are either weak or not seen in spectra of the Phe10 or Cyc10 domains.) The overall pattern of NOE intensities suggests that structural perturbations or fluctuations in the C-terminal helix of the Leu domain are largely repaired in the Cyc10 domain.⁷ By contrast, NOEs within the N-terminal β -hairpin are similar in the three fingers.

NOE intensities in the helix of the Leu10 domain may be attenuated either by a spatial mechanism (substates in which the distances are larger than in the wild-type structure) and/or by a dynamic mechanism (fluctuations on a time scale near the Lamor frequency leading to zero-

crossing of the NOE). Rare fluctuations in an activated process (such as that leading to amide–proton exchange on a time scale of seconds or minutes) would not be expected to affect NOE intensities.

2. *Amide proton exchange.* The Phe10 domain contains eight protected amide protons in freshly prepared D₂O solutions at 4° and 25°C (Fig. 4B, spectra i and ii), corresponding to β -sheet–related hydrogen bonds (residues 5 and 12), amide–sulfur hydrogen bonds in the metal-binding site (residues 7, 8, and 10), and α -helix–related hydrogen bonds (Qian and Weiss 1992) (residues 22–24) (Fig. 4A). The Leu10 domain lacks protected amide protons at 25°C (Lachenmann et al. 2002) and exhibits only weak protection of amide–sulfur hydrogen bonds at 4°C (Fig. 4B, spectrum iv). The Cyc10 domain exhibits an intermediate pattern of protection: At 25°C the amide–sulfur-related resonances of residues 7, 8, and 10 are observed as well as the helix-related resonance of residue 22 (Fig. 4B, spectrum iii). The β -sheet–related resonance of residue 5 is regained at 4°C. The time course of exchange at these sites at 4°C is shown in Figure 4, C–F. In each case protection that is attenuated in the Leu domain is partially restored in the Cyc10 domain. Because the thermodynamic stability of the Leu10 domain is greater than that of the Cyc10 domain (see below), these observations indicate that the non-standard side chain damps conformational fluctuations associated with the Leu10 packing defect. Given that the canonical volume “decrement” between Leu and Phe (21 Å³ exclusive of any adjoining crevices in the protein) is similar to the volume “increment” between Phe and Cyc (32 Å³; see above), the NMR results demonstrate that the dynamic stability of the Zn finger, although exquisitely sensitive to a packing defect, readily tolerates a comparable increase in side-chain volume. The draped Cyc10 ring creates only a minor protuberance on the protein surface; its volume presumably exceeds a critical threshold required to damp fluctuations.

ITC studies indicate that the affinity of the Cyc10 domain for Zn²⁺ is less than that of the Leu and Phe domains (Table 1). (The present affinities for Zn²⁺ are lower than those described in consensus Zn fingers, presumably due to the lower pH [pH 6.5] and effects of the “swapped” aromatic framework.) The decrement in free energy between the Phe10 and Cyc10 peptides ($\Delta\Delta G$ 1.2 kcal/mol; Table 1; Fig. 5A) suggests that the aromaticity of the core side chain indeed contributes to but is not required for stable packing. The magnitude of the decrement is in accord with loss of displaced aromatic stacking over the proximal histidine (Kochoyan et al. 1991), but we cannot exclude the possibility that relative stabilities reflect an integrated effect of

⁷The Cyc10 domain also exhibits a weak helix-related 22–26 $d_{\alpha\beta(i,i+4)}$ contact not present in the Phe or Leu domains.

Figure 4. Amide-proton exchange in wild-type, Leu10 variant, and Cyc10 “repaired” Zn fingers (pD 6.5 and 4°C). (A) Positions of peptide and amide-sulfur hydrogen bonds (white lines) in wild-type Zn finger. Zn^{2+} is represented as a pink sphere, sulfur atoms in yellow, and F10 in green. (B) ¹H-NMR spectra in H_2O and freshly prepared D_2O of Phe domain at 4 and 25°C (i and ii, respectively); corresponding initial D_2O spectra of Cyc domain at 25°C (iii), and Leu domain at 4°C (iv). (C–F) Amide-proton exchange is shown by residue (Tyr7 in C, Cys8 in D) or structural class (β -hairpin in E, α -helix in F). In each panel amide proton exchange in the Phe10 finger is depicted by open circles connected with a red line; exchange in the Leu finger is depicted by open circles connected with a green line (absent in E,F) and exchange in the Cyc10 finger by solid triangles connected with a blue line. Asterisks in C and D indicate brief residual protection of amide-sulfur hydrogen bonds in fluctuating Leu10 finger.

multiple additional differences between domains. (The lower affinity of the Cyc10 domain for Zn^{2+} is consistent with loss of an aromatic-aromatic interaction in model systems [Burley and Petsko 1988; Serrano et al. 1991]. The magnitude of $\Delta\Delta H$ [-0.7 ± 0.2 kcal/mol] is consistent with loss of this favorable enthalpic interaction but is in part

compensated by a favorable change in entropy [$T\Delta\Delta S$ 0.5 ± 0.2 kcal/mol], whose sign is opposite from changes in configurational entropy implied by NMR studies.) Apparent values of ΔH and inferred value of $T\Delta S$ are similar to those of the consensus domain. Although these values contain contributions from several sources (including Zn binding,

Table 1. Thermodynamic analysis of zinc binding

Domain	K_b (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
Zn-F10	3.5×10^6	-8.9	-9.4 ± 0.1	-0.5 ± 0.1
Zn-X10	4.6×10^5	-7.7	-8.7 ± 0.1	-1.0 ± 0.1
Zn-L10	8.8×10^6	-9.5	-17.7 ± 0.3	-8.2 ± 0.3
Differences ^a		$\Delta\Delta G$	$\Delta\Delta H$	$T\Delta\Delta S$
Zn-X10	—	-1.2	-0.7 ± 0.2	0.5 ± 0.2
Zn-L10	—	0.6	8.3 ± 0.4	7.7 ± 0.4

X indicates cyclohexanylanaline. Values were obtained in 50 mM HEPES buffer (pH 6.5 and 25°C). Data are derived from the mean of two individual experiments (see Materials and Methods). The errors shown are those associated with the nonlinear least-squares fitting of the enthalpy. The error from the fit of ΔG was less than the reported precision; hence the error in $T\Delta S$ tracks with ΔH . The observed peptide: Zn^{2+} stoichiometries were unity, and the determined heats of dilution, when subtracted, gave a baseline reading of zero enthalpy.

^a Differences in thermodynamic state function $\Delta\Delta J$ are defined as $\Delta J(wt) - \Delta J(\text{variant})$.

the coupled folding reaction, changes in the ionization buffer, and extent of residual structure in the unfolded-state ensembles), values of $\Delta\Delta H$ and $T\Delta\Delta S$ reflect changes in the solvent-coupled folding reaction as contributions from direct Zn^{2+} coordination and changes in buffer ionization are presumably the same for each peptide (see Materials and Methods). The results of ITC analysis have three implications: First, the large and compensating swings in enthalpy ($\Delta\Delta H$) and entropy ($T\Delta\Delta S$) exhibited by the Leu10 variant (black bars in Fig. 5A) are almost completely attenuated by the nonstandard substitution (red bars in Fig. 5A). Second, the calorimetric entropies of metal-dependent folding of the Phe10 and Cyc10 domains ($T\Delta S$ -0.5 ± 0.1 and -1.0 ± 0.1 kcal/mol, respectively) are less unfavorable than that of the Leu10 domain ($T\Delta S$ -8.2 ± 0.3 kcal/mol). Thus, unrelated to the physics of side-chain packing in the core (i.e., whether the Leu10-associated crevice is repaired by an aromatic or aliphatic side chain), changes in calorimetric entropy have opposite signs from inferred changes in configurational entropies. Finally, despite the weakened or altered side-chain packing in the Leu10 domain, its affinity for Zn^{2+} is rescued by increased enthalpy (ΔH -17.7 ± 0.3 kcal/mol). Such an accentuated change in enthalpy is not observed in the more native-like Cyc10 domain (ΔH -8.7 ± 0.1 kcal/mol).

We cannot exclude that Leu10 relieves unfavorable interactions in the Phe10 domain (thus providing an enthalpic compensation), and that by coincidence unfavorable interactions of a similar magnitude are introduced by Cyc10. This seems unlikely in light of their otherwise similar structures and small size (i.e., lack of a surrounding structure to encage an unfavorable contact). Further, coincidental perturbations by Phe10 and Cyc10 seem unlikely due to their physicochemical differences.

Discussion

The present study focuses on the Zn finger, a ubiquitous motif of eukaryotic protein–nucleic acid recognition (Klug and Rhodes 1987). This minidomain provides a model for analysis of protein stability as demonstrated by Berg and coworkers (Kim and Berg 1993; Blasie et al. 2002). Advantages of this system include its small size (which facilitates chemical synthesis), robustness of structure (which permits maintenance of a common metal-binding site among variants), and lack of ordered structure in the unfolded state (which provides a common thermodynamic reference state among variants). In this and a previous study (Lachenmann et al. 2002) we have investigated the role of the central aromatic residue (Phe10), which overlies the proximal histidine in the metal-binding site. Substitution of this side chain by leucine was found to permit high-affinity Zn^{2+} binding despite marked dynamic perturbations. Unlike the native domain, a Leu10 variant finger lacks protected amide resonances in D_2O , and its NOESY spectrum exhibits a subset of attenuated helix-related and long-range NOEs. The overall structure of the Leu10 analog is nonetheless similar to that of the native finger. Surprisingly, calorimetric analysis of Zn^{2+} binding demonstrated that the thermodynamic stability of the variant domain is maintained by ‘para-

Figure 5. ITC analysis and solvent-reorganization hypothesis. (A) Histogram showing marked EEC in Leu analog (black bars) and attenuation of perturbations in enthalpy ($\Delta\Delta H$) and entropy ($T\Delta\Delta S$) in Cyc analog (red bars). Thermodynamic double-differences ($\Delta\Delta J$) are defined as $\Delta J_{\text{wild-type}} - \Delta J_{\text{variant}}$. (B–D) Schematic outline of zinc finger “repair.” Whereas the central Phe seals the hydrophobic core (B), the Phe→Leu substitution creates a fluctuating packing defect (C) (Lachenmann et al. 2002). This crevice is repaired by Cyc (D). Blue spheres in C represent bound water molecules.

doxical' EEC: The sign of the change in entropy ($\Delta\Delta S$) is opposite to the apparent change in configurational entropy as inferred from NMR studies (Lachenmann et al. 2002). The goal of the present study was to test by nonstandard mutagenesis whether the marked perturbations observed in the Leu10 variant are due to loss of side-chain volume or lack of aromaticity. We therefore sought to "repair" the variant finger by cyclohexanylalanine, a larger aliphatic side chain predicted to restore core packing.

The Cyc10 domain was found to exhibit a native-like structure in which the cyclohexanyl moiety adopts a well-defined chair configuration. Although this nonplanar structure necessitates local adjustments in core packing, the overall structure is similar to that of the parent finger. The larger volume of Cyc relative to Phe is readily accommodated by draping of the distal ring over H21 to contribute to a convex protein surface. Reduced Zn^{2+} affinity ($\Delta\Delta G$ 1.2 kcal/mol) is observed consistent with loss of an aromatic–aromatic interaction (Kochoyan et al. 1991). Significantly, however, packing of the Cyc10 side chain attenuates thermodynamic excursions in enthalpy and entropy ($\Delta\Delta H$ and $T\Delta\Delta S$; red bars in Fig. 5A) despite fundamental physical differences between packing interactions within the native and repaired domains (aromatic or aliphatic) (Burley and Petsko 1988). Why does the Leu10 domain exhibit such marked dynamic and thermodynamic perturbations, and why are these perturbations attenuated in the Cyc10 domain? It is likely that the smaller volume of the leucine side chain is associated with a packing defect, leading to a crevice at the protein surface and fluctuations in the positions of surrounding side chains. Extensive crystallographic analyses of engineered cavities and crevices in model globular proteins (such as barnase and T4 phage lysozyme) have been undertaken (Buckle et al. 1993; for review, see Matthews 1995). Such cavities, although destabilizing (Kellis Jr. et al. 1988), are generally compatible with a native-like overall structure. The extent and dimensions of cavities depend both on the sizes of individual side chains (i.e., substitution of a larger side chain by a smaller one) and on their structural environment. Large-to-small substitutions in the core of a globular protein often lead to the confluence of adjoining packing defects, leading to cavities larger than would be suggested by standard tables of side-chain volumes (Matthews 1995). Although generally contained within a hydrophobic environment, such cavities may in part be lined by polar atoms in contact with one or more structural water molecules (Buckle et al. 1996). The thermodynamic contributions of such cavity-associated water molecules have not been resolved.

Because of the small size of the Zn finger, few side chains are entirely buried. The aromatic ring of Phe10, for example, is exposed at one edge and contributes to a convex protein surface free of packing defects (Fig. 6A). Its substitution by Leu therefore does not lead to a cavity but

Figure 6. Analysis of packing defects in a Zn finger. (A) Close packing of F10 in the core of parent domain precludes formation of cavities or crevices adjacent to the Zn^{2+} coordination sites. Although crevices are observed elsewhere between surface side chains (blue regions), these are variable among DG/RMD models. (B) CPK model of the Cyc10 Zn finger illustrating draping of Cys ring at a convex protein surface. Cyc10 is shown in red; His21, in green; and contiguous residues, in dark blue.

instead alters the topography and dynamics of this surface (Lachenmann et al. 2002). Insight into possible alterations may be obtained by analysis of the DG/RMD models. Although the standard volume of Leu is only $21 \text{ \AA}^3$ smaller than that of Phe (as isolated amino acids), application of the SURFNET algorithm (which enables cavities and crevices in proteins to be visualized) (Laskowski 1995) suggests the substitution can perturb the convexity or concavity of a contiguous surface spanning residues 10, 17, and 21 as previously described (Lachenmann et al. 2002). The imprecision of the Leu10 ensemble in this region leads to a wide range of possible crevice sizes ($85 \pm 53 \text{ \AA}^3$ among 20 models). The smallest is near the lower bound imposed by the standard volumes of the isolated amino acids; the largest could accommodate a network of at least five water molecules ($30 \text{ \AA}^3$ per water molecule). By contrast, the related surfaces of the Cyc10 models are consistently convex due to the larger size of cyclohexanylalanine, and in particular, to draping of the distal portion of the nonplanar ring over H21 (red and green regions in Fig. 6B). As in the Phe10 domain, no cavities or crevices are predicted by the SURFNET algorithm. Although we have not explicitly visualized bound water molecules in the present structures (such NMR experiments are confounded by the presence of multiple exchangeable sites near residue 10), the anomalous ITC results (and, in particular, the opposite signs between changes in calorimetric entropy and configurational entropy in relation to Leu10) suggest that EEC arises through solvent reorganization rather than through multiple adjustments within the fingers themselves. We speculate that such reorganization

arises in response to changes in the protein surface associated with the volume of residue 10 (Fig. 5B–D). In particular, the “hidden” thermodynamic perturbations in the Leu10 domain may reflect the partial polarity of an associated crevice, which contains possible docking sites for a network of water molecules (Fig. 5C). This feature contrasts with the hydrophobicity of previously characterized cavities in globular proteins (Jackson et al. 1993; Yamada et al. 1994; Matthews 1995; Steif et al. 1995; Takano et al. 1995). Cyc10 is thus proposed to repair the protein surface, desolvating a native-like core (Fig. 5D).

EEC occurs in a wide range of chemical and biochemical processes (Lumry and Rajender 1970; Dunitz 1995; Grunwald and Steel 1995; Liu and Guo 2001). Two divergent perspectives inform discussion of its mechanism. One focuses on the structure and dynamics of the nominal system (Searle and Williams 1992; Dunitz 1995; Calderone and Williams 2001); the other on coupling between this system and its environment (Lumry and Rajender 1970; Grunwald and Steel 1995; Liu and Guo 2001). The first perspective envisages a trade-off between the strength of interactions at a noncovalent interface (such as a protein–ligand complex or within a hydrophobic core) and the entropic cost of its immobilization. This mechanism, essentially a gas-phase view, is intuitive and readily rationalized by consideration of “tight” and “loose” potential wells (Supplemental Material; Dunitz 1995; Searle et al. 1995). Because this view seems inconsistent with the type of EEC observed here, we turn to a complementary perspective, which was originally motivated by solvation phenomena (Lumry and Rajender 1970; Grunwald and Steel 1995; Searle et al. 1995). Compensation arises naturally in theoretical models of solvent–solvent and solvent–solute interactions (Yu and Karplus 1988; Lee 1991; Smithrud et al. 1991; Chervenak and Toone 1994; Grunwald and Steel 1995). Changes in solvent reorganization lead to compensating terms in general expressions for partial molar entropy and enthalpy, and these contributions are proposed to dominate changes within the apparent object of study, which may be noncompensating (Grunwald and Steel 1995). Environmental compensation has been extensively described in the binding of ligands (including divalent metal cations) to crown ethers and other small host–guest systems (Smithrud et al. 1991; Searle and Williams 1992; Dunitz 1995; Searle et al. 1995). Elementary models of environmental coupling exhibit compensation independently of the details of the nominal system and solvent molecules (Supplemental Material; Qian and Hopfield 1996; Qian 1998; Liu and Guo 2001). Our results suggest that the variant fingers provide a physical realization of a fluctuating boundary ensemble (Qian 1998) in which solvation provides environmental coupling: In molecular terms, the enthalpic benefit of a putative Leu10–crevice-associated network of water molecules would match or exceed its entropic cost, and these terms would dominate

nominal perturbations (whether compensating or noncompensating) within the variant finger (Lachenmann et al. 2002). This hypothesis would rationalize why the signs of the thermodynamic perturbations are opposite to those observed in desolvated and destabilizing cavities previously characterized within globular proteins (i.e., in which the direction of $T\Delta\Delta S$ is in accord with changes in configurational entropy) (Takano et al. 1995). It would be of interest in the future to obtain quantitative estimates of changes in configurational entropy as inferred from heteronuclear NMR relaxation studies.

An elegant thermodynamic study of the binding of metal ions to the zinc metalloenzyme carbonic anhydrase II by Toone and colleagues has demonstrated how complex and subtle changes in the structure and dynamics of the folded state lead to uncertainties in interpretation of ITC results (DiTusa et al. 2001a,b; Supplemental Material). Despite the ubiquity of EEC, noncompensation can nevertheless occur if the assumptions of the fluctuating boundary ensemble (Qian and Hopfield 1996; Qian 1998) are not honored (that is, in the absence of fluctuations between the site of perturbation at the protein–ligand interface and its environment, including the rest of the protein and its solvation). (Changes in entropy and enthalpy are second-order derivatives of free energy and so functions of environmental constraints [Qian and Hopfield 1996]. Whereas in the presence of environmental coupling only a part of the total change in entropy and enthalpy contribute to the change in free energy [Lumry 1995] [“Benzinger’s discovery”], in the absence of fluctuations this need not be the case.) Examples of absent or incomplete compensation are well known (Gallicchio et al. 1998). Indeed, the frequent empirical success of medicinal chemistry in enhancing the affinity of lead compounds for target protein structures suggests approaches to circumvent compensation (Dullweber et al. 2001). An enhanced physical understanding of protein dynamics and solvation may make possible virtual screening algorithms (Green 2003). We suggest that even in the absence of crystallographic evidence for bound water molecules (as in the present case), ITC data can provide a signature for their implicit participation in ligand binding when EEC is opposite in sign from apparent changes in configurational entropy.

The present model finger is based on an even-numbered domain in the human male transcription factor ZFY (Qian and Weiss 1992). Such domains contain an aromatic side chain at position 10 of the finger rather than at position 12, the consensus position. Whereas Phe10 exhibits displaced-horizontal stacking over the imidazole ring of proximal histidine His21 (Kochoyan et al. 1991), in a consensus finger Phe12 assumes an edge-to-face orientation (Lee et al. 1989). These geometric differences are associated with distinct patterns of solvent accessibilities and weakly polar interactions (Burley and Petsko 1988). Although Phe→Leu substitutions in either framework induce analogous dynamic stability

(Mortishire-Smith et al. 1992; Lachenmann et al. 2002), their respective packing defects would be expected to differ in shape and solvation. Accordingly, it would be of future interest to investigate whether the present results generalize to the consensus framework. In particular, Cyc repair of a putative Leu12-associated cavity in the WT1 tumor suppressor protein (Kohsaka et al. 1999) would be predicted to restore dynamic stability, and in turn, high-affinity DNA recognition. Such effects, if observed, would demonstrate the functional importance of the framework of the Zn finger in providing a well-organized platform for sequence-specific DNA contacts.

Conclusions

The present study illustrates the utility of nonstandard mutagenesis in studies of protein stability. Cyclohexanylalanine has been employed to repair a packing defect without restoration of native-like aromatic–aromatic interactions (Kochoyan et al. 1991; Qian and Weiss 1992). The non-standard side chain damps conformational fluctuations otherwise associated with a smaller aliphatic side chain (leucine). Interestingly, the magnitudes of thermodynamic excursions ($\Delta\Delta H$ and $T\Delta\Delta S$) were observed to correlate with formation or repair of a convex protein surface rather than with changes in configurational entropy or the type of interaction (aromatic or aliphatic) within the domains. These results provide insight into the physics of a Zn finger and highlight the importance of solvent reorganization as a mechanism of isothermal EEC in a protein.

Materials and methods

Peptide synthesis

The Zn finger (Fig. 1A) is derived from human ZFY (exon residues 162–191) (Page et al. 1987). The peptide was obtained by solid-phase synthesis (Merrifield 1986) as described (Kochoyan et al. 1991).

Isothermal titration calorimetry

Data were obtained using an Omega ITC or MCS ITC, both from Microcal, Inc., as described (Lachenmann et al. 2002). Titrations were performed at 25°C in 50 mM HEPES-HCl (pH 6.5). Precautions were taken to ensure that the peptide remained reduced throughout the ITC experiments. The peptide was dialysed in a buffer that had been saturated with argon gas to displace dissolved O₂. Titrations were performed immediately after dialysis under airtight conditions. Despite the small diameter of the ITC fill tube (~2 mm) and seal between the syringe and cell mountings being almost airtight, the opening was sealed during calorimeter equilibration with parafilm. A flow of N₂ gas was applied across the cell mounting to reduce exposure to air. Data were fitted as described (Wiseman et al. 1989; Ladbury and Chowdhry 1996) after subtraction of respective heats of dissolution of salt into buffer and buffer into peptide solution as obtained in separate control experiments. Results represent the mean of two individual titrations.

Theory of thermodynamic coupling

The thermodynamics of Zn²⁺ binding reflect (1) its desolvation and protein coordination, (2) coupled peptide folding, and (3) associated changes in buffer ionization. Because the metal-binding sites in the native and variant domains are essentially identical (see Results) and because the substitutions do not affect ionizable groups in the peptide, we assume that aspects of folding determine thermodynamic differences between fingers ($\Delta\Delta G$, $\Delta\Delta H$, and $T\Delta\Delta S$). Justification for this procedure has been described (Lachenmann et al. 2002). In brief, the apparent enthalpy change, ΔH_{obs} , contains contributions from the intrinsic enthalpy of binding and the enthalpy of ionization of the buffer:

$$\Delta H_{\text{obs}} = \Delta H_{\text{binding}} + N_{\text{H}^+} \Delta H_{\text{ioniz}} \quad (1)$$

where N_{H^+} designates the number of protons released (if $N_{\text{H}^+} > 0$) or bound (if $N_{\text{H}^+} < 0$) by the buffer upon metal-dependent peptide folding. Because ITC studies employ the same buffer in titration of consensus and variant peptides, ΔH_{ioniz} terms are constant. We assume as above that N_{H^+} is also the same. With this assumption, observed thermodynamic differences simplify to reflect buffer-independent quantities.

$$\Delta\Delta H_{\text{obs}} = \Delta\Delta H_{\text{binding}} + \Delta N_{\text{H}^+} \Delta H_{\text{ioniz}} = \Delta\Delta H_{\text{binding}} \quad (2)$$

We therefore simplify our nomenclature by omitting subscripts: Differences between nominal ITC values are ascribed to the process of metal-dependent peptide folding. ITC analysis, thus exploits thermodynamic coupling between binding of the guest (Zn²⁺) and folding of the host (peptide). To account for the anomalous signs of EEC ($\Delta\Delta H$ and $T\Delta\Delta S$), we generalize the approach of Berg and coworkers (Kim and Berg 1993) to include solvation (Lachenmann et al. 2002). The nominal association reaction is accompanied by transfer of water molecules from the hydration shell of the metal ion to bulk solvent and by folding of the peptide, itself associated with changes in solvent reorganization. The overall thermodynamics of metal binding, as measured by ITC, thus reflects the net effects of coupled “nominal” (the polypeptide and metal ion) and “environmental” (changes in solvation) reactions (Grunwald and Steel 1995). Because coordination of Zn²⁺ is not directly affected by the substitutions, common terms cancel in comparison of consensus and variant peptides; i.e., the contributions of direct metal-ion binding and desolvation to $\Delta\Delta G$, $\Delta\Delta H$, and $T\Delta\Delta S$ are negligible. With these assumptions (implicit in the studies of Kim and colleagues; Kim and Berg 1993) comparison of peptides permits dissection of relative thermodynamic driving forces in the coupled peptide-folding reaction.

NMR spectroscopy

Peptides were dissolved in 0.7 mL of NMR buffer containing 50 mM deuterated Tris-HCl (pH 6.0) and 2.2 mM ZnCl₂. 2D experiments were performed as described (Lachenmann et al. 2002). Spectra and assignments are provided in the supplement. NOE and J-coupling (dihedral angle) restraints were used for molecular modeling as described (Lachenmann et al. 2002). NMR studies of the apo-peptides were conducted in 50 mM deuterated Tris-HCl (pD 8.0) and 5 mM deuterated dithiothreitol.

NMR analysis

Sequential assignments were obtained as described (Lachenmann et al. 2002). The marked dispersion of the ¹H-NMR spectrum of

the Cys analog enables key motif-specific NOEs to be resolved (Supplemental Material). Nonetheless, packing between Cyc10 and Leu18 (although indicated by an NOE between one of the δ -methylene resonances and δ 1-CH₃ resonance of Leu18) is primarily determined by the overall network of NOEs rather than resolved cross-peaks. Interpretation of the NOEs between these residues was delimited as follows. The δ 1-CH₃ resonance of Leu18 overlaps with one of the ϵ -methylene resonances of Cyc10, whereas the δ 1-CH₃ resonance of Leu18 overlaps with its γ resonances. Additional possible NOEs between these side chains are obscured near the diagonal. Despite these limitations, the DG/RMD ensemble predicts multiple contacts between Cyc10 and Leu18 in accord with the canonical Zn finger structure. Because the surface near residue 10 (and, in particular, the L10-associated packing defect) contains multiple exchangeable protons, it was not possible to obtain unambiguous NMR evidence for bound water molecules.

Molecular modeling

The volume and location of possible protein cavities and crevices were obtained using the program SURFNET (Fig. 2A; Laskowski 1995). Analysis of polar and nonpolar surfaces was obtained using the Connolly surface feature in InsightII (Biosym, Inc.). Accessible surface areas were obtained with a probe sphere size 1.4 Å using the program ACCESS (Lee and Richards 1971).

Electronic supplemental material

Eight tables of chemical shifts, statistical parameters, NOE intensities, thermodynamic parameters, and DG/SA restraints. Nine figures providing ¹H-NMR and CD spectra, a summary of nomenclature for the cyclohexanyllalanine side chain, sequential- and medium-range NOEs and main-chain coupling constants in Wüthrich formats and ¹H-NMR secondary shifts between variant Leu10 finger and cyclohexanyllalanine-repaired finger, and schematic mechanisms of EEC. All Supplemental Material is incorporated into a single pdf file.

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References

Berg, J.M. 1988. Proposed structure for the zinc-binding domains from transcription factor TFIIIA and related proteins. *Proc. Natl. Acad. Sci.* **85**: 99–102.

Blasie, C.A. and Berg, J.M. 2002. Structure-based thermodynamic analysis of a coupled metal binding–protein folding reaction involving a zinc finger peptide. *Biochemistry* **41**: 15068–15073.

Buckle, A.M., Cramer, P., and Fersht, A.R. 1996. Structural and energetic responses to cavity-creating mutations in hydrophobic cores: Observation of a buried water molecule and the hydrophilic nature of such hydrophobic cavities. *Biochemistry* **35**: 4298–4305.

Buckle, A.M., Henrick, K., and Fersht, A.R. 1993. Crystal structural analysis of mutations in the hydrophobic cores of barnase. *J. Mol. Biol.* **234**: 847–860.

Bumbulis, M.J., Wroblewski, G., McKean, D., and Setzer, D.R. 1998. Genetic analysis of *Xenopus* transcription factor IIIA. *J. Mol. Biol.* **284**: 1307–1322.

Burley, S.K. and Petsko, G.A. 1988. Weakly polar interactions in proteins. *Adv. Protein Chem.* **39**: 125–189.

Calderone, C.T. and Williams, D.H. 2001. An enthalpic component in cooperativity: The relationship between enthalpy, entropy, and noncovalent structure in weak associations. *J. Am. Chem. Soc.* **123**: 6262–6267.

Chervenak, M. and Toone, E.J. 1994. A direct measure of the contribution of solvent reorganization to the enthalpy of ligand binding. *J. Am. Chem. Soc.* **116**: 10534–10539.

Daniil de Namor, A.F., Ritt, M.C., Schwing-Weill, M.J., Arnaud-Neu, F.A., and Lewis, D.F.V. 1991. Solution thermodynamics of amino acid–18-crown-6 and amino acid–cryptand 222 complexes in methanol and ethanol. *J. Chem. Soc. Faraday Trans.* **87**: 3231–3239.

DiTusa, C.A., Christensen, T., McCall, K.A., Fierke, C.A., and Toone, E.J. 2001a. Thermodynamics of metal ion binding. 1. Metal ion binding by wild-type carbonic anhydrase. *Biochemistry* **40**: 5338–5344.

DiTusa, C.A., McCall, K.A., Christensen, T., Mahapatro, M., Fierke, C.A., and Toone, E.J. 2001b. Thermodynamics of metal ion binding. 2. Metal ion binding by carbonic anhydrase variants. *Biochemistry* **40**: 5345–5351.

Dullweber, F., Stubbs, M.T., Musil, D., Sturzebecher, J., and Klebe, G. 2001. Factorising ligand affinity: A combined thermodynamic and crystallographic study of trypsin and thrombin inhibition. *J. Mol. Biol.* **313**: 593–614.

Dunitz, J.D. 1995. Win some, lose some: Enthalpy–entropy compensation in weak intermolecular interactions. *Chem. Biol.* **2**: 709–712.

Elrod-Erickson, M., Rould, M.A., Nekludova, L., and Pabo, C.O. 1996. Zif268 protein–DNA complex refined at 1.6 Å: A model system for understanding zinc finger–DNA interactions. *Structure* **4**: 1171–1180.

Fairall, L., Schwabe, J.W., Chapman, L., Finch, J.T., and Rhodes, D. 1993. The crystal structure of a two zinc-finger peptide reveals an extension to the rules for zinc-finger/DNA recognition. *Nature* **366**: 483–487.

Gallicchio, E., Kubo, M.M., and Levy, R.M. 1998. Entropy–enthalpy compensation in solvation and ligand binding revisited. *J. Am. Chem. Soc.* **120**: 4526–4527.

Gao, J., Kuczera, K., Tidor, B., and Karplus, M. 1989. Hidden thermodynamics of mutant proteins: A molecular dynamics analysis. *Science* **244**: 1069–1072.

Gibson, T.J., Postma, J.P.M., Brown, R.S., and Argos, P. 1988. A model for the tertiary structure of the 28 residue DNA-binding motif (“zinc finger”) common to many eukaryotic transcriptional regulatory proteins. *Protein Eng.* **2**: 209–218.

Green, D.V. 2003. Virtual screening of virtual libraries. *Prog. Med. Chem.* **41**: 61–97.

Grunwald, E. and Steel, C. 1995. Solvent reorganization and thermodynamic enthalpy–entropy compensation. *J. Am. Chem. Soc.* **117**: 5687–5692.

Jackson, S.E., Moracci, M., elMasry, N., Johnson, C.M., and Fersht, A.R. 1993. Effect of cavity-creating mutations in the hydrophobic core of chymotrypsin inhibitor 2. *Biochemistry* **32**: 11259–11269.

Jamieson, A.C., Miller, J.C., and Pabo, C.O. 2003. Drug discovery with engineered zinc-finger proteins. *Nat. Rev. Drug Discov.* **2**: 361–368.

Kehres, D.G., Subramanian, G.S., Hung, V.S., Rogers, G.W.J., and Setzer, D.R. 1997. Energetically unfavorable interactions among the zinc fingers of transcription factor IIIA when bound to the 5 S rRNA gene. *J. Biol. Chem.* **272**: 20152–20161.

Kellis Jr., J.T., Nyberg, K., Sali, D., and Fersht, A.R. 1988. Contribution of hydrophobic interactions to protein stability. *Nature* **333**: 784–786.

Kim, C.A. and Berg, J.M. 1993. Thermodynamic β -sheet propensities measured using a zinc-finger host peptide. *Nature* **362**: 267–270.

———. 1996. A 2.2 Å resolution crystal structure of a designed zinc finger protein bound to DNA. *Nat. Struct. Biol.* **3**: 940–945.

Klug, A. and Rhodes, D. 1987. Zinc fingers: A novel protein motif for nucleic acid recognition. *Trends Biochem. Sci.* **12**: 464–469.

Kochoyan, M., Keutmann, H.T., and Weiss, M.A. 1991. Architectural rules of the zinc-finger motif: Comparative two-dimensional NMR studies of native and “aromatic-swap” domains define a “weakly polar switch.” *Proc. Natl. Acad. Sci.* **88**: 8455–8459.

Kohsaka, T., Tagawa, M., Takekoshi, Y., Yanagisawa, H., Tadokoro, K., and Yamada, M. 1999. Exon 9 mutations in the WT1 gene, without influencing

- KTS splice isoforms, are also responsible for Frasier syndrome. *Hum. Mutat.* **14**: 466–470.
- Lachenmann, M.J., Ladbury, J.E., Phillips, N.B., Narayana, N., Qian, X., and Weiss, M.A. 2002. The hidden thermodynamics of a zinc finger. *J. Mol. Biol.* **316**: 969–989.
- Ladbury, J.E. and Chowdhry, B.Z. 1996. Sensing the heat: The application of isothermal titration calorimetry to thermodynamic studies of biomolecular interactions. *Chem. Biol.* **3**: 791–801.
- Laskowski, R.A. 1995. SURFNET: A program for visualizing molecular surfaces, cavities and intermolecular interactions. *J. Mol. Graph.* **13**: 323–330.
- Lee, B. 1991. Solvent reorganization contribution to the transfer thermodynamics of small nonpolar molecules. *Biopolymers* **31**: 993–1008.
- Lee, B. and Richards, F.M. 1971. The interpretation of protein structure: Estimation of static accessibility. *J. Mol. Biol.* **55**: 379–400.
- Lee, M.S., Gippert, G.P., Soman, K.V., Case, D.A., and Wright, P.E. 1989. Three-dimensional solution structure of a single zinc finger DNA-binding domain. *Science* **245**: 635–637.
- Lee, M.S., Mortishire-Smith, R.J., and Wright, P.E. 1992. The zinc finger motif. Conservation of chemical shifts and correlation with structure. *FEBS Lett.* **309**: 29–32.
- Liu, L. and Guo, Q.X. 2001. Isokinetic relationship, isoequilibrium relationship, and enthalpy–entropy compensation. *Chem. Rev.* **101**: 673–695.
- Lumry, R. 1995. On the interpretation of data from isothermal processes. *Methods Enzymol.* **259**: 628–720.
- Lumry, R. and Rajender, S. 1970. Enthalpy–entropy compensation phenomena in water solutions of proteins and small molecules: A ubiquitous property of water. *Biopolymers* **9**: 1125–1227.
- Matthews, B.W. 1995. Studies on protein stability with T4 lysozyme. *Adv. Protein Chem.* **46**: 249–279.
- Merrifield, B. 1986. Solid phase synthesis. *Science* **232**: 341–347.
- Morii, H., Uedaira, H., Ogata, K., Ishii, S., and Sarai, A. 1999. Shape and energetics of a cavity in c-Myb probed by natural and non-natural amino acid mutations. *J. Mol. Biol.* **292**: 909–920.
- Mortishire-Smith, R.J., Lee, M.S., Bolinger, L., and Wright, P.E. 1992. Structural determinants of Cys2His2 zinc fingers. *FEBS Lett.* **296**: 11–15.
- Nolte, R.T., Conlin, R.M., Harrison, S.C., and Brown, R.S. 1998. Differing roles for zinc fingers in DNA recognition: Structure of a six-finger transcription factor IIIA complex. *Proc. Natl. Acad. Sci.* **95**: 2938–2943.
- Page, D.C., Mosher, R., Simpson, E.M., Fisher, E.M., Mardon, G., Pollack, J., McGillivray, B., de la Chapelle, A., and Brown, L.G. 1987. The sex-determining region of the human Y chromosome encodes a finger protein. *Cell* **51**: 1091–1104.
- Pavletich, N.P. and Pabo, C.O. 1991. Zinc finger–DNA recognition: Crystal structure of a Zif268–DNA complex at 2.1 Å. *Science* **252**: 809–817.
- . 1993. Crystal structure of a five-finger GLI–DNA complex: New perspectives on zinc fingers. *Science* **261**: 1701–1707.
- Qian, H. 1998. Entropy–enthalpy compensation: Conformational fluctuation and induced-fit. *J. Chem. Phys.* **109**: 10015–10017.
- Qian, H. and Hopfield, J.J. 1996. Entropy–enthalpy compensation: Perturbation and relaxation in thermodynamic systems. *J. Chem. Phys.* **105**: 9292–9298.
- Qian, X. and Weiss, M.A. 1992. Two-dimensional NMR studies of the zinc finger motif: Solution structures and dynamics of mutant ZFY domains containing aromatic substitutions in the hydrophobic core. *Biochemistry* **31**: 7463–7476.
- Searle, M.S. and Williams, D.H. 1992. The cost of conformational order: Entropy changes in molecular associations. *J. Am. Chem. Soc.* **114**: 10690–10697.
- Searle, M.S., Westwell, M.S., and Williams, D.H. 1995. Application of a generalised enthalpy–entropy relationship to binding co-operativity and weak associations in solution. *J. Chem. Soc. Perkin Trans.* **2**: 141–151.
- Serrano, L., Bycroft, M., and Fersht, A.R. 1991. Aromatic–aromatic interactions and protein stability. Investigation by double-mutant cycles. *J. Mol. Biol.* **218**: 465–475.
- Smithrud, D.B., Wyman, T.B., and Diederich, F. 1991. Enthalpically driven cyclophane–arene inclusion complexation: Solvent-dependent calorimetric studies. *J. Am. Chem. Soc.* **113**: 5420–5426.
- Steif, C., Hinz, H.-J., and Cesareni, G. 1995. Effects of cavity-creating mutations on conformational stability and structure of the dimeric 4- α -helical protein ROP: Thermal unfolding studies. *Proteins* **23**: 83–96.
- Takano, K., Ogasahara, K., Hidetomo, K., Yamagata, Y., Fujii, S., Kanaya, E., Kikuchi, M., Oobatake, M., and Yutani, K. 1995. Contribution of hydrophobic residues to the stability of human lysozyme: Calorimetric studies and x-ray structural analysis of the five isoleucine to valine mutants. *J. Mol. Biol.* **254**: 62–76.
- Wang, B.S., Grant, R.A., and Pabo, C.O. 2001. Selected peptide extension contacts hydrophobic patch on neighboring zinc finger and mediates dimerization on DNA. *Nat. Struct. Biol.* **8**: 589–593.
- Wiseman, T., Williston, S., Brandts, J.F., and Lin, L.N. 1989. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **179**: 131–137.
- Yamada, H., Kanaya, E., Inaka, K., Ueno, Y., Ikehara, M., and Kikuchi, M. 1994. Stabilization and enhanced enzymatic activities of a mutant human lysozyme C77/95A with a cavity space by amino acid substitution. *Biol. Pharm. Bull.* **17**: 192–196.
- Yu, H.-A. and Karplus, M. 1988. A thermodynamic analysis of solvation. *J. Chem. Phys.* **89**: 2366–2379.