
FOR THE RECORD

Importance of α -helix N-capping motif in stabilization of $\beta\beta\alpha$ fold

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Abstract

FSD-1 (full sequence design 1) is a protein folded in a $\beta\beta\alpha$ motif, designed on the basis of the second zinc finger domain of Zif268 by a substitution of its metal coordination site with a hydrophobic core. In this work, we analyzed the possibility of introducing the DNA recognition motif of the template zinc finger ($S^{13}RSDH^{17}$) into FSD-1 sequence in order to obtain a small DNA-binding module devoid of cross-link(s) or metal cofactors. The hybrid protein was unfolded, as judged by CD and NMR criteria. To reveal the role of each of the five amino acids, which form the N-capping motif of the α -helix, we analyzed conformational and stability properties of eight FSD-1 mutants. We used a shielded methyl group of Leu 18 and a CD signal at 215 nm as a convenient measure of the folded state. Glu 17 \rightarrow His substitution at the N_3 in $S^{13}NEKE^{17}$ variant decreased the folded structure content from 90% to 25% (equivalent to 1.8 kcal \cdot mole $^{-1}$ destabilization) by disruption of N-capping interactions, and had the most significant effect among single mutants studied here. The N_{cap} Asn 14 substitution with Arg considerably decreased stability, reducing structure content from 90% to 40% (1.4 kcal \cdot mole $^{-1}$ destabilization) by disruption of a helix-capping hydrogen bond and destabilization of a helix macrodipole. The N_1 Glu 15 \rightarrow Ser mutation also produced a considerable effect (1.0 kcal \cdot mole $^{-1}$ destabilization), again emphasizing the significance of electrostatic interactions in α -helix stabilization.

Keywords: Zinc finger; Zif268; FSD-1; helix capping; NMR; CD

Supplemental material: See www.proteinscience.org.

Zinc fingers are independently folded small domains representing the most abundant protein domain in the human genome (Venter et al. 2001). Cys₂His₂ transcription factors belong to the classical zinc finger family. They are most often arranged tandemly, and individual domains serve to recognize neighboring 3 to 4 bp along the major groove of double-stranded DNA. In recent years, there has been major

progress in designing the classical zinc fingers to be able to recognize a desired DNA sequence (Pabo et al. 2001). Classical Cys₂His₂ zinc fingers are of particular biophysical interest, as they have served to establish stereochemical constraints of metal binding and thermodynamics of protein folding, or to design novel metal-binding sites (Berg and Godwin 1997).

The $\beta\beta\alpha$ fold of Cys₂His₂ zinc fingers is composed of ~30 amino acid residues, and it is stabilized by zinc ion. The role of zinc is pivotal for the fold stability, as in its absence, the domain is unfolded and essentially devoid of DNA-binding properties (Berg and Godwin 1997). Using an advanced computational screening of a very large rotamer library, Dahiyat and Mayo (1997) showed that it is possible to obtain a stable fold of the classical zinc finger without stabilization by a metal ion or a covalent cross-link. In the

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Abbreviations: CD, circular dichroism; FSD-1, full sequence design 1; NMR, nuclear magnetic resonance; Zif268.2, the second zinc finger domain of murine transcription factor Zif268.

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designed peptide, called FSD-1, only 6 amino acids are identical and 11 are similar, as compared with the sequence of the second zinc finger domain of murine transcription factor Zif268 (Zif268.2), which was used as a template. Among the most essential differences between Zif268.2 and FSD-1 is a difference in the region that is involved in DNA recognition by Zif268.2 (residues 13–17). This segment forms the first turn of the α -helix, and in Zif268.2, its sequence is S¹³RSDH¹⁷, whereas in FSD-1 it is R¹³NEKE¹⁷. We explored the possibility of obtaining a small DNA-binding module devoid of metal stabilization by introducing a S¹³RSDH¹⁷ sequence into the FSD-1 framework. This hybrid peptide, however, appeared to be significantly unfolded, as judged from CD and NMR spectra. To reveal the source of instability, we constructed eight mutants in the N-capping motif of the α -helix and measured their conformational properties.

Results and Discussion

We studied R¹³NEKE¹⁷ (FSD-1), S¹³RSDH¹⁷ and eight additional mutants that differed in the five-residue sequence (positions 13 to 17) involved in DNA recognition (Elrod-Erickson et al. 1998). All of the mutants (shown in Table 1) will be abbreviated by single-letter amino acid codes of this sequence, and FSD-1 (RNEKE) serves in the present study as the wild-type protein. The structure of FSD-1 has been determined by NMR (Dahiyat and Mayo 1997) and consists of a short β -hairpin motif followed by an α -helix. The stable fold of FSD-1 has been designed replacing the Cys₂His₂ zinc-binding site of Zif268.2 with a small hydrophobic core. FSD-1 and Zif268.2 show completely unrelated modes of structure stabilization. Both structures, however, superimpose well and, despite different sequences, the position of the α -helix N-capping motif is preserved.

Table 1. Structure content of FSD-1 mutants measured by CD and NMR techniques

FSD-1 variant	Percent folded by CD		Percent folded by NMR
	pH 5.5	pH 7.5	
RNEKE	100	94	100
SNEKE	90	85	83
SNEDE	73	69	75
SNSKE	59	61	51
SNSDE	53	55	49
SREKE	40	45	nd
SNEKH	25	47	nd
SRSDE	25	30	nd
SNSDH	6	19	nd
SRSDH	0	12	0

CD spectra were recorded at 4°C and pH 5.5 and pH 7.5, whereas NMR spectra were collected at 5°C and pH ~5; (nd) Not determined.

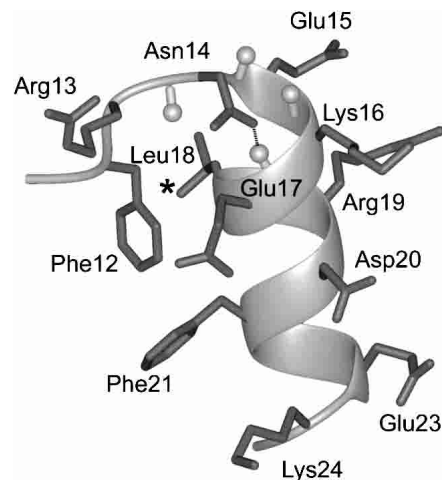


Figure 1. The N-capping motif of the representative conformation of FSD-1 (PDB code 1fsd). Amide protons of the residues in the first turn of α -helix are shown in ball-and-stick representation. Hydrogen bond between N_{cap} Asn 14 and N₃ Glu 17 is shown as a broken line. (*) Position of a shielded methyl group of Leu 18.

The amino-terminal part of the α -helix of the representative conformer of FSD-1 (the most similar to an average structure) is presented in Figure 1. The structure of the α -helix is stabilized by a hydrophobic interaction between Phe 12 and Leu 18, and it can be classified as a N'' \rightarrow N₄ capping motif (Aurora and Rose 1998). This motif is further stabilized by a hydrogen bond formed between a side chain of the N_{cap} residue, Asn 14 and the backbone amide of Glu 17. To identify the most crucial residues responsible for the decrease of SRS DH stability in relation to RNEKE, several mutations in the first turn of the α -helix were introduced.

Quantification of structure content

Thermal denaturation of RNEKE and other FSD-1 variants is weakly cooperative (Fig. 2), and the free energies of unfolding for all of the variants could not be determined reliably. However, we were able to determine the structure content of the peptides using CD and ¹H NMR techniques. Far UV CD spectra of all of the variants measured at pH 5.5 and 4°C are as shown in Figure 3. Progressive decrease of spectra intensity in the 210–230 nm range reflects the decrease of a folded state population resulting from mutations introduced in the N-capping motif of the α -helix. To quantify effects of the mutations, we used mean residue ellipticity measured at 215 nm for a given variant and compared it with the respective value for RNEKE (Table 1).

Because proton chemical shifts are very sensitive indicators of a protein's structural integrity, NMR spectroscopy is a reliable method for detection of tertiary interactions in folded proteins. Due to low stabilities of FSD-1 variants, NMR spectra were measured at 5°C. A series of ¹H NMR

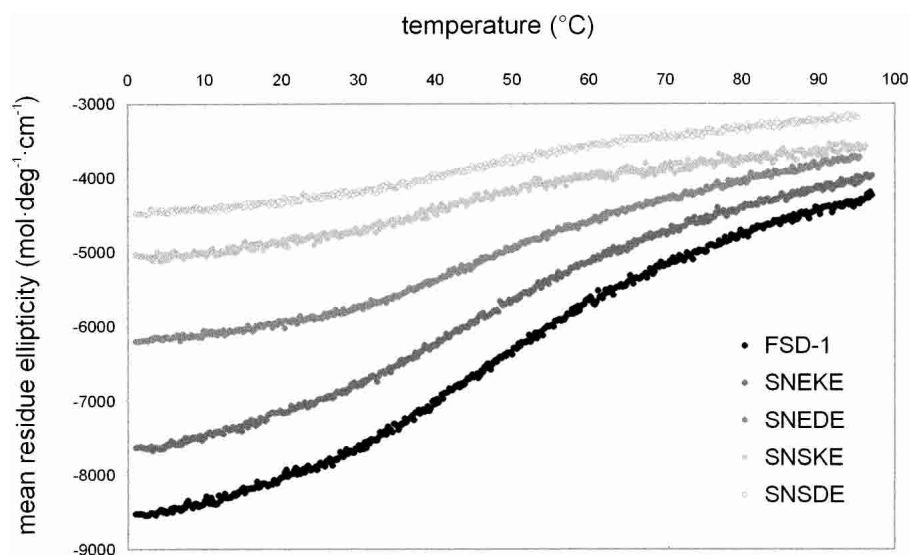


Figure 2. Thermal unfolding of FSD-1 variants monitored by CD. The temperature dependence of 215 nm ellipticity was measured at $5 \cdot 10^{-6}$ M peptide in 10 mM sodium acetate (pH 5.5), in 10-mm cuvette.

spectra of the most stable mutants is shown in Figure 4. Dispersion of signals in the aliphatic region reflects the population of the folded state. The chemical shift of the Leu 18 CH_3 group in FSD-1 is moved upfield, due to the hydrophobic interaction with Phe 12 that bridges the N-capping motif of the α -helix (Fig. 2). Thus, the degree of Leu 18 CH_3 group shielding was a convenient measure of the folded state and served to calculate structure content of the mutants relative to RNEKE (see Materials and Methods; Table 1). Comparison of H^α and H^N chemical shifts of the three most stable FSD-1 variants (RNEKE, SNEKE, and SNEDE) revealed small differences and excluded the possibility of significant structural changes upon mutations in

the N-capping motif (see Electronic Supplemental Material).

Positions of the four resolved methyl groups in ^1H NMR spectra of the most stable FSD-1 variants are shown in Figure 4. Two features reflect clearly an extent of unfolding of FSD-1 variants; the methyl group of Leu 18 tends to be less shielded, and the difference between Thr 4 and Thr 11 methyl groups decreases. Structure contents calculated from CD and ^1H NMR spectra are in good agreement (Table 1); however, chemical shifts could only be measured if the folded state was populated in more than $\sim 50\%$. ^1H NMR spectrum of SNEKH (Fig. 4) reflects a lack of Leu 18 CH_3 shielding and nearly degenerate chemical shifts of Thr 4 and

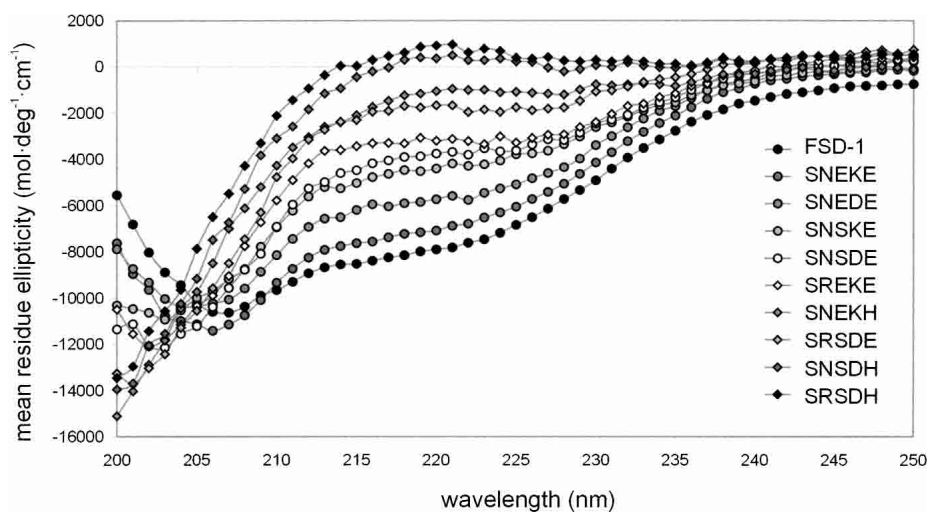


Figure 3. Circular dichroism spectra of FSD-1 variants. The spectra were measured at $5 \cdot 10^{-5}$ M peptide in 10 mM sodium acetate (pH 5.5), in a 1-mm cuvette, at 4°C.

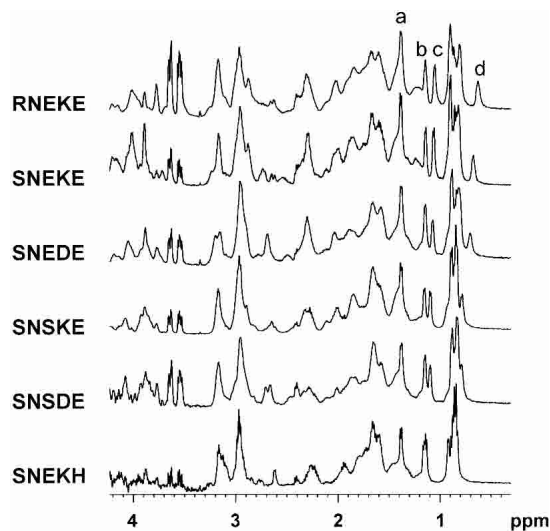


Figure 4. Aliphatic region of ^1H NMR spectra of the most stable FSD-1 variants. The four resolved methyl groups are as follows: (a) Ala 5; (b) Thr 4; (c) Thr 11; (d) Leu 18. All spectra were measured at 5°C (pH \sim 5).

Thr 11 methyl groups. This indicates that SNEKH is unfolded significantly and according to CD spectra population of structured state, is barely 25% (Table 1). This is also manifested in sharper signals of ^1H NMR spectrum of SNEKH relative to the more stable mutants (Fig. 4).

SNEKE: Arg 13 \rightarrow Ser mutation in FSD-1

Substitution of Arg 13, a residue that directly precedes the α -helix (the N' position), with Ser, leads to relatively small, 10% decrease of the FSD-1 stability (Table 1). The resulting FSD-1 variant, SNEKE, was a subject of further modifications and introduction of single substitutions into the first turn of the α -helix. Because there are charged side chains located at positions 14–17, in further studies we decided to keep Ser at position 13 to exclude a possibility of long-range electrostatic effect. Below, we rationalize effects of the substitutions of residues in the N_{cap} , N_1 , N_2 , and N_3 positions.

SREKE: The N_{cap} mutation (Asn 14 \rightarrow Arg)

The N_{cap} residue occupies one of the most important positions that stabilize the α -helix. Previous analysis showed that this site is very selective for 6 amino acid residues (Ser, Thr, Asp, Asn, Pro, Gly), whereas 11 are avoided (Val, Ile, Phe, Ala, Lys, Leu, Tyr, Arg, Glu, Met, Gln; Kumar and Bansal 1998). Small and polar residues, which can form hydrogen bond with amide protons from the first turn of α -helix, are preferred at the N_{cap} position (Doig et al. 1997; Kumar and Bansal 1998). Mutation of Asn 14 to Arg considerably decreased stability of SNEKE and comparison of

CD spectra (Fig. 3) shows that it is accompanied by a reduction of structure content from 90% to 40% (Table 1), equivalent to destabilization by $1.4 \text{ kcal} \cdot \text{mole}^{-1}$. Furthermore, analysis of ^1H NMR spectrum of SREKE failed to detect a folded structure (data not shown). Substitution of Asn 14 with Arg might cause two unfavorable effects, disruption of a helix-capping hydrogen bond, which involves a side chain carbonyl oxygen of the N_{cap} and a backbone amide of Glu 17 at the N_3 site, and destabilization of helix macrodipole by positively charged Arg.

SNSKE: The N_1 mutation (Glu 15 \rightarrow Ser)

Glutamate is one of the most favorable and commonly found residues at the N_1 position (Kumar and Bansal 1998; Cochran et al. 2001). Side chain of Glu 15, which occupies the N_1 site, is solvent exposed and carries a negative charge, which allows favorable interaction with a helix macrodipole. Furthermore, there are two positively charged residues Lys 16 and Arg 19 adjacent to Glu 15. Mutation of Glu 15 \rightarrow Ser should not introduce significant strain, as Ser is also a relatively common N_1 residue (Penel et al. 1999). We observed that this mutation resulted in a decrease of structure content from 90% to 59%, equivalent to destabilization by $1.0 \text{ kcal} \cdot \text{mole}^{-1}$ (Table 1). This is a considerable effect and emphasizes the significance of electrostatic interactions stabilizing α -helical structure.

SNEDE: The N_2 mutation (Lys 16 \rightarrow Asp)

The N_2 position of α -helix is usually solvent exposed and prefers a polar side chain (Penel et al. 1999). Because Lys is positively charged and cannot accept hydrogen bond, it has a moderate propensity for the N_2 site (Kumar and Bansal 1998; Cochran and Doig 2001). In contrast, Asp is a very common residue at this position, and it may potentially interact with main chain N_2 and N_3 amides (Kumar and Bansal 1998; Penel et al. 1999). Our measurements, however, showed that the Lys 16 \rightarrow Asp mutation led to destabilization of FSD-1 and decreased population of the folded state by 17% (destabilization of $0.7 \text{ kcal} \cdot \text{mole}^{-1}$; Table 1). Because N_2 is surrounded by three acidic residues (Glu 15, Glu 17, and particularly closely located Asp 20), the presence of Asp 16 might create destabilizing electrostatic strain. Furthermore, Lys at N_2 can form side chain to side chain interactions with N_6 , which is occupied by Asp 20 (Penel et al. 1999).

SNEKH: The N_3 mutation (Glu 17 \rightarrow His)

Glu residue has a very high propensity to occur at the N_3 position (Kumar and Bansal 1998; Penel et al. 1999). Its side chain is often found to be involved in the N-capping hydrogen bond with a backbone amide of the N_{cap} residue

(Penel et al. 1999). Glu 17→His substitution in SNEKE has the most significant effect among single mutants studied here. It leads to decrease of structure content from 90% to 25% ($1.8 \text{ kcal} \cdot \text{mole}^{-1}$ of destabilization) and it was not possible to detect any folded structure in SNEKH using ^1H NMR spectra (Fig. 4). The effect of the mutation cannot be fully explained as a consequence of the presence of a destabilizing positive charge of His at the N_3 position. The CD spectrum measured at a higher pH, which assured deprotonation of His side chain, showed that structure content is increased to only 47% (Table 1), which is equal to $1.0 \text{ kcal} \cdot \text{mole}^{-1}$ destabilization relative to SNEKE. We expect that another effect takes place upon Glu 17→His mutation. Solution structure of FSD-1 shows that in 2 of 41 conformers, the side chain of relatively disordered Glu 17 forms a hydrogen bond with the amide proton of Asn 14. Weak temperature gradient of Asn 14 amide proton strongly suggests that it is hydrogen bonded (see below). The arrangement of two hydrogen bonds involving a side chain of N_{cap} and a backbone of N_3 and, reciprocally, a side chain of N_3 and a backbone of N_{cap} supported by hydrophobic interaction between N'' and N_4 was found to be present in other proteins and is called a “big box” (Seale et al. 1994). Loss of this highly probable hydrogen bond upon Glu 17→His substitution may result in significant destabilization of the protein. Interestingly, the effect of this mutation is more destructive than loss of the N-capping hydrogen bond upon Asn 14 substitution with Arg.

Increasing the stability of SRSDH

Analysis of both CD and NMR spectra showed that SRSDH is almost completely unfolded. Chemical shifts of this variant are very close to random-coil values (data not shown). As shown above, two mutations: Asn 14→Arg (at N_{cap}) and Glu 17→His (at N_3), have the strongest effect, decreasing the population of the folded state of FSD-1. To verify this, we introduced reciprocal substitutions, Arg 14→Asn and His 17→Glu, into SRSDH variant. The single mutation of the N_{cap} residue, Arg 14→Asn, produced a small, 6% increase of the folded state population of SRSDH relative to SRSDH. A slightly larger effect was observed upon single substitution of the N_3 residue and structure content of His 17→Glu variant, SRSDE, increased to 25%. This mutation further emphasizes an important role of the N_3 position in α -helix stabilization, as it corresponds to both significant destabilization of SNEKE and stabilization of SRSDH. Simultaneous introduction of two mutations, Arg 14→Asn and His 17→Glu, provided a SRSDE variant that is 53% folded, and this could be detected by ^1H NMR analysis (Fig. 4). Our data show that positions of N_{cap} and N_3 are essential for stabilization of the α -helix, and lack of favorable interactions in the N-capping motif leads to a fully unfolded variant of FSD-1.

Effect of pH on FSD-1 stability

Analysis of CD spectra of FSD-1 variants revealed that, in most cases, the difference in the folded state population between pH 5.5 and pH 7.5 did not exceed 5%. However, the peptides containing His residue at the N_3 position showed meaningful stabilization of the structure upon increase of pH (Table 1). Deprotonation of His 17 at a higher pH leads to elimination of a positive charge located in the amino-terminal fragment of the α -helix. The largest effect was observed for SNEKH, and an increase in pH raised structure content by 22% ($0.8 \text{ kcal} \cdot \text{mole}^{-1}$ stabilization). The effect observed emphasizes that electrostatic interactions with helix macrodipole cannot be neglected in designing α -helices.

Amide proton temperature coefficients of SNEKE

Our previous study showed that temperature coefficients could be used to judge hydrogen bonds formed by amide protons in proteins (Cierpicki and Otlewski 2001; Cierpicki et al. 2002). Values of $\Delta\sigma_{\text{HN}}/\Delta T$ more positive than -4.6 ppb/K indicate the presence of a hydrogen bond with high probability. It was also shown that, unlike proteins, short protein fragments and peptides exhibit significant correlation between temperature coefficients of amide protons ($\Delta\sigma_{\text{HN}}/\Delta T$) and their conformational shifts ($\Delta\sigma_{\text{HN}}$; Andersen et al. 1997). In such cases, values of temperature gradients are affected by a decrease in the population of the structured state upon warming.

Analysis of the thermal denaturation curve of SNEKE (Fig. 2) shows lack of a cooperative unfolding process typical for globular proteins. Thus, an increase in temperature should result in a decrease of a population of a folded state, affecting chemical shifts of amide protons. In turn, the correlation between $\Delta\sigma_{\text{HN}}/\Delta T$ and $\Delta\sigma_{\text{HN}}$ should be expected.

The relationship between $\Delta\sigma_{\text{HN}}/\Delta T$ and $\Delta\sigma_{\text{HN}}$ for SNEKE is shown in Figure 5. The linear dependence (correlation coefficient $R = 0.90$) is observed upon excluding four amides, which belong to residues 13–15 and carboxy-terminal Arg 28. The three consecutive residues, Arg 13, Asn 14, and Glu 15 are located within the initial fragment of the α -helix. The linear correlation is in agreement with the poor unfolding cooperativity of SNEKE. However, lack of the correlation observed for the three amides preceding the α -helix suggests that this short fragment may unfold in a noncooperative manner relative to the $\beta\beta\alpha$ fold of SNEKE. This compares well with several other cases, in which much better correlation was observed for secondary structure elements than for a full-length peptide (Andersen et al. 1997).

We observe reasonable correlation between values of temperature coefficients and the presence of hydrogen bonds in SNEKE (Fig. 5). The most pronounced exception is the amide proton of Asn 14, which has the weakest tem-

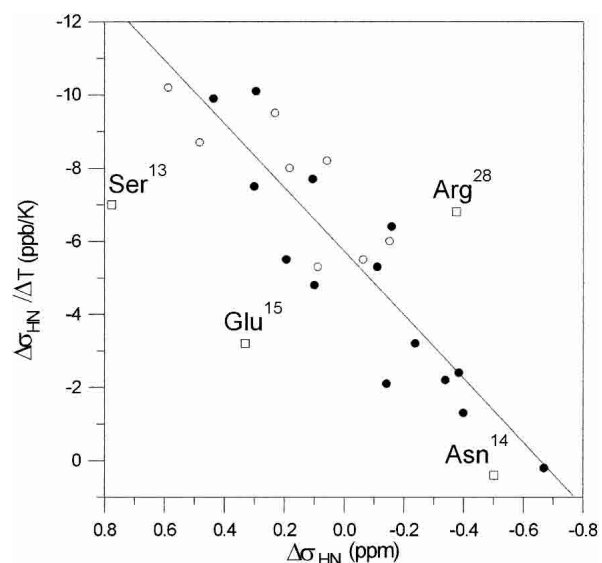


Figure 5. Correlation between amide proton temperature coefficients ($\Delta\sigma_{\text{HN}}/\Delta T$) and conformational shifts of amide protons ($\Delta\sigma_{\text{HN}}$) of SNEKE ($R = 0.90$). (Filled circles) Amides forming hydrogen bonds; (open circles) amides not involved in hydrogen bonds; (squares) four amides excluded from correlation.

perature coefficient ($\Delta\sigma_{\text{HN}}/\Delta T = 0.2$ ppb/K), but according to the NMR structure, it fails to form a hydrogen bond. Our observation regarding the effect of Glu 17→His substitution led us to conclude that this amide might be involved in a hydrogen bond with the side chain of Glu 17. This example emphasizes that the values of $\Delta\sigma_{\text{HN}}/\Delta T$ may also be useful to predict hydrogen bonds in proteins of relative low stability.

In this work, we carried out mutational analysis of an amino-terminal fragment of α -helix and its impact on structure content of FSD-1. Recent study of a coiled-coil dimerization domain within the human kinesin neck region also emphasized the significance of the N-capping motif in overall structure stability (Tripet and Hodges 2002). Our study revealed that the N-capping motif of Zif286.2 is far from being optimal. Contrary, the presence of a “big box” motif in FSD-1 provides an optimal network of interactions such as hydrogen bonds and electrostatics. The most crucial residues, N_{cap} and N_3 , are involved in hydrogen bonds, and their substitution has the strongest effect. The two other positions, N_1 and N_2 , are solvent exposed and should be optimized to enable favorable interactions with both helix macrodipole and neighboring residues. The analysis presented here shows that the optimal design of the α -helix N-capping motif is crucial for obtaining structured proteins, and even trivial mutation may cause a significant effect.

Materials and methods

Bacterial strain *Escherichia coli* BL21(DE3)pLysS was purchased from Stratagene. pAED4 expression vector was a kind gift of

Professor P.S. Kim (MIT, Cambridge, Massachusetts, USA). Restriction enzymes were supplied by New England Biolabs. Other DNA-modifying enzymes and the Sequenase v.2.0 DNA sequencing kit were from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by MWG-Biotech. Acetonitrile was supplied by Roth; TFA and cyanogen bromide were obtained from Fluka. Other chemicals used were from Sigma.

Peptide expression and purification

The coding sequence of the FSD-1 variant, SRSDH, was annealed from two pairs of partially overlapping oligonucleotides and converted to fully double stranded using Sequenase v. 2.0. The resulting cassettes were digested with *Hind*III + *Bcl*I and *Bcl*I + *Bam*HI and ligated into *Hind*III + *Bam*HI-digested pAED4 expression vector. FSD-1 and its other variants were obtained through site-directed mutagenesis using synthetic oligonucleotides and a single-stranded template of SRSDH containing vector, and confirmed by DNA sequencing.

The peptides were expressed in *E. coli* BL21(DE3)pLysS from pAED4 vector as fusion with a hydrophobic leader sequence. Fusion proteins were purified from inclusion bodies, cleaved with cyanogen bromide, and the free peptides were double purified with high-performance liquid chromatography on C_{18} column (Vydac) with acetonitrile gradient in 0.1% TFA. The peptide identity was verified using MALDI mass spectrometry.

CD measurements

CD spectra of the peptides were recorded in the 190–250-nm wavelength range in 10 mM sodium acetate (pH 5.5), or 10 mM sodium phosphate (pH 7.5) at 4°C on a Jasco J-715 spectropolarimeter. Measurements were performed at a protein concentration of $5 \cdot 10^{-5}$ M using a 1-mm cuvette. Spectra were averaged from 10 separate scans. Thermal stability of the peptides was measured in 10 mM sodium acetate (pH 5.5) at a protein concentration of $5 \cdot 10^{-6}$ M using a 10-mm cuvette. Thermal denaturation was monitored following the ellipticity at 215 nm, using a band slit of 2 nm and a 16-sec response time. Automatic Peltier accessory, PFD 350S, allowed continuous monitoring of the thermal transition at a constant rate of $45\text{deg} \cdot \text{hour}^{-1}$. Temperature of the sample was monitored directly using a probe immersed in the cuvette and controlled with PFD-350S/350L Peltier type FDCD attachment.

Structure content for a given FSD-1 variant was calculated from its CD spectrum, according to the following formula:

$$\% \text{folded}_{\text{CD}} = \frac{\theta_{\text{obs}}}{\theta_{\text{RNEKE}}} * 100\%,$$

in which θ_{obs} and θ_{RNEKE} are the mean residue ellipticity at 215 nm for a given variant and RNEKE, respectively.

¹H NMR spectroscopy

All protein samples were dissolved in 50 mM phosphate buffer and 90% H_2O , 10% D_2O mixture. Final concentrations of FSD-1 variants were ~1 mM and the pH ranged between 5.2 and 5.4. NMR spectra were acquired on a Bruker DRX500 spectrometer. One-dimensional ¹H spectra were measured at various temperatures ranging from 5 to 30°C and were referenced to DSS signal. Chemical-shift assignment of RNEKE, SNEKE, SNEDE, and SRSDH was carried out from the combination of two-dimensional TOCSY (Braunschweiler and Ernst 1983) and NOESY (Kumar et al.

1980) experiments. All NMR data were processed using Bruker Xwinnmr software and analysis of two-dimensional spectra was performed using the Sparky program (Goddard and Kneller 2002).

Structure content relative to FSD-1 (RNEKE) was calculated from chemical shifts of the upfield shifted methyl group of Leu 18 ($\sigma_{\text{RNEKE}} = 0.673$ ppm). Chemical shift of Leu 18 CH_3 of the least stable variant, SRSDH, was determined from two-dimensional TOCSY ($\sigma_{\text{SRSDH}} = 0.86$ ppm). Structure content was calculated according to the following formula:

$$\% \text{folded}_{\text{NMR}} = \frac{\sigma_{\text{obs}} - \sigma_{\text{SRSDH}}}{\sigma_{\text{RNEKE}} - \sigma_{\text{SRSDH}}} * 100\%$$

in which σ_{obs} is the observed Leu 18 CH_3 group chemical shift.

Electronic supplemental material

Comparison of conformational shifts of amide protons and H^α protons of FSD-1, SNEKE, and SNEDE is available.

Acknowledgments

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