The *Escherichia coli* mazEF addiction module plays a crucial role in the cell death program that is triggered under various stress conditions. It codes for the toxin MazF and the antitoxin MazE, which interfere with the lethal action of the toxin. To better understand the role of various conformations of MazE in bacterial life, its order-disorder transitions were monitored by differential scanning calorimetry, spectropolarimetry, and fluorimetry. The changes in spectral and thermodynamic properties accompanying MazE dimer denaturation can be described in terms of a compensating reversible process of the partial folding of the unstructured C-terminal half (high mean net charge, low mean hydrophobicity) and monomerization coupled with the partial unfolding of the structured N-terminal half (low mean net charge, high mean hydrophobicity). At pH \( \leq 4.5 \) and \( T < 50 \, ^\circ\text{C} \), the unstructured polypeptide chains of the MazE dimer fold into (pre)molten globule-like conformations that thermally stabilize the dimeric form of the protein. The simulation based on the thermodynamic and structural information on various addiction modules suggests that both the conformational adaptability of the dimeric antitoxin form (binding to the toxins and DNA) and the reversible transformation to the more flexible monomeric form are essential for the regulation of bacterial cell life and death.

Programmed cell death, defined as an active process that results in cell suicide, refers to any form of cell death or stasis mediated by an intracellular death program, no matter what triggers it (1–3). In bacteria, programmed cell death is mediated through a unique genetic system. This system, called an “addiction module,” consists of a pair of genes that specify for two components: a toxin and an antitoxin that interfere with the lethal action of the toxin. The toxin and the antitoxin are coexpressed. Their expression is autoregulated at the level of transcription either by the toxin-antitoxin complex or by the antitoxin alone (4–19). Until recently, such genetic systems for programmed bacterial cell death have been found mainly in *Escherichia coli* on low copy number plasmids. When bacteria lose the plasmid(s) (or other extrachromosomal elements), the cured cells are selectively killed because the unstable antitoxin is degraded faster than the more stable toxin (4–7). The cellular target of the toxin is known only in the *ccdB* and *kid-kis* systems. In *ccdB*, CcdB on plasmid F attacks the A subunit of gyrase, whereas in the *kid-kis* system, Kid on plasmid R1 targets DnaB (20–22). Thus, the cells are “addicted” to the short-lived antitoxin because its presence is essential for cell survival (4).

Toxin-antitoxin systems, some of which are homologous to these extrachromosomal addiction modules, have been found on the chromosomes of several bacteria. For example, in *E. coli*, there are several such pairs of genes, including *mazEF* (23–25), *cbbB* (24), *relBE* (26–28), *yeF-myoB* (29–31), and *dinJ-yaqQ* (32). The first toxin-antitoxin system contained in a bacterial chromosome that was described as regulatable and responsible for programmed cell death was the *E. coli* *mazEF* module (25), located in the *relA* operon (23). The product of *mazF* (MazF) is a stable toxin, and the product of *mazE* (MazE) is a labile protein, degraded by the protease ClpAP (25). Therefore, preventing MazF-mediated death requires a continuous cellular production of MazE. In contrast to the addiction modules that are triggered by the loss of the element, death mediated by chromosomal *mazEF* is achieved by several stressful conditions that prevent *mazEF* expression. Initially, the *mazEF* module was found to be under the control of ppGpp (25), the amino acid starvation signal molecule produced by the RelA protein (33). Overproduction of ppGpp leads to inhibition of the expression of *mazEF* and thereby to cell death (25, 34). However, as reported recently, inhibiting the expression of *mazEF* and thus inducing cell death can also be achieved by several additional stressful conditions. These include the inhibition of transcription and/or translation (35–37), DNA damage (38, 39), and oxidative stress (39).

In the last few years, the chromosomal *mazEF* toxin-antitoxin systems have attracted additional attention, in particular in the following four directions. (a) The first is studies on the mechanism of MazF action. MazF has been shown to inhibit translation by cleaving mRNAs at specific sites (40, 41). How-
ever, two laboratories have reported contradictory results for the mechanism of cleavage. According to one group, MazF inhibits translation by a ribosome-dependent mechanism (40), and according to a second group, MazF is a sequence-specific endoribonuclease that cleaves mRNA at ACA sequences in a codon- and ribosome-independent manner (41). This second version was further confirmed by a more recent report from the same laboratory showing that, at least in vitro, MazF functions enzymatically in a similar manner to RNase A, although with different sequence specificity (42). (b) The second direction is studies on the mazEF-mediated cell death network. There is a “point of no return” in MazF-mediated cell death; overexpression of MazE can only reverse MazF lethality over a short window of time (43), suggesting that MazF is a mediator rather than an executioner of cell lethality (44). (c) The third direction is studies on the function of mazEF showing that it can prevent spreading of phage infection (45). (d) The fourth is molecular studies characterizing the forces that drive the unfolding (folding) of proteins (MazE, MazF) and the interactions that drive MazE-MazF, MazE-DNA, and MazE-MazF-DNA complex formation. The first step toward this goal was achieved by crystallization of the antitoxin MazE and by determination of part of its three-dimensional structure (46). The resulting structure is the first of any addiction antitoxin, the crystallization of which had been hampered because of its susceptibility to proteases and its high content of unstructured polypeptide. We circumvented this problem by crystallizing MazE in complex with the specific dromedary antibody fragment VH1. Furthermore, the energetics of MazE interactions with VH1 was investigated in detail. It has been suggested that, under physiological conditions in solution, a significant part of the MazE polypeptide chain may be unstructured, as in the crystal of the MazE-VH1 complex (47). Almost simultaneously, Kamada et al. (48) determined the crystal structure of the MazE-MazF complex, in which MazE chains appear to be less unstructured than in the complex with VH1.

It is known that proteins that lack intrinsic globular structure (intrinsically flexible, intrinsically unstructured, natively unfolded, pliable) are frequently involved in some of the most important regulatory functions in the cell (30, 49–55). Numerous examples of domains that are unstructured in solution but become structured upon binding to the target have been identified. Thus, the intrinsic lack of structure can offer functional advantages to a protein, including its ability to bind several different targets (30, 49–57).

In this study, we investigated to what extent MazE belongs to this category of proteins by studying its conformational changes induced by variation in temperature, pH, protein concentration, and urea concentration. The energetics of MazE order-disorder transitions was studied by various techniques (differential scanning calorimetry, spectropolarimetry, and fluorometry) and is discussed in terms of its three-dimensional structures (46, 48). An attempt was made to predict some functional features of the addiction modules from the equilibrium thermodynamic model, which involves antitoxin and toxin molecules and DNA. To the best of our knowledge, the research described here represents for the first time a complete thermodynamic study of structural transitions of a protein with an intrinsically flexible nature.

**EXPERIMENTAL PROCEDURES**

**Preparation of MazE Solutions**

The expression and purification of MazE have been described previously (46). MazE solutions for calorimetric and spectroscopic measurements were prepared by diluting the stock solution of MazE in water to the appropriate concentrations in the appropriate buffer solutions. Depending on the pH, the buffer solutions used in our experiments were as follows: 50 mM HCl (pH 1.5), 50 mM citrate (pH 3.1), 50 mM acetate (pH 4.5), 50 mM cacodylate (pH 6.1), 50 mM phosphate (pH 7.1), and 50 mM HEPPS (pH 8.1), and 20 mM borate (pH 9.4). Unless stated otherwise, all buffer solutions contained 150 mM NaCl. For urea denaturation, 21 solutions with the same MazE concentration and varying urea concentrations (0–8 M) were prepared from stock 10 mM urea and stock MazE solutions in the same buffer. The pH of all solutions was checked and adjusted to the appropriate value by NaOH or HCl. Before each experiment (except those involving urea), the protein buffer solution was extensively dialyzed against the corresponding buffer solution and degassed for 15 min. The concentrations of the 98-residue-long His-tag fused MazE protein (molecular mass of 12111.5 g mol⁻¹) were determined spectrophotometrically by measuring the absorbance at 280 nm. The corresponding extinction coefficient of MazE in 6 M guanidinium chloride and 20 mM phosphate buffer (pH 6.5) was obtained from the amino acid composition by the method introduced by Gill and von Hippel (58) (available at www.expasy.ch). Finally, an extinction coefficient of 1.27 ± 0.05 mg⁻¹ ml⁻¹ cm⁻¹ was calculated by combination of the measured absorbance of the guanidinium chloride-free MazE solution of the same concentration at the same wavelength (280 nm) as the one measured in guanidinium chloride.

**Differential Scanning Calorimetry (DSC)**

The thermally induced transitions of MazE were measured between pH 1.5 and 9.4 using a Nano-II DSC differential scanning calorimeter (Calorimetry Sciences Corp.). The heating rate was 1 °C/min⁻¹, and the concentration of proteins in the measuring cell was ~1.2 mg ml⁻¹. To obtain the presented thermograms (Cp (T) versus temperature curves) (see Fig. 2), the heat capacity of the protein in the initial (native) state was subtracted from the raw signal corrected for buffer contribution. The transition enthalpies (ΔH(ex)) were obtained by integration of the C p(T) versus temperature curves.

**CD Spectropolarimetry**

CD spectra were recorded using a Model 62A DS spectropolarimeter (Aviv, Lakewood, NJ) equipped with a programmable thermostatically controlled cell holder. The presented mean residue ellipticities (θl), obtained from raw data (ellipticities) by subtracting the corresponding spectra of the buffer solution and taking into account the mass concentration (c), molecular mass (Mw, number of MazE amino acid residues (N), and optical path length (l)) through the relation [θl] = θMw/(Ncλ).

**Thermal Denaturation**—Changes in MazE secondary (far-UV, 205–255 nm; 1-nm cuvette) and tertiary (near-UV, 255–310 nm; 1-cm cuvette) structures were followed by recording CD spectra at a MazF concentration of 0.5 mg ml⁻¹ (see Fig. 3, a and b) at different temperatures with a temperature step of 3 °C. At a lower concentration (0.05 mg ml⁻¹), temperature scans in the far-UV region were performed also at λ = 230 nm in a 1-cm cuvette.

**Urea Denaturation**—The changes in MazE secondary structure upon increasing urea concentrations (0–8 M) were followed by measuring the ellipticity at 225 nm in a 1-cm cuvette at a MazE concentration of 0.05 mg ml⁻¹ (see Fig. 5, b and c). The measurements were performed at a number of different temperatures.

**Fluorescence Spectroscopy**

Fluorescence emission spectra were recorded using a PerkinElmer Life Sciences LS 50 luminescence spectrometer equipped with a thermally controlled cell holder and a cuvette with a 1-cm path length. Thermal denaturation of MazE was followed by the emission fluorescence of the external fluorescent probe 1-anilino-8-naphthalenesulfonic acid ammonium salt (ANS; Fluka, Buchs, Switzerland). The emission spectra (415–615 nm) were recorded at different temperatures in the range 5–90 °C using λex = 400 nm (see Fig. 4). The concentrations of MazE and ANS in the buffer solutions were 0.05 and 0.126 mg ml⁻¹, respectively.

Urea-induced denaturation of MazE was followed by measuring the changes in MazE intrinsic (Tryp) emission fluorescence between 310 and 410 nm (λex = 280 nm) at urea concentrations of 0–8 M. The concentration of MazE was 0.05 mg ml⁻¹ (see Fig. 5b), and the measurements were performed at a number of different temperatures.

2 The abbreviations used are: HEPPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DSC, differential scanning calorimetry; ANS, 1-anilino-8-naphthalenesulfonic acid ammonium salt.
Energetics of MazE Structure

Model Analysis of Thermally Induced and Urea-induced Transitions

Our results reveal that the majority of the monitored structural transitions can be adequately described in terms of a reversible two-state process in which the protein molecule can exist either in macro state N or in macro state D. Such process can be presented as shown in Equation 1.

\[
K = \frac{(1/n)N_o}{D}, \Delta G^0 = -RT\ln K = -(RT/\ln(a\sigma C_\sigma^{1/2}(1 - a)))
\]

where \(N_o\) represents the MazE dimer (\(n = 2\)) in the initial (native) state, whereas \(D\) corresponds to MazE in the final monomeric (denatured) state; \(K\) is the equilibrium constant of denaturation; \(\Delta G^0\) is the corresponding standard Gibbs free energy change per mol of monomer; \(n\) is the number of subunits to which each protein dissociates upon denaturation; \(a\) is the fraction of protein in the denatured state, and \(C_\sigma\) is the total protein concentration given in moles of monomer \(\text{l}^{-1}\). It should be noted that, under some specific conditions, two successive transitions that do not overlap significantly were observed ("Results"). In such cases, the two transitions can be treated separately: the first one that corresponds to the transition of the native dimer \(N_2\) into the intermediate dimer \(I_2\) (\(1/2N_2 \leftrightarrow 1/2I_2\)) is a special case of Equation 1 with \(n = 1\), and the second one that describes the transition of the intermediate dimer \(I_2\) into the denatured monomer \(D\) (\(1/2I_2 \leftrightarrow D\)) is a common example of Equation 2 with \(n = 2\). The thermodynamic parameters of denaturation obtained for the two successive transitions are practically the same as those obtained from the model that takes into account all three states simultaneously (\(1/2N_2 \leftrightarrow 1/2I_2 \leftrightarrow D\)) (see the supplemental material).

\[\Delta G^0\] can always be expressed by the integrated Gibbs-Helmholtz equation (Equation 2),

\[
\Delta G^0 = \int_{T_0}^{T} \frac{\Delta H^0(T)}{T} \, dT + \Delta H^0(T_0) \left(1 - \frac{T}{T_0}\right) + \Delta C_\sigma^0(1 - \ln(T/T_0)) + \Delta C_p^0 \left(1 - \ln(T/T_0)\right)
\]

where \(\Delta H^0(T_0)\) is the standard enthalpy of denaturation at the reference temperature \(T_0\) (transition temperature at \(a = 0.5\)) and \(\Delta C_\sigma^0\) is the corresponding standard heat capacity change assumed to be independent of temperature. According to the model (Equation 1), one can express an average of a physical property, \(\bar{F}\) (in our case, \(P\)), in terms of the corresponding contributions \(F_N\) and \(F_p\), which characterize states N and D (47, 59), respectively (Equation 3).

\[
\bar{F} = (1 - a)F_N + aF_p = F_N + a(F_p - F_N)
\]

In the case of transitions followed by spectroscopic methods (CD and fluorescence), \(F_N\) and \(F_p\) are assumed to be linear functions of temperature (thermal denaturation) or urea concentration (urea denaturation). The model function \((C_p - C_N)^a\) for the DSC signal is the partial molar heat capacity of the protein relative to state N. It can be derived from the first partial derivative of Equation 3 on temperature at constant pressure (60–62) (Equation 4).

\[
(C_p - C_N) = a\Delta C_p^0 + \frac{(n(1 - a)(n - a(n - 1)) - \Delta H^0(T_0))}{(RT_0)^2}
\]

Taking into account Equations 1–4, the observed temperature profiles (melting curves) can be described in terms of the parameters \(\Delta H^0(T_0), \Delta C_p^0,\) and \(T_0\). Their values were obtained from fitting the model function (CD = Equation 3 and DSC = Equation 4) to the experimental temperature profiles using the Levenberg-Marquardt nonlinear \(\chi^2\) regression procedure (63). In the case of urea-induced denaturation at a given temperature, the denaturation profiles were calculated by Equations 1 and 3 combined with the well known empirical relation \(\Delta G^0 = \Delta G^0 - m\sigma\), where \(\Delta G^0\) is the standard Gibbs free energy in the absence of urea and \(m\) is the proportionality coefficient. At each temperature, the parameters \(\Delta G^0\) and \(m\) were adjusted according to the \(\chi^2\) regression procedure mentioned above. These values were then fitted by Equation 2 (for \(\Delta G^0 = \Delta G^0\)) to obtain the corresponding parameters \(\Delta H^0(T_0), \Delta C_p^0,\) and \(T_0\) in the absence of urea.

Structure-based Thermodynamic Calculations

The non-polar (\(A_p\)) and polar (\(A_p\)) solvent-accessible surface areas of proteins were calculated with NACCESS Version 2.1 (64). \(A_p\) and \(A_p\) of native MazE were obtained from the crystal structure of the MazE-VHII complex (probe size of 1.4 Å), and the unstructured residues were assumed to be exposed in the same way as they are in the extended Ala-X-Ala tripeptide. \(A_p\) and \(A_p\) contained by the denatured MazE were estimated as the sum of the corresponding accessibility of the protein residues in an extended Ala-X-Ala tripeptide. \(\Delta C_p^0\) accompanying MazE denaturation and dissociation (\(1/2N_2 \leftrightarrow D\)) was calculated from the corresponding changes in non-polar and polar accessible areas using the expression introduced by Murphy and Freire (65) (Equation 5).

\[\Delta C_p^0 = 0.45 \text{(cal mol}^{-1}\text{K}^{-1}\text{Å}^{-2}) \cdot (A_p - 0.26 \text{(cal mol}^{-1}\text{K}^{-1}\text{Å}^{-2}) \cdot A_p
\]

The same type of relations introduced by Makhatadze and Privalov (66), Spolar and Record (67), and Myers et al. (68) result in very similar \(\Delta C_p^0\) values for MazE denaturation and dissociation (\(1/2N_2 \leftrightarrow D\)). The enthalpy change accompanying the dissociation and denaturation of MazE (\(1/2N_2 \leftrightarrow D\)) was calculated using the parameterized expression for \(\Delta H^D\) introduced by Xie and Freire (69) (Equation 6).

\[\Delta H^D = -8.44 \text{(cal mol}^{-1}\text{Å}^{-2}) \cdot A_p + 31.4 \text{(cal mol}^{-1}\text{Å}^{-2}) \cdot A_p + \Delta C_p^0(T - 333.15)
\]

where the sum of the first two terms on the right-hand side represents the \(\Delta H^D\) value observed with most globular proteins at their median transition temperature of 60°C. The corresponding entropy change was calculated as a sum of four contributions (69–71) (Equation 7).

\[\Delta S^0 = \Delta S_{pol}^0 + \Delta S_{pol}^0 + \Delta S_p^0 + \Delta S_{pol}^0
\]

The solvation term \(\Delta S_{pol}^0\), which describes the exposure of polar and non-polar groups to the solvent upon protein denaturation and dissociation, was obtained as \(\Delta S_{pol}^0 = \Delta C_p^0 \ln(7385.15)\) (71, 72). The second term that reflects the change in the side chain conformational entropy upon dissociation (\(1/2N_2 \leftrightarrow N\)) was calculated as \(\Delta S_{pol}^0 = 4.3 \text{cal K}^{-1}\text{mol}^{-1}\) of Ala residues (66). The entropy change accompanying the unfolding of MazE was normalized to its A in the corresponding Ala-X-Ala tripeptide (70). The \(\Delta S_p^0\) values were those reported by Lee et al. (73). The third contribution \(\Delta S_p^0\) is due to mixing of the solvent and solute molecules when the number of solute molecules in the initial state differs from that in the final state. For the reaction \(1/2N_2 \leftrightarrow N\) carried out in a hypothetical 1 M standard state, this contribution amounts to \(\Delta S_{pol}^0 = -3.28 \text{cal K}^{-1}\text{mol}^{-1}\) (74). There is a considerable debate in the literature whether this contribution accounts for the entropy change due to the increase in translational/ rotational degrees of freedom upon dissociation (69, 70, 71, 75–85). Nevertheless, its value has been found as the most appropriate for description of protein-protein rigid body dissociations (70, 71, 75). The fourth contribution \(\Delta S_{rot}^0\) refers to the configurational entropy change that accompanies the unfolding of the protein (N \(\leftrightarrow D\)). It was estimated as \(\Delta S_{rot}^0 = (N)\cdot 4.3 \text{cal K}^{-1}\text{mol}^{-1}\) residue), where \(N\) is the number of amino acid residues participating in the unfolding process and 4.3 cal K\(^{-1}\) mol residue\(^{-1}\) is the average configurational entropy change associated with the passage of an amino acid from a buried to a solvent-exposed state (65). To correlate the energetics of denaturation of MazE with its structural features, the \(\Delta A_p\) and \(\Delta A_p\) values were estimated also from Equations 5 and 6 using the experimentally obtained \(\Delta H^D\) and \(\Delta C_p^0\) values (see Fig. 6c). Moreover, \(N\) was estimated from Equation 7 as \(N = (\Delta S_{rot}^0 + \Delta S_{pol}^0 + \Delta S_p^0)/4.3 \text{cal K}^{-1}\text{mol}^{-1}\) residue\(^{-1}\), where \(\Delta S^0\) is the measured standard entropy change (see Fig. 9d).

Mean Hydrophobicity and Mean Net Charge

The hydrophobicity of each amino acid sequence was calculated by the Kyte and Doolittle approximation (68) using a window size of five amino acids. The hydrophobicity of individual residues was normalized to a scale of 0–1 in these calculations. The mean hydrophobicity (\(H_p\)) is defined as the sum of the normalized hydrophobicities of all residues divided by the number of residues in the polypeptide. The mean net charge (\(R\)) is defined as the net charge at pH 7 divided by the total number of residues. The calculations for various addiction antigen and toxin sequences were performed using the Swiss Institute of Bioinformatics server ExPASy (available at www.expasy.ch) (67).
Energetics of MazE Structure

Distribution of Molecular Species Involved in Addiction Modules

The model for calculation of fractions of antitoxin and toxin species is defined by the postulates that were extracted from the thermodynamic and structural information on addiction modules (10, 11, 46–48, 88–92) and from the physical properties of an E. coli cell (available at redpoll.pharmacy.ualberta.ca/CCDB/cgi-bin/STAT_NEW.cgi). The postulates and the details on the numerical procedure are given in the Supplemental Material.

RESULTS

As shown by its crystal structure (Fig. 1a), MazE exists in a solid state as a dimer. We have proved by gel filtration that it exists as a dimer also in buffer solutions and that its dimeric state around room temperature remains unchanged under the experimental conditions applied in our studies.

The extent of reversibility of the observed thermally induced transitions was checked by performing two consecutive temperature scans (DSC and CD) and/or by measuring spectra (CD, intrinsic, and ANS fluorescence) after cooling down the MazE sample to the pre-transitional temperature. Reversibility of urea denaturation was examined by dialyzing concentrated urea solutions against the appropriate buffer and measuring an optical property (CD and fluorescence) before and after the dialysis. The extent of reversibility of all monitored transitions (except for the high temperature one at pH 1.5) is >80% which makes the laws of reversible thermodynamics applicable for their description. By contrast, the model-dependent parameters (Equation 1) that describe the high temperature transition at pH 1.5 (reversibility of ~30%) can serve only for comparative purposes (Table I).

pH Dependence of Thermally Induced Transitions

DSC—Thermograms between pH 1.5 and 9.4 show one transition at T > 60 °C, whereas at pH 1.5 and 3.1, we observed another transition at ~25 °C (Fig. 2). The high temperature transitions can be described in terms of the equilibrium two-state model (Equations 1, 2, and 4) with n = 2, whereas for the description of low temperature transitions, a two-state model with n = 1 should be used. The low temperature transitions are characterized at pH 1.5 by the model-independent (ΔHΔ = 9 kcal mol⁻¹) and model-dependent (ΔHΔ(Tb) = 9 kcal mol⁻¹ and Tb = 24 °C) quantities, whereas at pH 3.1, the corresponding values are ΔHΔ = 12 kcal mol⁻¹, ΔHΔ(Tb) = 13 kcal mol⁻¹, and Tb = 28 °C. ΔCp values were too small to be determined accurately and were therefore neglected in our model analysis. The successive low and high temperature transitions of MazE observed at pH 1.5 and 3.1 were described also by a three-state model (1/2N2 ↔ 1/2I2 ↔ D; see the Supplemental Material). The resulting ΔHΔ(Tb), ΔCp, and Tb values for both transitions were practically the same as those obtained when each transition was considered as an independent two-state transition. This agreement clearly indicates that the overlap of the two successive transitions is insignificant, and therefore, MazE denaturation can be discussed in terms of one or two independent two-state processes. Considering these facts, the thermodynamic stability of MazE at different conditions was described by the corresponding parameters (ΔHΔ(Tb), ΔCp, and Tb) derived from the model analysis of the measured DSC data (see Figs. 6 and 7 and Table 1).

CD Spectropolarimetry—The far-UV CD spectra measured at pH 7.1 and T = 37 °C (Fig. 1c) show that the fractions of α-helix, β-sheet, and the remainder of the structure estimated according to Provencher and Glöckner (93) are consistent with those observed in the structure of MazE within the crystal of the MazE-VHH complex (Fig. 1a). Furthermore, the far-UV CD spectra of MazE (Figs. 1c and 8a) show an increase in the CD signal upon lowering the pH from 7.1 to 1.5, which is typical for intrinsically flexible proteins (51). One may speculate that the increase in the CD signal at pH 1.5 observed at λ < 205 nm can be ascribed to the increased amount of the coil-like conformation, whereas at λ > 210, the increased CD intensity may be
The concentrations of MazE used in these experiments were 1.2 mg/ml (DSC), 0.5 mg/ml (far- and near-UV CD), and 0.12 mg/ml at pH 4.5.

Table I

| pH  | Tb, °C | ΔCp(Tb), kcal mol⁻¹ | ΔCp, kcal mol⁻¹ °C⁻¹ |
|-----|--------|----------------------|----------------------|
| 1.5 | 101    | 13                   | 0.00                 |
| 3.1 | 96     | 22                   | 0.14                 |
| 4.5 | 82     | 23                   | 0.25                 |
| 6.1 | 79     | 29                   | 0.32                 |
| 7.1 | 79     | 29                   | 0.25                 |
| 8.1 | 74     | 21                   | 0.12                 |
| 9.4 | 74     | 20                   | ±0.04                |

a Model-independent ΔH,ΔN values were determined by integration of the corresponding DSC thermograms.

b Values were determined for the high temperature transition (1/2I 2 N2 -> D; see “Results”).

The errors were estimated from repetitive experiments by variation of possible base-line positions defining the initial (native) and final (denatured) states. The errors obtained as square roots of diagonal elements of the variance-covariance matrix by fitting the corresponding model function (Equation 3 or 4) to the individual melting curves are much lower.

The far- and near-UV CD temperature profiles constructed at λ = 230 and 280 nm, respectively, show at pH 1.5–9.4 one transition at T > 60 °C, whereas at pH ≤4.5, another transition at ~25 °C was detected (Fig. 3, c and d). The melting curves describing the high temperature transitions show concentration dependence (Fig. 3, a, c, and d), indicating that these processes are not monomolecular (n > 1; see Equations 1–3). By contrast, low temperature transitions observed at pH ≤4.5 seem to be concentration-independent and can be characterized as monomolecular (n = 1). Some melting curves measured at higher concentrations do not level off at T < 100 °C (limitation of the method), which made the determination of the end (denatured) state as a linear function of temperature questionable (Equation 3). Consequently, the end states determined at lower concentrations were used in the model analysis of these curves. Fig. 3c shows a typical temperature profile at pH ≤4.5 measured at two different concentrations. The analysis of the three-state model (1/2N2 -> 1/2L2 -> D; see the Supplemental Material) confirms the observations from DSC measurements that the two transitions are rather independent (Fig. 3d). Wherever possible, the melting curves were analyzed in terms of an appropriate model (Equations 1–3: low temperature transitions, n = 1; and high temperature transitions, n = 2). In these cases, the model function (Equation 3) displays good agreement with experimentally measured mean residue ellipticities (Fig. 3), and the resulting ΔH(Tb), ΔCp,D, and Tb values agree well with those derived from DSC experiments (Table I).

**ANS Fluorescence**—Changes in ANS fluorescence are frequently used to detect non-native intermediate conformations of globular proteins (94–96). This is because such intermediates are characterized by the presence of solvent-exposed non-polar clusters that bind ANS molecules. As a result, an increase in ANS fluorescence intensity accompanied by a pronounced blue shift of the emission maximum is observed (95, 96). At pH ≤4.5, the observed changes in the intensity at T < 50 °C (Fig. 4) show that, under these conditions, at least a part of the MazE molecule exists in a (pre)molten globule-like conformation (52, 97). Furthermore, at pH 1.5, temperature-induced transitions at ~25 °C (red shift of ~3 nm) and 65 °C (red shift of ~5 nm) were observed that correspond well to the low and high temperature transitions observed with DSC and CD (Fig. 4b). At pH >4.5, no significant changes in the intensity or position of the emission maximum with increasing temperature were observed, which indicates that, at pH >4.5, neither the native dimeric state nor the denatured monomeric state of MazE contains exposed clusters of non-polar residues.
Thermal denaturation of MazE monitored by CD spectropolarimetry. a, far-UV CD spectra of MazE recorded between 26 and 98 °C at pH 7.1. The thick line represents the spectrum measured at 26 °C after heating to 98 °C. Inset, the corresponding melting curves constructed at a single wavelength (230 nm) measured at concentrations of 0.12 (solid line) and 0.05 (dotted line) mg ml⁻¹. Solid lines represent graphs of the best fit model function (Equation 3). b, near-UV CD spectra of MazE recorded between 26 and 98 °C at pH 7.1. The thick line represents the spectrum measured at 26 °C after heating to 98 °C. Inset, the corresponding melting curve constructed at a wavelength of 280 nm. The solid line represents the best fit model function (Equation 3). c, melting curves measured at pH 4.5 at concentrations of 0.12 (solid line) and 0.05 (dotted line) mg ml⁻¹. Solid lines represent graphs of the best fit model function (three-state, 1/2N₂ ↔ 1/2I₁ ↔ D; Equation 9 in the Supplemental Material). d, fractions of the intermediate dimeric (I₁) and denatured monomeric (D) forms at concentrations of 0.12 mg ml⁻¹ (solid line I₁; 1/2N₂ ↔ 1/2I₁ ↔ T₁₀ = 23 °C, ΔHᵣ(T₁₀) = 23 kcal mol⁻¹) and 0.04 mg ml⁻¹ (dotted line I₁; 1/2N₂ ↔ 1/2I₁ ↔ T₁₀ = 24 °C, ΔHᵣ(T₁₀) = 23 kcal mol⁻¹) and ΔCₚ = 0.01 kcal mol⁻¹ K⁻¹ and 0.04 mg ml⁻¹ (dotted line D; 1/2N₂ ↔ D ↔ T₁₀ = 82 °C, ΔHᵣ(T₁₀) = 23 kcal mol⁻¹) and ΔCₚ = 0.04 mg ml⁻¹ K⁻¹; and 0.05 mg ml⁻¹ K⁻¹); and dotted line D: 1/2I₁ ↔ D ↔ T₁₀ = 82 °C, ΔHᵣ(T₁₀) = 22 kcal mol⁻¹ and ΔCₚ = 0.1 kcal mol⁻¹ K⁻¹) calculated from the stated best fit parameters. deg, degrees.

**DISCUSSION**

**Energetics of the MazE Structure**—The stability curves (ΔG⁰ versus temperature) of MazE at pH 7.1 obtained from thermal and urea-induced denaturation are presented in Fig. 6 together with the corresponding enthalpy and entropy contributions. At T = 5–85 °C, the ΔG⁰ values obtained from urea-induced denaturation (ΔG⁰ = ΔG⁺, see “Experimental Procedures”) are slightly higher than those obtained from thermal denaturation. Because, in the urea-denatured state, the fraction of secondary and tertiary structure is substantially lower than in the thermally denatured state (Fig. 3), the corresponding decrease in ΔG⁰ may be ascribed mainly to the enhanced hydrophobic effect.

**Urea-induced Transitions**

Urea-induced denaturation of MazE at pH 7.1 was monitored by intrinsic fluorescence and far-UV CD. In Fig. 5a, an insert of the MazE structure is presented that shows that Trp is the only aromatic amino acid residue in the structured part of the MazE molecule (there are two more Trp residues in the unstructured part) that is exposed to a large extent to the solvent. This is reflected in the MazE fluorescence emission spectra, which display, in the measured solution, (λₘ₉ₐₓ)max values that are close to those observed with pure Trp in water. Our measurements also show that urea-induced denaturation of MazE is characterized, contrary to many globular proteins, by an increase in the intrinsic fluorescence intensity accompanied by a small blue shift of ~3 nm (Fig. 5b). This result is consistent with the measured CD spectra of MazE in urea solutions (Fig. 3, a and b), which show that, even in 8 M urea, neither the secondary nor tertiary structure of MazE disappears (98). Moreover, the observed blue shift and increase in fluorescence intensity may be indicative of a local structuring of residues around one, two, or all three Trp residues. Interestingly, a similar blue shift was observed also with the thermal denaturation of MazE in urea-free aqueous solutions. Fig. 5b also shows that the far-UV CD intensity decreased with increasing urea concentration and that urea-induced transitions, followed by either CD or fluorescence, can be described in terms of an appropriate two-state model (Equations 1 and 3 with n = 2).
accompanying the urea-induced denaturation, i.e. to the increased number of unfolded residues accompanied by an increase in the solvent-accessible surface area. This explanation is supported by the experimental observation that $\Delta C_p^0$ obtained from the urea denaturation (0.46 kcal mol$^{-1}$ K$^{-1}$) is significantly higher than $\Delta C_p^0$ determined from the thermal denaturation (0.23 kcal mol$^{-1}$ K$^{-1}$). In their excellent publications, Makhatadze and Privalski (66), Makhatadze and co-workers (99), Murphy and Freire (65), Xie and Freire (69), Spolar and Record (67), and Myers et al. (68) correlated protein structural features to the corresponding thermodynamics of unfolding.

In their structure-based calculations of thermodynamic quantities of unfolding, they used empirical parameterization based largely upon changes in polar ($\Delta \Delta P$) and non-polar ($\Delta \Delta N$) surface areas exposed to the solvent. $\Delta \Delta N$ and $\Delta \Delta P$ are usually calculated from the structural data. Thus, their values depend on the structural definition of the initial (native) and final (unfolded) states of the protein. The crystal structure of MazE (46) may be a good approximation of the free MazE dimer native form in solution because VHH does not interrupt MazE-DNA association (46, 47) and does not interfere with MazE binding (see the figure in the Supplemental Material). This approximation is supported by the analysis of the far-UV CD spectra, which, under physiological conditions, predict a MazE structure (46) that is close to the one observed in the crystal of the MazE-VHH complex (Fig. 1). The final (unfolded) state of MazE may be defined as a state in which each MazE residue ($x$) is exposed to the solvent in the same way as in the corresponding Ala-X-Ala tripeptide. Using the described initial and final states, we obtained the thermodynamic profile (Equations 5–7) for the complete unfolding and dissociation of MazE (1/2N$_U$$\rightarrow$ D) (Fig. 6), in which all 44 structured residues are involved in the changes of the solvent-exposed surfaces ($\Delta A_{\Delta N} = 2907 \, \text{Å}^2$, $\Delta A_P = 1498 \, \text{Å}^2$, and $\langle N \rangle = 44$). a, $\Delta G^0$ as a function of temperature. The symbols represent $\Delta G^0$ extrapolated to [urea] = 0 obtained from CD (■) and fluorescence (❖) measurements ($T_{m1}$). The dashed line is the best fit of Equation 2 to the CD data ($T_{m1} = 90.6 \, ^\circ\text{C}$, $\Delta H^0(T_{m1}) = 40.5 \, \text{kcal mol}^{-1}$, $\Delta C_P^0 = 0.46 \, \text{kcal mol}^{-1}$, and $C_T = 1.2 \, \text{mg ml}^{-1}$). b, $\Delta H^0$ as a function of temperature. c, $T \Delta S^0$ as a function of temperature.
In an attempt to correlate the structural and thermodynamic data, we also used an opposite approach in which ΔΛυ, ΔΛp, and Δ(N) that accompany MazE denaturation were calculated from experimental thermodynamic data (Equations 5–7). The calculations show that, upon denaturation and monomerization of MazE induced by urea (ΔΛυ = 1793 Å², ΔΛp = 1320 Å², and Δ(N) = 31), more residues unfolded, and more non-polar surface was exposed to solvent than in the case of thermal denaturation (ΔΛυ = 1113 Å², ΔΛp = 1035 Å², and Δ(N) = 21). The estimates are in complete agreement with our explanation of the discrepancy between ΔG°(urea) and ΔG°(thermal) and show that, at physiological pH, both thermally denatured and urea-denatured states are far from being completely unfolded (⟨N⟩ < the total number of structured residues, ⟨N⟩υ = 44). We would like to point out that these ΔΛυ, ΔΛp, and Δ(N) values comprise errors of the empirical parameterization and those of the measured quantities and can therefore be considered only as reasonably good approximations (see Fig. 8, c and d). Finally, we estimated the average number of unfolded residues (⟨N⟩) simply as (⟨N⟩ = (ΔΛυ + ΔΛp)υ/ΔΛυ(ΔΛυ+ΔΛp)(ΔΛυ+ΔΛp), where ΔΛυ and ΔΛp refer to the thermal or urea-induced denaturation of MazE, and ΔΛυ(U), ΔΛp(U), and Δ(N)υ are defined as described above. Fig. 8d shows that (⟨N⟩) values determined in this way are surprisingly close to the corresponding ⟨N⟩ values calculated from Equation 7, in which the configurational entropy change upon actual unfolding is taken as ΔSconf = (⟨N⟩-4.3 cal K⁻¹ (mol residue)⁻¹ (65). We believe that the observed agreement between the (⟨N⟩) values obtained from independent parameterization of ΔC°p and ΔH° on the one side and ΔS° on the other strongly supports the parameterization of the experimental quantities ΔC°p, ΔH°, and ΔS° suggested by Equations 5–7. In other words, the suggested parameterization seems to be successful provided that the “true” ΔΛυ and ΔΛp values are used.

As shown in Fig. 7a, the stability of the MazE dimer conformation strongly depends on pH. In the studied temperature range, the measured ΔG° versus pH curve exhibits a maximum at pH ~ 4.5. Such behavior may be explained in terms of different structural characteristics of the final (high temperature) monomeric state and the initial (low temperature) dimeric state. At physiological pH, the low temperature dimeric state contains the unstructured C-terminal half characterized by low mean hydrophobicity and low negative mean net charge (see Fig. 10). Lowering the pH results in reduction of the net charge, and consequently, the unstructured MazE chains start organizing themselves into a sort of compact molten globule-like conformation. This structuring effect is opposed by a simultaneous change in the conformation of the structured N-terminal half (low mean net charge and high mean hydrophobicity) (see Fig. 10), which is responsible for dimer formation. Because of the strong favorable contribution of the hydrophobic effect (mainly hydrophobic residues are involved in dimer formation) (Fig. 1b), a surplus of a positive charge on the N-terminal half at low pH does not break the dimer apart; however, it reduces the fraction of the secondary and tertiary structure. Inspection of data describing the high temperature monomeric state of MazE shows that, relative to the low temperature state, the secondary structure complexity of the high temperature state is about the same at all measured pH values (Fig. 8a). By contrast, the amount of the high temperature tertiary structure to the low temperature state is reduced to about the same extent only at pH 4.5. At lower pH values, this reduction appears to be much smaller (Fig. 8b). It appears that the thermodynamic parameters of denaturation result from the compensating effect of the partial folding of the unstructured C-terminal half and monomerization coupled with the partial unfolding of the structured N-terminal half. Inspection of Table I shows that lowering the pH thermally stabilizes the dimeric form of MazE (Tς, at a given MazE concentration increases with decreasing pH). Because, at pH 1.5–7.1, ΔC°p and ΔH° values decrease upon lowering the pH, the maximum stability reached at pH ~ 4.5 is most likely due to the simultaneous lowering of ΔS°conf. The suggested explanation is in full agreement with the calculations of ΔΛυ, ΔΛp, and ⟨N⟩ (Fig. 8, c and d) based on the experimental ΔC°p, ΔH°, and ΔS° values. They show that the net effect of pH and temperature is such that, at high temperature, fewer residues unfold, and fewer non-polar and polar surfaces become exposed to the solvent at pH ≈ 4.5 than at physiological pH. Moreover, ΔΛυ, ΔΛp, and ⟨N⟩ values are much lower than those calculated for the complete unfolding of MazE, thus indicating that, irrespective of the pH, the high temperature state of MazE is not a fully unfolded state.

Because of ~50% of the structured polypeptide chains that contain a substantial amount of tertiary structure at physiological temperature and pH, we cannot characterize the MazE dimer strictly as an intrinsically flexible (natively unfolded) protein (49–55, 97). However, to describe the changes in its spectral and thermodynamic properties, we have to take into account the intrinsically flexible nature of its C-terminal half (49–55, 97, 100). Furthermore, the flexible nature of MazE is hidden in the dimer-monomer equilibrium because the fractions of dimer and monomer depend on the total (dimer + monomer) MazE concentration, C重要意义（Fig. 1b）

Functional Stability of the Addiction Antitoxins—Addiction antitoxins and toxins can exist in the cell as free monomers and
inhibited either by low affinity binding of the antitoxin to the promoter/operator DNA (10, 11, 47, 89, 90) or by binding of the antitoxin-toxin complexes to the same part of the DNA with higher affinity (10, 48, 90, 92). According to these observations, one may expect that the strength of the specific interactions (binding affinities) within various addiction modules would be of similar magnitude. This led us to the construction of the equilibrium model that is able to predict some features essential for understanding the function of addiction modules (for details, see the Supplemental Material). The first aim of its application was to estimate the total concentrations of antitoxin \([A_p]\) and toxin \([T_p]\) under ordinary cellular conditions (in the absence of stress). It was assumed that, under steady-state conditions (the rate of antitoxin and toxin synthesis is the same as the rate of their degradation), the total concentrations of the antitoxin and toxin are about the same and that no toxin is bound to its cellular target. Because the total concentration of DNA (encoding for the antitoxin and toxin) in the cell can be estimated as 2.5 nm, the antitoxin and toxin concentrations were varied simultaneously from \(10^{-8}\) to \(10^{-5}\) M. Fig. 9e shows that the modeled addition module showed the highest sensitivity to the changes in \([A_p]\) and \([T_p]\) in the \(10^{-7}\) to \(10^{-6}\) M range (maximum sensitivity \((d\log[A_p])/(d\log[A_T])\) is at \(d\log[A_p] = \log([T_T]_{\text{max}}) \approx 6.6 \Rightarrow [A_p] = [T_T] \approx 2.5 \times 10^{-5}\) M). Consequently, it is not reasonable to expect that the cell would function at \([A_p] > [T_T] = 10^{-6}\) M, where practically all antitoxin- and toxin-antitoxin-binding sites on DNA are occupied (inhibition of expression). On the other hand, in the absence of stress, the cell would probably not function at \([A_T] \approx [T_T] < 10^{-7}\) M, where almost all antitoxin and toxin molecules are in the unbound state and their total number \((N)\) is very small \((N = 4)\) at \([A_T] \approx [T_T] = 10^{-8}\) M. Fig. 9b shows that, in this range, the most populated antitoxin-toxin complexes contain more \(T_T\) than \(A_p\) molecules (\(A_p T_T\) and \(A_p T_T\)). The \(A_p T_T\) form was also observed experimentally (48, 90, 92). By contrast, DNA is in the \(10^{-7}\) to \(10^{-6}\) M range occupied mainly by complexes in which \(A_p\) and \(T_T\) molecules are at a 1:1 ratio (Fig. 9c) and favors binding of longer \(A_p T_T\) associates, which is in accordance with recently proposed structural models (46, 48). Fig. 9d indicates how the fractions of various species change with a \([T_T]/[A_T]\) ratio at fixed \([T_T] = 2.5 \times 10^{-7}\) M. This simulation approximately shows the response of the addiction module under stress conditions when antitoxin and toxin expression is inhibited. With increasing \([T_T]/[A_T]\) ratios, the concentration of free dimeric toxin rises, and thus, it becomes available for interaction with its cellular target. If the rate of degradation of the antitoxin were known (for CcdA, \(T_{30} \approx 60\) min in the presence of CcdB) (101), it would not be difficult to convert the \([T_T]/[A_T]\) ratio to the time scale and to monitor the dying of the cell.

Our numerical simulation is based on the thermodynamic parameters estimated from in vitro experiments. To obtain measurable concentration-dependent signals, the number of antitoxin, toxin, and DNA molecules in the monitored systems has to be very large. Only under these conditions can we describe the system behavior in terms of macroscopic populations (fractions) of various species. Because the volume of the cell is very small (volume of the Escherichia coli cytoplasm of \(6.7 \times 10^{-16}\) liter), we are at “normal conditions” \((d\log[A_p])/(d\log[A_T]) \approx 2.5 \times 10^{-7}\) M) dealing with \(\approx 100\) antitoxin and \(\approx 100\) toxin molecules. If this is the case, practically every single molecule is important. To keep the cell alive, the regulation of an addiction module has to be very precise and rapid because it must compensate for even very small fluctuations in the number of antitoxin and toxin molecules. Our model shows that, in a long enough period of time (cell division cycle of Escherichia coli of \(\approx 20\) min), a single antitoxin molecule spends \(\approx 40\%\) of the time in complexes with toxin and

![Fig. 8. pH dependence of MazE structural characteristics.](image)
DNA, ~50% of time as a free dimer, and ~10% of time in the monomeric form (Fig. 9a).

An important feature of intrinsically flexible proteins is that they undergo disorder-order transitions during or prior to their biological function (49–57). In the case of addiction antitoxins, such conformational alterations have been observed upon both toxin and DNA binding (11, 47, 89, 90, 92). Moreover, we have shown that the antitoxin molecules may spend a significant amount of time as monomers with a higher potential for structural adaptability than the dimeric form. It seems that the rapid response of an addiction module to fluctuations in the number of antitoxin and toxin molecules is achieved not only by conformational adaptability of the dimeric antitoxin form, but also by transformation to the more flexible monomeric form. In this light, the thermodynamic characterization of the dimer-monomer equilibrium and the monomeric forms themselves is essential for understanding the functioning of the addiction modules. Fig. 10 shows that, according to the criterion introduced by Uversky (51, 52, 54) and Uversky et al. (53), the addiction antitoxins in the monomeric form have a high tendency to be partially unstructured, whereas the corresponding toxins tend to be structured even as monomers. The prediction of structuring based on the mean hydrophobicity and mean net charge of the protein polypeptide chain is in good agreement with our observations and with recently reported results on addiction modules (11, 47, 89, 90, 102). Moreover, the large amount of the random-coil form found in free antitoxins in solutions (11, 47, 89, 90, 102) represents high vulnerability for their proteolytic cleavage (25, 101, 103).

To the best of our knowledge, the numerical simulation presented here is the first attempt to combine thermodynamic and structural information on addiction modules in terms of a mathematical model. We are aware that some properties defined in the model differ from module to module. From the numerical simulation point of view (convergence), it is not a numerical simulation point of view (convergence), it is not a
data problem to increase the size (number) of the A₂T₂ complexes and the number of DNA-binding sites or/and to include more specific binding modes (more binding constants) in the model. It is also possible to include the kinetics (degradation rates for antitoxin and toxin) and the binding of the toxin to the specific target. The model has been developed to incorporate specific properties of a particular addiction module and to simulate some of its actions. At present, there are too little thermodynamic and structural data available to propagate the model. However, we believe that even this limited size of the model comprehends the basic physical meaning of the interplay between the antitoxin, toxin, and DNA.

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