
Equilibrium denaturation studies of the *Escherichia coli* factor for inversion stimulation: Implications for in vivo function

SARAH A. HOBART,¹ SERGEY ILIN,^{1,3} DANIEL F. MORIARTY,¹ ROBERT OSUNA,²
AND WILFREDO COLÓN¹

¹Rensselaer Polytechnic Institute, Department of Chemistry, Troy, New York 12180, USA

²Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222, USA

(RECEIVED December 18, 2001; FINAL REVISION April 4, 2002; ACCEPTED April 10, 2002)

Abstract

The Factor for Inversion Stimulation (FIS) is a dimeric DNA binding protein found in enteric bacteria that is involved in various cellular processes, including stimulation of certain specialized DNA recombination events and transcription regulation of a large number of genes. The intracellular FIS concentration, when cells are grown in rich media, varies dramatically during the early logarithmic growth phase. Its broad range of concentrations could potentially affect the nature of its quaternary structure, which in turn, could affect its ability to function in vivo. Thus, we examined the stability of FIS homodimers under a wide range of concentrations relevant to in vivo expression levels. Its urea-induced equilibrium denaturation was monitored by far- and near-UV circular dichroism (CD), tyrosine fluorescence, and tyrosine fluorescence anisotropy. The denaturation transitions obtained were concentration-dependent and showed similar midpoints (C_m) and m values, suggesting a two-state denaturation process involving the native dimer and unfolded monomers ($N_2 \leftrightarrow 2U$). The ΔG_{H_2O} for the unfolding of FIS determined from global and individual curve fitting was 14.2 kcal/mole. At concentrations $<9 \mu\text{M}$, the FIS dimer began to dissociate, as noted by the change in CD signal and size-exclusion high-pressure liquid chromatography retention times and peak width. The estimated dimer dissociation constant based on the CD and size-exclusion chromatography data is in the micromolar range, resulting in a ΔG_{H_2O} of at least 5 kcal/mole less than that calculated from the urea denaturation data. This discrepancy suggests a deviation from a two-state denaturation model, perhaps due to a marginally stable monomeric intermediate. These observations have implications for the stability and function of FIS in vivo.

Keywords: Factor for inversion stimulation, FIS; equilibrium denaturation; dimer stability; transcription regulation; dissociation constant; DNA binding

Reprint requests to: Wilfredo Colón, Rensselaer Polytechnic Institute, Department of Chemistry, 110 8th Street, Troy, NY 12180, USA; e-mail: colonw@rpi.edu; fax: (518) 276-4887.

³Present address: Institute for Organic Chemistry, Center for Biological Magnetic Resonance, Johann Wolfgang Goethe-University, Marie-Curie-Strasse 11, N120-5 011 49 69 7982 9573, D-60439 Frankfurt am Main, Germany.

Abbreviations: CD, circular dichroism; far-UV, far ultraviolet; FIS, factor for inversion stimulation; HPLC, high-pressure liquid chromatography; K_d , dissociation constant; MW, molecular weight; near-UV, near ultraviolet; PB, potassium phosphate buffer; PCR, polymerase chain reaction; Tris-HCl, Tris hydroxymethyl aminomethane hydrochloride; WT, wild type.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.5050102>.

Regulation of protein function can generally be achieved by induced conformational changes resulting from chemical modifications or association with cofactors, or by efficient control of their intracellular levels (Laney and Hochstrasser 1999; Cohen 2000). Varying rates of protein synthesis and degradation can significantly affect the intracellular concentration, which in turn, can profoundly influence the ability of the protein to function in vivo. Rapid changes in intracellular concentrations are often critical for a suitable response to an environmental signal. In the case of DNA binding proteins, modulating their rates of synthesis and

degradation is of particular importance for the cell's ability to adapt to ongoing environmental changes (Ciechanover 1998; Ramos et al. 2001).

The Factor for Inversion Stimulation (FIS) is a dimeric DNA-binding and -bending protein found in *Escherichia coli* (*E. coli*) and other enteric bacteria (Thompson et al. 1987; Finkel and Johnson 1992; Osuna et al. 1995; Beach and Osuna 1998). It is involved in a number of cellular processes, including stimulation of site-specific DNA inversion reactions catalyzed by the Hin-family of recombinases (Johnson et al. 1986; Koch and Kahmann 1986; Haffter and Bickle 1987), stimulation of lambda and HKO22 phage DNA recombination (Thompson et al. 1987; Ball and Johnson 1991; Dorgai et al. 1993), stimulation of transcription of ribosomal and tRNA operons and other genes (Bosch et al. 1990; Ross et al. 1990; Xu and Johnson 1995a; Gonzalez-Gil et al. 1996), repression of its own transcription and that of several other genes (Ball et al. 1992; Xu and Johnson 1995b), and modulation of DNA topology (Schneider et al. 1997). FIS is composed of two identical 98-residue subunits, and several crystal structures have been solved, all revealing an intertwined dimer of predominantly α -helical structure (Fig. 1) (Kostrewa et al. 1991; Yuan et al. 1991; Safo et al. 1997). The N-terminus of FIS consists of a β -hairpin and helix A, and is required for the stimulation of Hin-mediated DNA inversion (Koch et al. 1991; Osuna et al. 1991; Safo et al. 1997). Helix B runs through the center of the protein, and is highly involved in monomer-monomer interactions, as it makes extensive contacts with helices A and B of the other subunit in the dimer. The C-terminal region contains the helix-turn-helix DNA binding motif, and is required for DNA-binding and -bending functions. It consists of helices C and D, which are also involved in

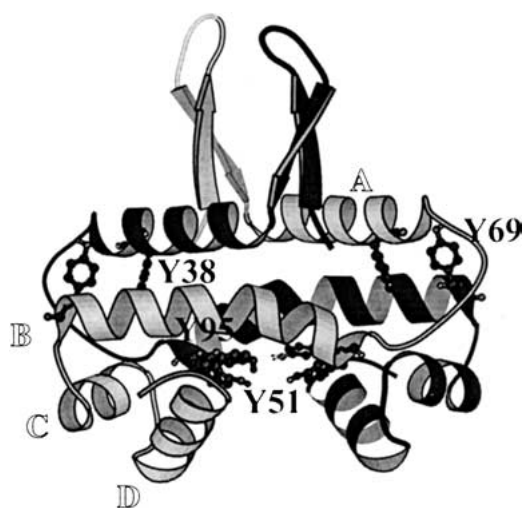


Fig. 1. A ribbon diagram of the 3D structure of FIS (Safo et al. 1997) showing the tyrosine residues. The four α -helices are labeled A through D. The program MOLSCRIPT (Kraulis 1991) was used to draw the figure.

intramolecular interactions with helix B of the same subunit.

The function of FIS is controlled by dramatic changes in its intracellular concentration in response to a nutritional up-shift (Thompson et al. 1987; Ball et al. 1992; Nilsson et al. 1992). Although FIS is not detected in cells during stationary phase, upon initiation of growth in rich medium, FIS levels rapidly accumulate from less than 500 to over 50,000 molecules per cell within 75 min of growth (early logarithmic growth phase) (Ball et al. 1992). Thereafter, FIS levels rapidly decrease, and become nearly undetectable by late logarithmic growth phase. The *fis* mRNA levels obey a similar expression pattern, indicating that much of the regulation occurs at the transcriptional level (Ball et al. 1992; Ninnemann et al. 1992; Pratt et al. 1997). However, the rapid removal of FIS during mid-logarithmic cell growth also suggests the existence of an efficient degradation process.

The large changes in FIS concentration can potentially affect its dimer stability and, consequently, its function and susceptibility to proteolytic enzymes. Thus, it was of interest to closely examine the stability of this protein at physiologically relevant concentrations. In this study, we used various methods to investigate the FIS dimer dissociation constant and its equilibrium denaturation at various concentrations. The results have implications for the regulation of the functions of FIS, and for the regulation and degradation of other transcription factors and repressors.

Results

Equilibrium denaturation experiments

The denaturation of FIS was monitored using various techniques to observe the loss of secondary, tertiary, and quaternary structure. The reversible (see inset in Fig. 2A) loss of helical structure with increasing urea concentrations was monitored by far-ultraviolet (far-UV) circular dichroism (CD) over a wide range of FIS concentrations (Fig. 2A) to determine if the loss of secondary structure is dependent on the dissociation of the dimer. The data shown in Figure 2A indicates that the unfolding of FIS, as shown by the loss of helical signal, is a concerted and concentration-dependent event. To probe for the presence of a dimeric intermediate with native-like secondary structure or a largely unfolded monomeric intermediate, we also monitored FIS denaturation using near-ultraviolet (near-UV) CD, tyrosine fluorescence, and fluorescence anisotropy.

The near-UV CD spectrum of a protein provides a "fingerprint" of the tertiary structure, as the specific environment of aromatic residues is unique for every protein (Mulkerin 1996). Because FIS has no tryptophan residues, the near-UV CD spectrum (see inset in Fig. 2B) arises from the different contributions of the four tyrosines located (Fig.

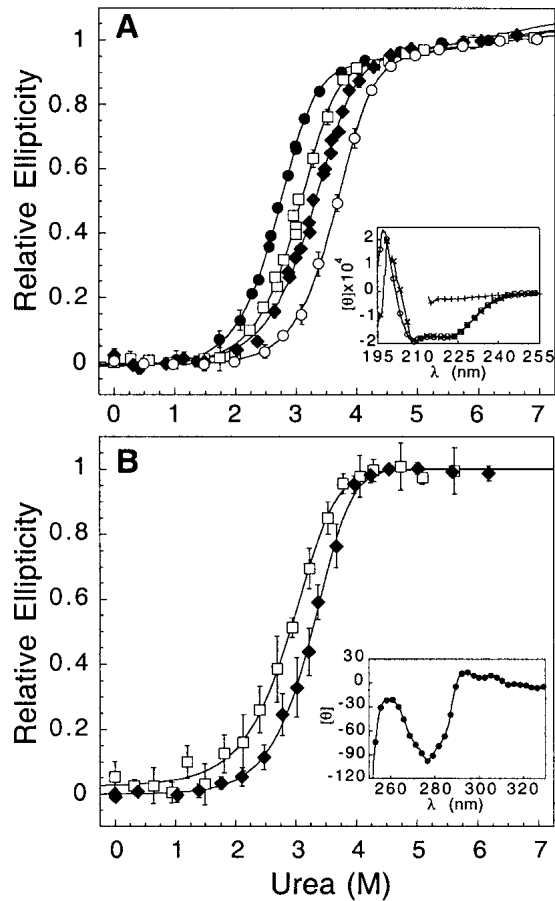


Fig. 2. Urea-induced denaturation of FIS monitored by (A) far-UV CD at 222 nm, and (B) near-UV CD at 277 nm. FIS concentrations were 107 μM (\circ), 35.6 μM (\blacklozenge), 8.9 μM (\square), or 1.8 μM (\bullet). The insets in (A) and (B) show the full wavelength scans in the far- and near-UV regions, respectively. The molar ellipticity, $[\theta]$, has units of degree-cm²-dmol⁻¹. The symbols in inset (A) represent the full spectrum of native (\circ), unfolded in 6 M urea ($+$), and refolded (\times) FIS. Error bars represent the average of at least three individual data sets. The solid lines on the denaturation curve indicate the fit for each data set as described in Materials and Methods.

1) at positions 38 (helix A), 51 (beginning of helix B), 69 (end of helix B), and 95 (helix D). The equilibrium unfolding studies monitored by near-UV CD (277 nm) at 36 and 107 μM FIS, also demonstrate a two-state, concentration-dependent unfolding transition (Fig. 2B). Because the error in the near-UV CD is larger due to the low signal, it was not practical to perform denaturation experiments at lower FIS concentrations. Although the relative contribution of the individual tyrosine residues to the native near-UV CD spectrum is not clear, preliminary results with a Y51F FIS mutant indicate that about 70% of the signal is due to the tyrosine at position 51 (unpublished data). Because this residue is located at the dimer interface, it appears that the loss of the near-UV CD signal arises predominantly from the loss of the quaternary structure.

The equilibrium unfolding mechanism of FIS was further explored by monitoring the changes in steady-state tyrosine fluorescence intensity and anisotropy (Fig. 3). The denaturation of FIS monitored by both methods displays concentration-dependent transitions, as did the CD data. The increase in fluorescence signal between native FIS and FIS unfolded in 5 M urea is ~ 1.25 fold (data not shown). This low signal prevented the collection of reliable data at FIS concentrations below 36 μM . Even at higher FIS concentrations, the steep baselines defining the dependence of the native and unfolded state signals on urea (Fig. 3A), made the analysis of this data less reliable. This baseline effect seen in relative fluorescence intensity was not an issue in steady-state anisotropy experiments (Fig. 3B). It is expected that the anisotropy will decrease as FIS dissociation occurs, largely, but not exclusively, due to a decrease in correlation time. Although changes in anisotropy cannot be directly correlated with fractional dissociation/unfolding be-

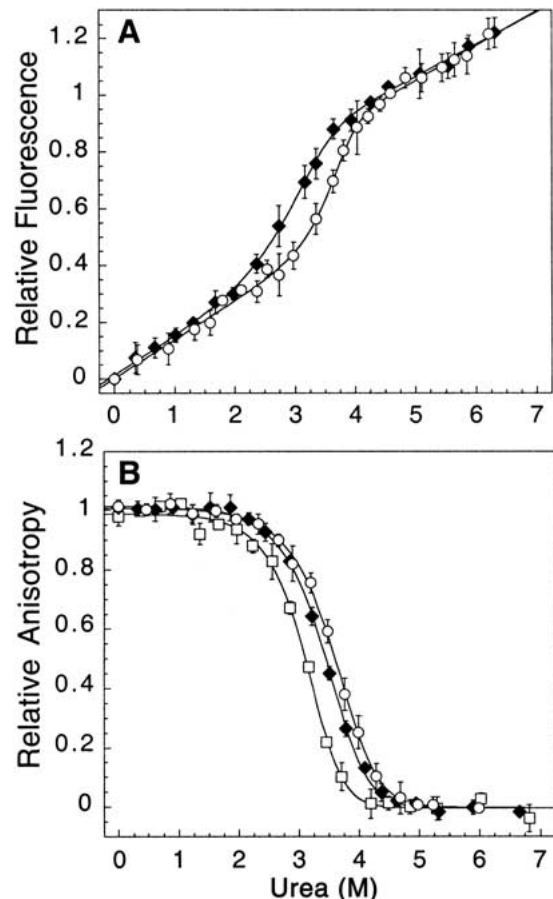


Fig. 3. Urea-induced denaturation of FIS monitored by (A) fluorescence and (B) steady-state anisotropy at 107 μM (\circ), 35.6 μM (\blacklozenge), or 8.9 μM (\square) FIS. The excitation wavelength was 276 nm, and the emission wavelength was 305 nm. Error bars represent the average of at least three individual data sets. The solid lines indicate the fit for each data set as described in Materials and Methods.

cause of the changes in fluorophore quantum yield upon unfolding (Eftink 1994), this effect is minimized here due to the similar fluorescence intensities of the native and unfolded FIS states. Anisotropy changes can also be caused by changes in the lifetime of the excited state or through increased mobility of the tyrosine side chains upon unfolding (Eftink 1994). However, these changes should also be minimal because the four tyrosines are all partially solvent exposed, and the most restricted tyrosines (51 and 95) are held within the dimer interface (Fig. 1); therefore, their rotational freedom should parallel dissociation. Overall, it can be inferred that the majority of the decrease in anisotropy arises from changes in quaternary structure upon addition of urea. Through this method, the dissociation/unfolding of the FIS dimers appear to proceed through a concerted concentration-dependent process (Fig. 3B).

By plotting together the apparent fraction of unfolded FIS (determined using equation 4) against urea concentration obtained by all methods thus far discussed, it is evident that at each FIS concentration used, the denaturation mechanism is consistent with a two-state process, whereby quaternary, tertiary, and secondary structure are lost in parallel (Fig. 4). Although the fluorescence data appears to deviate from the other data presented in Figure 4B and C, all of the data is within error. This data is best fit to a two-state denaturation pathway, as described in Materials and Methods. The parameters obtained from the global fitting and from the individual fit of each transition are in excellent agreement, yielding a $\Delta G_{\text{H}_2\text{O}}$ of around 14 kcal/mole (Table 1). The larger errors of the near-UV CD and fluorescence data, due to the low signal and the steep baseline slopes, respectively, are reflected in the thermodynamic parameters.

Estimating the FIS dimer dissociation constant

The ability to measure dimer and monomer populations using serially diluted FIS samples should allow for the calculation of the dimer dissociation constant (K_d). Although this is ideally accomplished using a method that directly monitors quaternary structure, CD can be used to measure a protein's K_d if the protein unfolds via a two-state mechanism (Azuma and Hamaguchi 1976; Franchini and Reid 1999; Steinmetzer et al. 2000). In this case, the disappearance of the CD signal should occur in parallel with subunit dissociation. In addition to far-UV CD, we used size-exclusion high-pressure liquid chromatography (HPLC) to monitor FIS dimer dissociation. We were not able to use fluorescence anisotropy due to artificial increases in fluorescence polarization. This is a common problem observed when using dilute samples, and is presumably caused by light scattering from solvent or optics, which becomes significant compared to the very weak fluorescence of the sample (Lakowicz 1999).

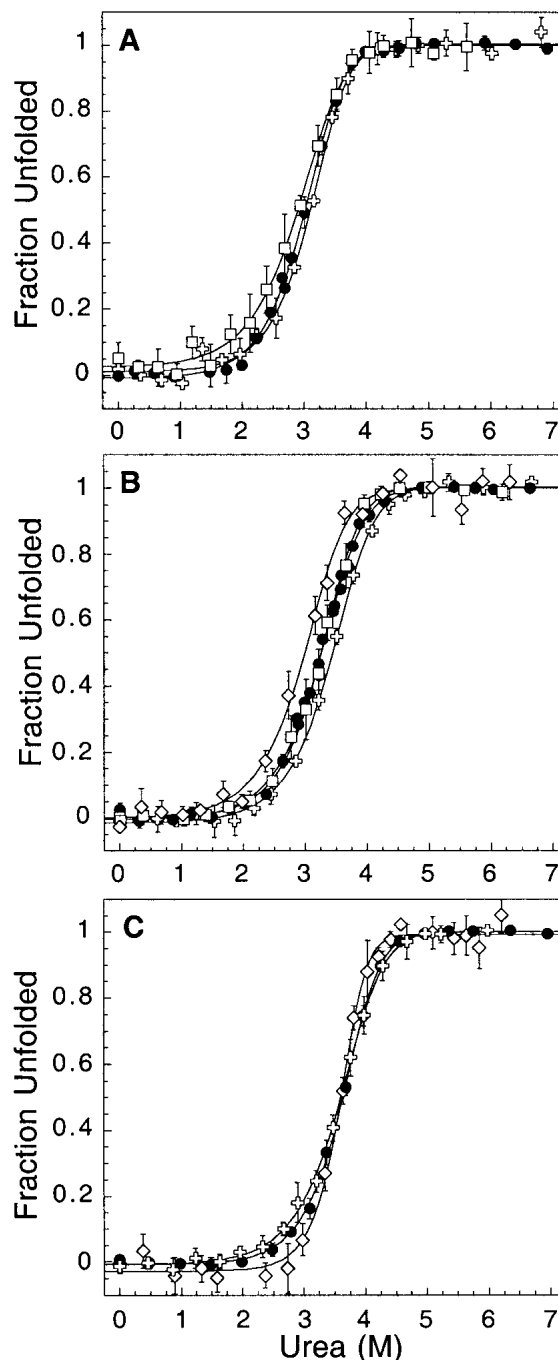


Fig. 4. An overlay of the urea denaturation curves of FIS determined by far-UV CD (●), near-UV CD (□), fluorescence (◇), and anisotropy (+) at FIS concentrations of 8.9 μM (A), 35.6 μM (B), or 107 μM (C). The data was normalized to apparent fraction unfolded using equation 4.

Results from the CD and HPLC experiments show that changes in signal begin to occur below 9 μM FIS (Fig. 5). When FIS is run on the HPLC at the highest protein concentrations ($\sim 26 \mu\text{M}$), it runs at the expected molecular weight of the dimer, determined according to a calibration curve based on molecular weight standards. Upon dilution

Table 1. Equilibrium denaturation thermodynamic parameters

Technique	[Pt] (μM)	C_m (M Urea)	m-value [kcal/(mol M)]	$\Delta G_{\text{H}_2\text{O}}$ (kcal/mol)
Far UV-CD	1.8	2.7 ± 0.05	2.6 ± 0.27	14.8 ± 1.6
	8.9	3.0 ± 0.05	2.6 ± 0.30	14.7 ± 1.7
	35.6	3.3 ± 0.05	2.4 ± 0.21	14.0 ± 1.2
	107.0	3.6 ± 0.07	2.6 ± 0.17	14.8 ± 1.0
Near UV-CD	8.9	2.9 ± 0.13	2.2 ± 0.65	13.3 ± 4.0
	35.6	3.3 ± 0.12	2.5 ± 0.48	14.3 ± 2.8
Anisotropy	8.9	3.1 ± 0.07	2.7 ± 0.49	15.3 ± 2.8
	35.6	3.4 ± 0.05	2.4 ± 0.33	14.2 ± 2.0
Fluorescence	107.0	3.6 ± 0.05	2.3 ± 0.27	13.8 ± 1.6
	35.6	3.0 ± 0.34	1.8 ± 0.94	11.5 ± 6.2
Global analysis	107.0	3.6 ± 0.17	3.5 ± 2.0	18.0 ± 10
			2.4 ± 0.06	14.2 ± 0.2

The $\Delta G_{\text{H}_2\text{O}}$ is defined by: $m \cdot C_m - [RT \ln[\text{Pt}]]$. Urea denaturation profiles were analyzed according to the linear free-energy model. Errors are based on averaged data from at least three individual experiments. Global analysis was performed with SAVUKA version 5.1., a nonlinear least-squares fitting program (Zitzewitz et al. 1995; Bilsel et al. 1999).

of FIS, the HPLC data (Fig. 5B) clearly show an increase in both the retention time and the peak width at half height. However, this data (Fig. 5B, inset) could not be analyzed to obtain an estimation of the K_d because the ending retention time for monomeric FIS in the absence of urea was unknown. In contrast, the CD data could be fit to estimate the K_d of FIS using results from urea denaturation experiments, which indicated that unfolded FIS elicits $\approx 11\%$ of the native CD signal at 222 nm. It is important to emphasize that for this K_d analysis we assumed that FIS is dissociating to an unfolded monomer (two-state assumption), as suggested by the urea denaturation experiments. If our assumption is correct, the resulting K_d would be a good approximation of the real value, and the corresponding ΔG of dissociation would match the ΔG obtained from the urea denaturation studies. Under this assumption, we fit the CD data to the same two-state denaturation mechanism used to fit the urea denaturation data (see Materials and Methods) and calculated the K_d of FIS to be about $0.4 \mu\text{M}$, which yields a $\Delta G_{\text{H}_2\text{O}}$ of ≈ 9 kcal/mole for dissociation/unfolding. This is approximately 5 kcal/mole lower than the $\Delta G_{\text{H}_2\text{O}}$ calculated by urea denaturation. Although this is only an estimated K_d , the log relationship between K_d and ΔG means that relatively rough approximations in K_d will give relatively accurate ΔG s. These results imply that in the absence of urea FIS does not dissociate into the globally unfolded monomer as one would expect for a two-state system, but dissociates into a compact monomer. Thus, the two-state assumption that we made in calculating the K_d of FIS is not justified, and the actual K_d of FIS should be larger than the value calculated. The HPLC data (Fig. 5B) supports the CD results, suggesting a FIS K_d higher than $0.4 \mu\text{M}$, and the population of a monomeric intermediate upon dilution.

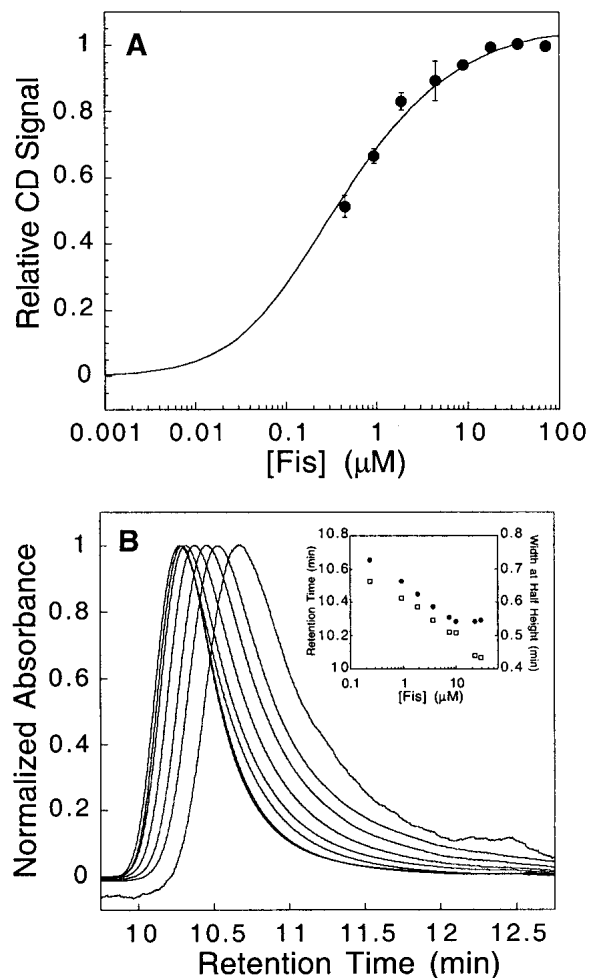


Fig. 5. The change in signal upon FIS dilution as monitored by (A) CD and (B) size-exclusion HPLC. The solid line in (A) indicates the fit of the data as described in Materials and Methods. The chromatographic traces (B) show a representative elution profile at 29, 22, 9.9, 7.4, 3.7, 1.9, 0.93, and $0.02 \mu\text{M}$ FIS (from left to right). The absorbance at 222 nm was normalized to the maximum peak height for comparison of data at different protein concentrations. The inset in (B) shows a corresponding plot of the change in retention time (\bullet) and width at half height (\square) versus FIS concentration.

Discussion

FIS denaturation pathway

In our equilibrium denaturation experiments involving the small dimeric protein FIS, we observe superimposable denaturation curves from different methods, suggesting a two-state unfolding process. Although some deviation is seen with the less reliable fluorescence data (Fig. 4B,C), resulting in larger errors in the calculated ΔG , the consistency of all the denaturation data across a broad concentration range (9–107 μM) strongly supports the two-state model. Individual and global data fitting yields a $\Delta G_{\text{H}_2\text{O}}$ of 14.2 kcal/

mole, which is consistent with the stability of other dimeric proteins of similar size (Neet and Timm 1994). Most equilibrium denaturation studies of dimeric proteins have shown that their denaturation mechanism is described by either a concerted two-state process ($N_2 \leftrightarrow 2U$) involving a native dimer (N_2) and an unfolded monomer ($2U$), or a three-state process, involving a dimeric ($N_2 \leftrightarrow I_2 \leftrightarrow 2U$) or monomeric ($N_2 \leftrightarrow 2I \leftrightarrow 2U$) intermediate (Neet and Timm 1994). Small dimeric proteins usually exhibit a concerted two-state equilibrium unfolding mechanism (Neet and Timm 1994; Xu et al. 1998) similar to that of many small monomeric proteins. Thus, the observed two-state denaturation of FIS is not surprising, considering both its small size and intertwined structure (Fig. 1). Moreover, 29% of the total surface area calculated for the individual FIS monomers is buried in the dimer interface, whereas proteins known to exhibit a three-state denaturation mechanism usually only bury about 10% of their total surface area (Xu et al. 1998). Thus, it appears that most of the stability of FIS arises from the monomer–monomer interactions rather than the folded monomers.

Is there a monomeric intermediate at low FIS concentration?

The disagreement between the ΔG_{H_2O} calculated from urea denaturation (14.2 kcal/mole) and dilution (≈ 9 kcal/mole) experiments suggest that, at very low concentrations, FIS denaturation deviates from a two-state process. If dissociation and unfolding were concerted processes, at low FIS concentrations the ΔG_{H_2O} calculated from urea denaturation experiments would match that obtained from the estimated K_d (Neet and Timm 1994). The observed deviation could be explained by the existence of an unstable monomeric intermediate that becomes populated when FIS is highly diluted in the absence of urea. Both the increase of HPLC retention times and the band broadening seen upon successive dilution of a FIS sample (inset, Fig. 5B) are consistent with the presence of a monomeric species in rapid equilibrium with the dimer (Woodbury et al. 2002). At the lower protein concentrations, the monomer becomes more populated, which leads to a shift in retention time and a trailing in the peak. A globally unfolded monomer would be expected to have a higher hydrodynamic radius than the native dimer, and therefore, would elute with shorter retention times. Indeed, the HPLC retention times when FIS is denatured in urea are shorter than that of the native dimer in the absence of urea (data not shown). Taken together, these observations suggest that at low FIS concentrations, a marginally stable monomeric intermediate is present.

These observations have general implications for denaturation studies of oligomeric proteins, many of which are performed (due to instrument sensitivity) at concentrations significantly higher than those present in vivo. Dimeric pro-

teins with unstable monomeric intermediates may denature via a two-state mechanism in vitro, when studied at protein concentrations much higher than their K_d s, where the dimer is further stabilized (i.e., higher C_m) relative to the monomeric intermediate (Neet and Timm 1994). At lower and perhaps more physiologically relevant protein concentrations (approaching the K_d), it may be possible to observe monomeric intermediates. This is particularly pertinent for small single-domain dimeric proteins, which are unlikely to exhibit stable monomeric equilibrium intermediates by urea denaturation. Thus, structure–function correlations of dimeric proteins based on in vitro denaturation experiments carried out at concentrations much higher than the protein's K_d , may not always hold biological relevance, especially if the monomeric state has altered functions in vivo.

Potential impact of K_d on FIS function

Although we are not able to calculate an accurate K_d for FIS, our CD and HPLC data clearly show that it is in the micromolar range. The relatively high K_d of FIS suggests that the amount of FIS protein present as dimers in *E. coli* could be affected by the drastic changes in its expression levels observed during growth in rich medium. During early exponential growth in rich medium, FIS levels rapidly accumulate to about 45 μM and then rapidly decline to less than 0.4 μM (if one assumes an average cell volume of approximately 1 μm^3) (Neidhardt 1987). Thus, it is likely that FIS exists exclusively as dimers during early logarithmic growth phase, when its levels are highest. However, as the FIS concentration decreases during mid and late logarithmic growth phase, an appreciable fraction of dissociated monomers can be expected to become populated. This could enhance the cell's ability to remove dimeric FIS during this growth phase. Moreover, because FIS monomers are likely to be significantly more susceptible to proteolytic degradation than the dimers, the rate of FIS turnover would also step up during this stage. Thus, it is conceivable that the high K_d for FIS is critical for its rapid removal during mid and late logarithmic growth phase. Timely removal of FIS is very important because cell viability significantly decreases if FIS is constitutively expressed during stationary phase (Osuna et al. 1995).

FIS-DNA binding studies have been performed in vitro using FIS concentrations in the 4–40 nM range (Bruist et al. 1987; Ball et al. 1992; Ninnemann et al. 1992; Gosink et al. 1993; Beach and Osuna 1998), suggesting that most of the FIS protein in these experiments was initially present as monomers, assuming a K_d in the micromolar range. Although it has been generally assumed that FIS dimer formation is a prerequisite for DNA binding, the possibility that monomeric FIS is capable of DNA binding should also be considered. DNA binding by monomeric FIS, albeit with reduced efficiency, has already been suggested based on

DNA binding results using a FIS mutant thought to be defective in dimerization (Deufel et al. 1997). Therefore, it is possible that partially folded and weakly stable FIS monomers could retain weak DNA binding activity. In this case, two monomers bound to adjacent half sites may be able to dimerize (given their high local concentration) and produce a stable nucleoprotein complex. Such a binding mechanism, if it exists, would probably rely on a higher number of monomer–DNA contacts per half-site than that of a preexisting dimer in solution. The observation that at lower FIS concentrations where monomer may be populated, FIS preferentially binds a few select DNA sequences, and at higher concentrations it can bind additional DNA sequences, in some cases with apparent nonspecificity (Schneider et al. 1997), might suggest that pre-formation of a stable dimer may be required for binding to other less-specific DNA sequences. Thus, FIS concentration-dependent affinities for different DNA sequences might be affected by the monomer–dimer dynamics that are possible because of its K_d .

Modulation of protein levels as a way of regulating protein function

There are examples of DNA-binding proteins that appear to be partially regulated by their levels of expression and the resulting effect on their oligomeric state (Sevenich et al. 1998; Palena et al. 1999). Among them is the nitrogen regulatory protein (NtrC), which is a transcription activator of genes involved in nitrogen metabolism (McFarland et al. 1981), and has been suggested to be an evolutionary precursor of FIS (Morett and Bork 1998). It is a dimeric protein of 102 kD, which contains a 20-kD DNA-binding domain with high structural homology to FIS (Rippe et al. 1998; Pelton et al. 1999). When phosphorylated, NtrC binds to two adjacent DNA sites with high cooperativity (Porter et al. 1993), and there is evidence that an octamer comprised of phosphorylated NtrC dimers is required for the formation of an active NtrC complex (Su et al. 1990; Wyman et al. 1997; Rippe et al. 1998). The concentration of NtrC required for octamer formation is >10 nM. The intracellular concentration of NtrC is estimated to be 10 nM when inactive and rises to ≈ 100 nM under nitrogen limiting conditions. This rise in concentration is an important part of the regulatory mechanism of NtrC (Sevenich et al. 1998). Similar to NtrC, it is probable that many oligomeric proteins require a drastic increase in expression levels to form a functional oligomer or a higher order oligomer possessing a different function.

Thus, the oligomeric state of many DNA-binding proteins in vivo may be important, not only for DNA binding, but for providing additional means for rapid regulation. Perhaps it is not coincidental that many transcription regulatory proteins have dissociation constants in the μM range (Farez-Vidal et al. 1996; Abril et al. 1997; Jana et al. 1997;

Palena et al. 1999; Sengchanthalangsy et al. 1999; Steinmetzer et al. 2000). Determinations of how the levels of DNA-binding proteins correlate with their K_d s and with their turnover rates should shed light on how these parameters may impact their function in vivo.

Materials and methods

Protein expression and purification

FIS was generally overexpressed in *E. coli* as described by Osuna et al. (1991), with some modifications. Differences to that procedure were that cells were grown until $\text{OD}_{600} = 0.8$, and expression was induced with 0.3 mM IPTG for 1 h. The cells were lysed in a volume (mL) five times the mass. After removal of the DNA, extracts were dialyzed against 0.3 M NaCl, 25 mM Tris hydroxymethyl aminomethane hydrochloride (Tris-HCl) at pH 7.0, and loaded onto a SP Sepharose Fast Flow column (Amersham Pharmacia) equilibrated with 25 mM Tris-HCl (pH 7.0). FIS was eluted from the column with a NaCl gradient of 0.3 to 1 M, and the crude FIS fractions were pooled, concentrated, and dialyzed against 25 mM Tris-HCl (pH 7.0), which results in precipitation of FIS. Precipitated FIS was resuspended in 1 M NaCl, 25 M Tris-HCl (pH 7.0), and purity was determined to be greater than 95%, based on SDS polyacrylamide gel electrophoresis.

Sample preparation

Protein concentration (in monomer units) was obtained in 1 M NaCl, 25 mM Tris-HCl, using an ϵ_{278} of $6340 \text{ M}^{-1} \text{ cm}^{-1}$, which was determined as described by Pace et al. (1995). All experiments were performed over a range of concentrations at 20°C in either 10 mM potassium phosphate buffer (PB) at pH 7.2 or 20 mM MOPS (pH 7.2), with 0.1 M NaCl, unless otherwise stated. Urea denaturation experiments using 107 μM FIS were carried out at 0.2 M NaCl to prevent the precipitation of FIS that occurs in low salt conditions.

Circular dichroism studies

CD spectra were recorded on an OLIS CD instrument equipped with a dual-beam optical system. All CD samples were prepared in 10 mM PB (pH 7.2). Unfolded structure was observed in 10 mM PB (pH 7.2), 6 M urea, at 20°C , and reversibility was verified by returning the sample to native state conditions through a six-fold dilution. The change in far-UV signal upon FIS dilution was measured by averaging three full wavelength scans at different concentrations, each derived from serial dilutions of a 71 μM stock. All FIS dilutions were in 10 mM PB (pH 7.2), 0.17 M NaCl, except the 71 μM stock, which was in 0.33 M NaCl. The use of varying protein concentration and cell sizes in the dilution experiments required that all scans be plotted in molar ellipticity:

$$[\theta] = (\theta_{\text{observed}})/(10lcN_p) \quad (1)$$

where l is the path length in cm, c is the molar concentration, and N_p is number of peptide bonds. All spectra were corrected by subtracting a buffer baseline in the appropriate cuvette.

Equilibrium urea denaturations in the far-UV were monitored at 222 nm to observe the α -helical content and subtracted from the baseline at 245 nm. Denaturation experiments in the near-UV re-

gion were monitored at 277 and 330 nm was used for a baseline. Scans were time-averaged over 3 min with each urea concentration used. Samples were prepared independently or by a codilution method, in which FIS samples of progressively higher urea concentrations were made by repetitive withdrawal of native FIS from the cell followed by addition of the corresponding volume from a FIS solution in 8 M urea. Urea concentrations for all experiments were determined from the refractive index measurement using an Abbe refractometer (Pace et al. 1990).

Fluorescence studies

Fluorescence measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer at 20°C using 1-cm path-length cuvettes. Excitation and emission wavelengths were 276 and 305 nm, respectively, with an excitation slit of 5 nm and an emission slit of 10 nm. Samples were prepared in 20 mM MOPS (pH 7.2). Equilibrium urea denaturations were performed using the dilution method described for the CD experiments. Samples were equilibrated for 4 min between measurements, and time scans were measured over 40 sec. The fluorescence signal of FIS was internally normalized.

Steady-state anisotropy was performed using Hitachi polarization accessories and carried out in 1-cm path-length cuvettes. Samples were prepared in 10 mM PB (pH 7.2). For each anisotropy value at least five measurements were recorded and averaged. The integration time was 1 sec. Anisotropy was calculated from the formulas:

$$A = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (2)$$

$$G = \frac{I_{hv}}{I_{hh}} \quad (3)$$

where I_{vv} , I_{vh} , I_{hh} , and I_{hv} are the fluorescence intensities when the excitation and emission polarizers, respectively, are set in the vertical (v) or horizontal (h) positions (Eftink 1994).

Size-exclusion HPLC

HPLC measurements were performed on a Beckman System Gold Nouveau with the 166NM single wavelength detector (1-cm path-length flow cell), using a 7.8 mm × 30 cm TOSOH BIOSEP TSK-GEL 2000SWXL column. Samples were prepared in 20 mM PB (pH 7.2), 0.5 M NaCl. The HPLC data had to be collected under higher salt conditions than our spectroscopic data to prevent interaction with the column and the collection of lower concentration data points. FIS samples injected onto the column were immediately diluted ~18-fold and rapidly came to equilibrium at the diluted concentration. The extent of sample dilution in the column was determined by monitoring at 276 nm and calculating the concentration of FIS from the absorbance at the peak maximum. To investigate the oligomerization state of FIS at different concentrations, the column was preequilibrated to the above conditions, and samples at varying FIS concentrations, achieved by serial dilution from a more highly concentrated stock, were injected onto the column. The elution of FIS was monitored at 220 nm, with the column running at a flow rate of 1 mL/min. The column was calibrated using the proteins: bovine serum albumin (67 kD), ovalbumin (43 kD), human Cu/Zn super oxide dismutase (32 kD), cytochrome C (17 kD), ribonucleaseA (13.7 kD), and

myoglobin (12.3 kD). The resulting calibration curve indicated that dimeric FIS ran on the column roughly as expected for a protein of its molecular weight (data not shown).

Data modeling

For comparison of data collected at different protein concentrations or by different spectral methods, the data was normalized to F_u , the apparent fraction of unfolded protein,

$$F_u = \frac{Y_o - Y_n}{Y_u - Y_n} \quad (4)$$

where Y_o is the signal at a given urea concentration, and Y_n and Y_u are the observed values for the native and unfolded protein, respectively. The terms Y_n and Y_u are defined as linear equations specifying the urea dependence of the native and unfolded baselines, respectively.

The data from the urea denaturation experiments were best fit to a two-state dimer model, with native dimer (N_2) in equilibrium with unfolded monomers ($2U$):



The equilibrium constant for concerted unfolding and dissociation, K_u , is defined as $K_u = [U]^2/[N_2]$. The total protein concentration, Pt , in terms of monomer is $Pt = 2N_2 + U$, and the fraction of native dimers (F_n) = $1 - F_u$, where $F_u = [U]/Pt$. Combining these equations and solving for F_u in terms of the equilibrium constant K_u and Pt , one obtains the following equation:

$$F_u = \frac{\sqrt{K_u^2 + 8K_uPt} - K_u}{4Pt} \quad (6)$$

Rearranging equation 4 yields the equation used for fitting the denaturation transitions:

$$Y_o = Y_n(1 - F_u) + Y_u(F_u) \quad (7)$$

The thermodynamic parameters listed in Table 1 were obtained by fitting the denaturation curves to equation 7, upon substitution of equation 6, by defining K_u according to the linear free-energy model, which states that the changes in free-energy (ΔG_u) that accompany protein unfolding are linearly dependent on the concentration of urea denaturant (Schellman 1978; Schellman 1987):

$$\Delta G_u = -RT \ln(K_u) = \Delta G_{H_2O} + m[\text{denaturant}] \quad (8)$$

Fits of individual data sets were performed with KaleidaGraph v 3.5 (Synergy Software). Global analysis of the data was done by simultaneously fitting all of the data at different FIS concentrations from the different methods to the two-state model described above. This analysis was carried out using the program SAVUKA version 5.1, a nonlinear least-squares fitting program (Zitzewitz et al. 1995; Bilsel et al. 1999).

The change in signal that occurs upon dilution of protein was also modeled to the two-state system shown in equation 5. In this case, we varied the FIS concentration and directly solved for the K_d (dimer dissociation/unfolding constant) instead of K_u , by using equations 6 and 7.

Acknowledgments

The authors are grateful to Osman Bilsel for help with the program Savuka. This work was supported by grants from the National Science Foundation (NSF MCB-9984913 to W.C.) and National Institutes of Health (NIH GM52051 to R.O.). S. I. and S. H. were partially supported by a Howard Hughes Medical Institute undergraduate research program. S.H. is a NSF Graduate Fellow.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Abril, A.M., Salas, M., Andreu, J.M., Hermoso, J.M., and Rivas, G. 1997. Phage phi29 protein p6 is in a monomer-dimer equilibrium that shifts to higher association states at the millimolar concentrations found in vivo. *Biochemistry* **36**: 11901–11908.
- Azuma, T. and Hamaguchi, K. 1976. The mechanism of reassembly of immunoglobulin G. *J. Biochem. (Tokyo)* **80**: 1023–1038.
- Ball, C.A. and Johnson, R.C. 1991. Efficient excision of phage lambda from the *Escherichia coli* chromosome requires the Fis protein. *J. Bacteriol.* **173**: 4027–4031.
- Ball, C.A., Osuna, R., Ferguson, K.C., and Johnson, R.C. 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. *J. Bacteriol.* **174**: 8043–8056.
- Beach, M.B. and Osuna, R. 1998. Identification and characterization of the fis operon in enteric bacteria. *J. Bacteriol.* **180**: 5932–5946.
- Bilsel, O., Zitzewitz, J.A., Bowers, K.E., and Matthews, C.R. 1999. Folding mechanism of the alpha-subunit of tryptophan synthase, an alpha/beta barrel protein: Global analysis highlights the interconversion of multiple native, intermediate, and unfolded forms through parallel channels. *Biochemistry* **38**: 1018–1029.
- Bosch, L., Nilsson, L., Vijgenboom, E., and Verbeek, H. 1990. FIS-dependent trans-activation of tRNA and rRNA operons of *Escherichia coli*. *Biochim. Biophys. Acta* **1050**: 293–301.
- Bruist, M.F., Glasgow, A.C., Johnson, R.C., and Simon, M.I. 1987. Fis binding to the recombinational enhancer of the Hin DNA inversion system. *Genes & Dev.* **1**: 762–772.
- Ciechanover, A. 1998. The ubiquitin-proteasome pathway: On protein death and cell life. *EMBO J.* **17**: 7151–7160.
- Cohen, P. 2000. The regulation of protein function by multisite phosphorylation—A 25 year update. *Trends Biochem. Sci.* **25**: 596–601.
- Deufel, A., Hermann, T., Kahmann, R., and Muskhelishvili, G. 1997. Stimulation of DNA inversion by FIS: Evidence for enhancer-independent contacts with the Gin-gix complex. *Nucleic Acids Res.* **25**: 3832–3839.
- Dorgai, L., Oberto, J., and Weisberg, R.A. 1993. Xis and Fis proteins prevent site-specific DNA inversion in lysogens of phage HK022. *J. Bacteriol.* **175**: 693–700.
- Eftink, M.R. 1994. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys. J.* **66**: 482–501.
- Farez-Vidal, M.E., Wilson, T.J., Davidson, B.E., Howlett, G.J., Austin, S., and Dixon, R.A. 1996. Effector-induced self-association and conformational changes in the enhancer-binding protein NTRC. *Mol. Microbiol.* **22**: 779–788.
- Finkel, S.E. and Johnson, R.C. 1992. The Fis protein: It's not just for DNA inversion anymore. *Mol. Microbiol.* **6**: 3257–3265.
- Franchini, P.L. and Reid, R.E. 1999. A model for circular dichroism monitored dimerization and calcium binding in an EF-hand synthetic peptide. *J. Theor. Biol.* **199**: 199–211.
- Gonzalez-Gil, G., Bringmann, P., and Kahmann, R. 1996. FIS is a regulator of metabolism in *Escherichia coli*. *Mol. Microbiol.* **22**: 21–29.
- Gosink, K.K., Ross, W., Leirmo, S., Osuna, R., Finkel, S.E., Johnson, R.C., and Gourse, R.L. 1993. DNA binding and bending are necessary but not sufficient for Fis-dependent activation of rrmB P1. *J. Bacteriol.* **175**: 1580–1589.
- Haffter, P. and Bickle, T.A. 1987. Purification and DNA-binding properties of FIS and Cin, two proteins required for the bacteriophage P1 site-specific recombination system. *J. Mol. Biol.* **198**: 579–587.
- Jana, R., Hazbun, T.R., Mollah, A.K., and Mossing, M.C. 1997. A folded monomeric intermediate in the formation of lambda Cro dimer-DNA complexes. *J. Mol. Biol.* **273**: 402–416.
- Johnson, R.C., Bruist, M.F., and Simon, M.I. 1986. Host protein requirements for in vitro site-specific DNA-inversion. *Cell* **46**: 531–539.
- Koch, C. and Kahmann, R. 1986. Purification and properties of the *Escherichia coli* host factor required for inversion of the G segment in bacteriophage Mu. *J. Biol. Chem.* **261**: 15673–15678.
- Koch, C., Ninnemann, O., Fuss, H., and Kahmann, R. 1991. The N-terminal part of the *E. coli* DNA binding protein FIS is essential for stimulating site-specific DNA inversion but is not required for specific DNA binding. *Nucleic Acids Res.* **19**: 5915–5922.
- Kostrewa, D., Granzin, J., Koch, C., Choe, H.W., Raghunathan, S., Wolf, W., Labahn, J., Kahmann, R., and Saenger, W. 1991. Three-dimensional structure of the *E. coli* DNA-binding protein FIS. *Nature* **349**: 178–180.
- Kraulis, P.J. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**: 946–950.
- Lakowicz, J.R. 1999. *Principles of fluorescence spectroscopy*. Kluwer Academic/Plenum, New York.
- Laney, J.D. and Hochstrasser, M. 1999. Substrate targeting in the ubiquitin system. *Cell* **97**: 427–430.
- McFarland, N., McCarter, L., Artz, S. and Kustu, S. 1981. Nitrogen regulatory locus "glnR" of enteric bacteria is composed of cistrons ntrB and ntrC: Identification of their protein products. *Proc. Natl. Acad. Sci.* **78**: 2135–2139.
- Morett, E. and Bork, P. 1998. Evolution of new protein function: Recombinational enhancer Fis originated by horizontal gene transfer from the transcriptional regulator NtrC. *FEBS Lett.* **433**: 108–112.
- Mulkerrin, M.G. 1996. Protein structure analysis using circular dichroism. In *Spectroscopic methods for determining protein structure in solution* (ed. H.A. Havel), pp. 5–15. VCH Publishers, Inc., New York.
- Neet, K.E. and Timm, D.E. 1994. Conformational stability of dimeric proteins: Quantitative studies by equilibrium denaturation. *Protein Sci.* **3**: 2167–2174.
- Neidhardt, F.C. 1987. Chemical composition of *Escherichia coli*. In *Escherichia coli and Salmonella: Cellular and molecular biology* (eds. F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger), pp. 3–6. American Society for Microbiology, Washington, DC.
- Nilsson, L., Verbeek, H., Vijgenboom, E., van Drunen, C., Vanet, A., and Bosch, L. 1992. FIS-dependent trans activation of stable RNA operons of *Escherichia coli* under various growth conditions. *J. Bacteriol.* **174**: 921–929.
- Ninnemann, O., Koch, C., and Kahmann, R. 1992. The *E. coli* fis promoter is subject to stringent control and autoregulation. *EMBO J.* **11**: 1075–1083.
- Osuna, R., Finkel, S.E., and Johnson, R.C. 1991. Identification of two functional regions in Fis: The N-terminus is required to promote Hin-mediated DNA inversion but not lambda excision. *EMBO J.* **10**: 1593–1603.
- Osuna, R., Lienau, D., Hughes, K.T., and Johnson, R.C. 1995. Sequence, regulation, and functions of fis in *Salmonella typhimurium*. *J. Bacteriol.* **177**: 2021–2032.
- Pace, C.N., Shirley, B.A., and Thomson, J.A. 1990. Measuring the conformational stability of a protein. In *Protein structure: A practical approach* (ed. T.E. Creighton), pp. 311–330. Oxford University Press, Oxford.
- Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Theronica, G. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**: 2411–2423.
- Palena, C.M., Gonzalez, D.H., and Chan, R.L. 1999. A monomer-dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA. *Biochem J.* **341**: 81–87.
- Pelton, J.G., Kustu, S., and Wemmer, D.E. 1999. Solution structure of the DNA-binding domain of NtrC with three alanine substitutions. *J. Mol. Biol.* **292**: 1095–1110.
- Porter, S.C., North, A.K., Wedel, A.B., and Kustu, S. 1993. Oligomerization of NTRC at the glnA enhancer is required for transcriptional activation. *Genes & Dev.* **7**: 2258–2273.
- Pratt, T.S., Steiner, T., Feldman, L.S., Walker, K.A., and Osuna, R. 1997. Deletion analysis of the fis promoter region in *Escherichia coli*: Antagonistic effects of integration host factor and Fis. *J. Bacteriol.* **179**: 6367–6377.
- Ramos, J.L., Gallegos, M.T., Marques, S., Ramos-Gonzalez, M.I., Espinosa-Urgel, M., and Segura, A. 2001. Responses of Gram-negative bacteria to certain environmental stressors. *Curr. Opin. Microbiol.* **4**: 166–171.
- Rippe, K., Mucke, N., and Schulz, A. 1998. Association states of the transcription activator protein NtrC from *E. coli* determined by analytical ultracentrifugation. *J. Mol. Biol.* **278**: 915–933.
- Ross, W., Thompson, J.F., Newlands, J.T., and Gourse, R.L. 1990. *E. coli* Fis protein activates ribosomal RNA transcription in vitro and in vivo. *EMBO J.* **9**: 3733–3742.
- Safo, M.K., Yang, W.Z., Corselli, L., Cramton, S.E., Yuan, H.S., and Johnson,

- R.C. 1997. The transactivation region of the fis protein that controls site-specific DNA inversion contains extended mobile beta-hairpin arms. *EMBO J.* **16**: 6860–6873.
- Schellman, J.A. 1978. Solvent denaturation. *Biopolymers* **17**: 1305–1322.
- Schellman, J.A. 1987. The thermodynamic stability of proteins. *Ann. Rev. Biophys. Biophys. Chem.* **16**: 115–137.
- Schneider, R., Travers, A., and Muskhelishvili, G. 1997. FIS modulates growth phase-dependent topological transitions of DNA in *Escherichia coli*. *Mol. Microbiol.* **26**: 519–530.
- Sengchanthalangsy, L.L., Datta, S., Huang, D.B., Anderson, E., Braswell, E.H., and Ghosh, G. 1999. Characterization of the dimer interface of transcription factor NFkappaB p50 homodimer. *J. Mol. Biol.* **289**: 1029–1040.
- Sevenich, F.W., Langowski, J., Weiss, V., and Rippe, K. 1998. DNA binding and oligomerization of NtrC studied by fluorescence anisotropy and fluorescence correlation spectroscopy. *Nucleic Acids Res.* **26**: 1373–1381.
- Steinmetzer, K., Hillisch, A., Behlke, J., and Brantl, S. 2000. Transcriptional repressor CopR: Amino acids involved in forming the dimeric interface. *Proteins* **39**: 408–416.
- Su, W., Porter, S., Kustu, S., and Echols, H. 1990. DNA-looping and enhancer activity: Association between DNA-bound NtrC activator and RNA polymerase at the bacterial *glnA* promoter. *Proc. Natl. Acad. Sci.* **87**: 5504–5508.
- Thompson, J.F., Moitoso de Vargas, L., Koch, C., Kahmann, R., and Landy, A. 1987. Cellular factors couple recombination with growth phase: Characterization of a new component in the lambda site-specific recombination pathway. *Cell* **50**: 901–908.
- Woodbury, R.L., Hardy, S.J., and Randall, L.L. 2002. Complex behavior in solution of homodimeric SecA. *Protein Sci.* **11**: 875–882.
- Wyman, C., Rombel, I., North, A.K., Bustamante, C., and Kustu, S. 1997. Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science* **275**: 1658–1661.
- Xu, D., Tsai, C.J., and Nussinov, R. 1998. Mechanism and evolution of protein dimerization. *Protein Sci.* **7**: 533–544.
- Xu, J. and Johnson, R.C. 1995a. Fis activates the RpoS-dependent stationary-phase expression of proP in *Escherichia coli*. *J. Bacteriol.* **177**: 5222–5231.
- . 1995b. Identification of genes negatively regulated by Fis: Fis and RpoS comodule growth-phase-dependent gene expression in *Escherichia coli*. *J. Bacteriol.* **177**: 938–947.
- Yuan, H.S., Finkel, S.E., Feng, J.A., Kaczor-Grzeskowiak, M., Johnson, R.C., and Dickerson, R.E. 1991. The molecular structure of wild-type and a mutant Fis protein: Relationship between mutational changes and recombinational enhancer function or DNA binding. *Proc. Natl. Acad. Sci.* **88**: 9558–9562.
- Zitzewitz, J.A., Bilsel, O., Luo, J., Jones, B.E., and Matthews, C.R. 1995. Probing the folding mechanism of a leucine zipper peptide by stopped-flow circular dichroism spectroscopy. *Biochemistry* **34**: 12812–12819.