Stability and DNA Binding of the Phd Protein of the Phage P1 Plasmid Addiction System

(Received for publication, November 2, 1998, and in revised form, November 17, 1998)

Ehud Gazit‡ and Robert T. Sauer§

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The plasmid addiction module of bacteriophage P1 encodes two proteins, Doc, a toxin that is stable to proteolytic degradation, and Phd, the toxin’s antidote that is proteolytically unstable. Phd has been shown to auto-regulate its expression by specific DNA binding. Here, we investigate the secondary structure and thermal stability of Phd, the effect of operator DNA binding on the structure and stability of Phd, and the stoichiometry, affinity, and cooperativity of Phd binding to operator subsites and intact operator DNA. Phd folds as a monomer at low temperatures or in the presence of osmolytes but exists predominantly in an unfolded conformation at 37 °C. The native state of Phd is stabilized by operator binding. Two Phd monomers bind to each operator subsite, and four monomers bind to the intact operator. The subsite binding reaction shows a second-order dependence on protein concentration and monomer-bound DNA species are unpopulated, suggesting that two Phd molecules bind cooperatively to each operator subsite. In intact operator binding experiments, both dimer-bound and tetramer-bound DNA species are populated, and binding occurs at protein concentrations similar to those required for subsite binding, suggesting that there is no significant dimer-dimer cooperativity.

The stable and efficient maintenance of low-copy plasmids within bacterial cells is ensured, in part, by addiction mechanisms mediated by specific proteins (1, 2). For example, when bacteriophage P1 lysogenizes Escherichia coli as a low copy plasmid, the rate of spontaneous plasmid loss is only about one per 10⁵ generations (3). Two proteins comprise the plasmid addiction system of bacteriophage P1: Doc (death on cure), a 126-residue toxic protein, and Phd (prevent host death), a 73-residue antidote (4). This system functions to kill cells that have been cured of the plasmid. The addiction mechanism depends on significant differences in the proteolytic stability of Doc, which is resistant to proteolysis, and Phd, which is degradable in part dependent on the host-encoded ClpXP protease complex (5). In P1 lysogens, a concentration of Phd sufficient to suppress Doc toxicity is maintained by a continuous synthesis of Phd molecules de novo. In a bacterial cell that has lost the P1 genome, existing Phd is degraded and because there is no further synthesis of new Phd, levels fall, and the cytotoxically inherited Doc kills the P1-free daughter cells.

Post-segregational killing of plasmid-cured cells is also used by other low-copy number plasmids, including the F (6), RK2 (7), and R1 (8) plasmids of E. coli, and similar systems in Streptomyces lividans and Klebsiella oxytoca (for review, see Refs. 1 and 9). In each case studied, a long-lived toxin and short-lived antidote are part of the addiction mechanism. Interestingly, a functionally similar two-protein module is encoded by the maeE and maeF genes of E. coli (10), which are regulated by the cellular level of ppGpp, an indicator of amino acid starvation. Functional homologues of the Phd and Doc proteins are also found upstream of the Ecoprii type IC DNA restriction and modification operon (11). These results suggest that addiction system homologues may function in the response to starvation and DNA restriction.

The Phd and Doc genes are expressed from the same operon (4), and Phd negatively regulates its own expression as well as that of Doc (12), ensuring a relatively low level of expression of both proteins in P1 lysogens. The DNA site bound by Phd is 23 base pairs in length and includes two 10-base pair subsites that are roughly palindromic and separated by 3 base pairs (12) (see Sequence 1). In footprinting experiments, Phd occupies the left subsite at roughly 10-fold lower concentrations than the right subsite (12). Studies in vivo show that Phd alone is sufficient to repress transcription from the plasmid addiction operon (12).

In this work, we characterize the secondary structure and thermal stability of the purified Phd protein, study the effect of operator DNA binding on the stability of Phd, and determine the stoichiometry, mechanism, and cooperativity of Phd binding to the intact operator DNA and to operator subsites. The results are discussed in the context of the plasmid addiction mechanism. Phd shows some functional and sequence similarities to Arc, MetJ, and other members of the ribbon-helix-helix family of DNA-binding proteins (12–14), but the studies reported here suggest that Phd has a different structure from Arc.

MATERIALS AND METHODS

Protein Purification—E. coli transformed with plasmid pHAL20 (encoding Phd under tac-promoter control) (12) was grown in LB broth containing 100 μg/ml ampicillin (LB-amp) at 37 °C to an A₆₀₀ of approximately 0.7, and protein expression was induced by the addition of 100 μg/ml isopropyl-1-thio-β-D-galactopyranoside for 30 min. Cells were harvested by centrifugation and stored frozen at −80 °C prior to purification. The cells were thawed at 4 °C, resuspended in buffer A (50 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA) with 50 μM phenylmethylsulfonyl fluoride, and lysed by sonication. Insoluble material was removed by centrifugation for 10 min at 13,000 × g, followed by a 0.22-μm filtration. The supernatant was applied to a Mono S HR 5/5 FPLC column (Amersham Pharmacia Biotech) equilibrated in buffer A, and proteins were eluted with a gradient from 50 to 1000 mM NaCl. A peak that included Phd eluted at approximately 400 mM NaCl. This peak was applied to a reverse-phase semipreparative C₄ column (Vydac) and was resolved by using a 0—80% acetonitrile gradient in water and 0.1% trifluoroacetic acid. At this point, Phd was greater than 95% pure as assessed by Coomassie staining of SDS-polyacrylamide gels.

* This work was supported by National Institutes of Health Grants AI-15706 and AI-16892. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by postdoctoral fellowships from the European Molecular Biology Organization and the Human Frontiers Science Foundation Program.

§ To whom correspondence should be addressed: MIT 68-571, Cambridge, MA 02139. E-mail: bobsauer@mit.edu.
matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry performed using a Voyager-DE STR Biospectrometry Workstation confirmed the molecular mass expected for purified Phd with no modifications (8128 daltons).

**Biophysical Studies**—Circular dichroism (CD) spectra were obtained by using an Aviv 606S spectropolarimeter equipped with a temperature-controlled sample holder and a 10-mm path length cuvette. Mean residue ellipticity, \( [\theta] \), was calculated as,

\[
[\theta] = \frac{100 \times \theta \times m}{c \times L}
\]

where \( \theta \) is the observed ellipticity, \( m \) is the mean residue weight, \( c \) is the concentration in mg/ml, and \( L \) is the path length in centimeters.

The concentration of Phd was determined by measuring tyrosine absorbance both at pH 7 and in 0.1 M KOH using a Hewlett-Packard 8452A diode array spectrophotometer. Protein concentrations were calculated by using extinction coefficients of 1394 M\(^{-1}\) cm\(^{-1}\) (274 nm at pH 7) and 2377 M\(^{-1}\) cm\(^{-1}\) (294 nm in 0.1 M KOH) for the single tyrosine of Phd. For thermal denaturation experiments, samples in a buffer containing 50 mM NaCl, 100 mM Tris-HCl (pH 7.4), 1 mM EDTA were equilibrated at each temperature for 1 min, and the CD ellipticity at 222 nm was averaged for 2 min. Experiments were performed at protein concentrations ranging from 0.5 to 15 \( \mu \)M. Melting curves were fit to a two-state transition between native and denatured protein by nonlinear least squares fitting using the program NONLIN for Macintosh (15).

**DNA Binding Assays**—Equilibrium binding of Phd to its operator DNA site and to DNA subsites was monitored by a polyacrylamide gel mobility shift assay as described (16–18). Single-stranded DNA oligomers were 5 end-labeled by incubation with \( [\gamma^3P]dATP \) and T4 polynucleotide kinase (New England Biolabs) for 45 min at 37 °C. Enzyme was removed from the reaction by phenol:chloroform extraction, and unincorporated nucleotides were removed by using a G-25 Sephadex Quick Spin column (Boehringer Mannheim). The second DNA strand was than annealed by mixing equimolar amounts of the complementary oligonucleotides, heating to 90 °C, and cooling slowly. Labeled oligomers were diluted to approximately 5 \( \times 10^5 \) counts/min (~10 ps). Synthetic DNA oligomers that were used included a 32-bp oligomer that contained the 23-bp operator DNA site of Phd (underlined) in binding buffer and incubated for 2 h. The gel-mobility assays were quantified using a Molecular Dynamics PhosphorImager and IMAGEQUANT software.

**Stoichiometry Assays**—To determine the molar ratio of Phd bound to its palindromic DNA subsites, increasing amounts of Phd were added to 1 \( \mu \)M left subsite DNA in binding buffer and incubated for 2 h. The concentration of DNA and protein in this experiment was much higher than the Phd-DNA dissociation constant, to ensure a nearly complete binding of the protein to the DNA. A small amount (~1 nm) of radiolabeled DNA was also added to quantify the ratio of bound to unbound DNA, following electrophoresis on a 8% polyacrylamide gel at 250 V in TBE buffer.

**RESULTS**

**Secondary Structure and Thermal Stability of Phd**—Phd was purified to homogeneity as described under “Materials and Methods.” Fig. 1A shows the CD spectra of Phd at 4 °C and 37 °C, and also at 37 °C in buffer plus trimethylamine N-oxide (TMAO). B, thermal denaturation. Thermal stability was determined by monitoring CD ellipticity as a function of temperature in 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA at protein concentrations of 1 or 15 \( \mu \)M.

**Fig. 1.** A, circular dichroism spectra. CD spectra of Phd were taken in a 0.1-cm cuvette in buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA at 4, at 37, and at 37 °C in the presence of 2 mM trimethylamine N-oxide (TMAO). B, thermal denaturation. Thermal stability was determined by monitoring CD ellipticity as a function of temperature in 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA at protein concentrations of 1 or 15 \( \mu \)M.

mixtures were incubated for at least 2 h. After this time, glycerol was added to each sample (to a final concentration of 5%), and the samples were loaded onto an 8% polyacrylamide gel and electrophoresed at 250 V in TBE buffer (90 mM Tris borate, 2 mM EDTA). The gel-mobility assays were quantified using a Molecular Dynamics PhosphorImager and IMAGEQUANT software.

**Stoichiometry Assays**—To determine the molar ratio of Phd bound to its palindromic DNA subsites, increasing amounts of Phd were added to 1 \( \mu \)M left subsite DNA in binding buffer and incubated for 2 h. The concentration of DNA and protein in this experiment was much higher than the Phd-DNA dissociation constant, to ensure a nearly complete binding of the protein to the DNA. A small amount (~1 nm) of radiolabeled DNA was also added to quantify the ratio of bound to unbound DNA, following electrophoresis on a 8% polyacrylamide gel at 250 V in TBE buffer.

**RESULTS**

**Secondary Structure and Thermal Stability of Phd**—Phd was purified to homogeneity as described under “Materials and Methods.” Fig. 1A shows the CD spectra of Phd at 4 °C and 37 °C, and also at 37 °C in buffer plus trimethylamine N-oxide, an osmolyte that stabilizes native structure (19). The amount of secondary structure was estimated from the CD spectrum (20, 21). At 37 °C in buffer alone, Phd seems to be in a largely

---

**TABLE 1**

| Sequence | Oligonucleotide Sequence |
|----------|--------------------------|
| 5′-TGTGT ACACA TAA CGAGT ACACG-3′ | 3′-ACACA TGTGT ATT GCTCA TGTGC-5′ |
| 5′-GGCTGTGTAACATACCGTACCGAGTTCG-3′ | 3′-CCGACACATGATGATGTTCGTGCTCCAC-5′ |
| 5′-GGACCGGCTTGACATACCATAGGGTG-3′ | 3′-CGTCCGCAACATGATGATTCCTCCAC-5′ |
| 5′-GGGAGGATGACCTACCGAGTTG-3′ | 3′-CTCCATATGATGATGATTCCTCCAC-5′ |

**Sequence 2**

or 26-bp oligomers that included the 10-bp left or right operator subsites (underlined).

**Sequence 3 and 4**

As a control, a 27 bp oligomer that contained the P22 Arc repressor operator was used.

5′-GCTATGACGATGCTCTATCATGATG-3′
3′-GCTATGACGATGCTCTATCATGATG-5′

**Sequence 5**

DNA binding assays were performed at room temperature in binding buffer containing 50 mM NaCl, 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μg/ml bovine serum albumin, and 0.02% Nonidet P-40. Serial dilutions of the Phd protein were added to the labeled DNA, and the

---

1 The abbreviation used is: bp, base pair(s).
unfolded, random-coil conformation. However, at 4 °C or at 37 °C in the presence of trimethylamine N-oxide, Phd has a CD spectra indicative of a folded protein containing approximately 45% α-helix (Fig. 1A).

Denaturation experiments monitored by CD were performed to determine the thermal stability of Phd (Fig. 1B). The thermal denaturation of Phd is cooperative, with a Tm of about 25 °C. As expected, Phd is predominantly unfolded at 37 °C. Superimposable denaturation curves were observed at Phd concentrations of 1 and 15 μM, suggesting that the native form of Phd is stable as a monomer (Fig. 1B). The thermal denaturation of Phd was fully reversible in a reverse melt, indicating that the protein can readily alternate between its folded and unfolded states.

Stabilization of Phd by DNA Binding—To study the effect of DNA binding on Phd structure, increasing amounts of a 32-bp oligomer containing the phd operator site were added to a solution containing Phd, and CD spectra were taken (Fig. 2A). DNA binding induces a dose-dependent increase in Phd structure, suggesting that the folding and site-specific DNA binding of Phd are coupled. The ellipticity at 228 nm (a wavelength at which B-DNA does not contribute to the CD signal, see Fig. 2B) is plotted as a function of DNA concentration in Fig. 2C. The slope of the initial part of the titration curve shows that the amount of operator DNA needed for induction of fully folded Phd is roughly one-quarter of the protein concentration, suggesting that Phd binds to its operator DNA as a tetramer. When nonspecific DNA (a 27-bp DNA oligomer containing the arc operator (22)) was added as a control, no increase in the CD signal of Phd at 228 nm was observed over the range of DNA concentrations tested (Fig. 2C).

Thermal denaturation of Phd was performed in the presence of increasing amounts of the phd operator DNA (Fig. 3). The Tm of Phd increases as a function of the concentration of operator DNA (Fig. 3B), demonstrating stabilization of the native Phd structure by DNA binding. No significant change in the Tm of Phd was observed when nonspecific DNA was added at concen-
Stoichiometry of Phd Binding to Operator Subsites—
To determine the oligomeric form of Phd that binds to an operator subsite, increasing amounts of Phd were added to a 1 μM solution of left subsite DNA, and binding was assayed by a gel-mobility-shift assay (Fig. 4A). A single bound complex was observed. At the DNA concentration used for this experiment, almost all of the added Phd becomes DNA-bound (see below), and the stoichiometry of binding can be calculated from the slope of the rising portion of the titration curve (Fig. 4B). In the experiment shown, this slope is 0.52, indicating that Phd binds as a dimer.

Equilibrium Binding of Phd to Operator DNA—
Fig. 5A and B, show a gel-mobility-shift assay of Phd binding to DNA fragments containing the left (L) operator subsite (A) or the right (R) operator subsite (B). C, Scatchard plot (bound DNA in arbitrary Phosphor Imager units) of Phd binding to the left and right operator subsites (~10 pM) at room temperature in 50 mM NaCl, 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μg/ml bovine serum albumin, and 0.02% Nonidet P-40. Designation: circles, left subsite; squares, right subsite.

plot of the binding data is concave downward as expected for a reaction displaying positive cooperativity (Fig. 5C). Fits of this binding data reveal that subsite DNA binding shows an approximate second-order dependence on Phd concentration as expected for the model,

\[
2P + S \rightleftharpoons P_S K_d = [P]^2/[P_S]
\]

MODEL 1

where P represents a mixture of folded and unfolded Phd monomers and S represents subsite DNA. At half-maximal binding, \(K_d = [P]^2\) or about 6×10^{-16} M^2 for the left subsite and 1×10^{-15} M^2 for the right subsite binding experiments shown in Fig. 5.

Gel-mobility-shift assays were also used to assay Phd binding to the intact operator site (Fig. 6). In these experiments, both Phd dimer-bound and tetramer-bound DNA species are observed in the gel. The dimer-bound species are observed at lower Phd concentrations than the tetramer-bound species but continue to persist as populated intermediates as the Phd concentration is raised. Moreover, binding to the full-site DNA is observed over the same general range of Phd concentrations up to 500 nM.

Stoichiometry of Phd Binding to Operator Subsites—
To determine the oligomeric form of Phd that binds to an operator subsite, increasing amounts of Phd were added to a 1 μM solution of left subsite DNA, and binding was assayed by a gel-mobility-shift assay (Fig. 4A). A single bound complex was observed. At the DNA concentration used for this experiment, almost all of the added Phd becomes DNA-bound (see below), and the stoichiometry of binding can be calculated from the slope of the rising portion of the titration curve (Fig. 4B). In the experiment shown, this slope is 0.52, indicating that Phd binds as a dimer. This value is consistent with the finding that four molecules of Phd are stabilized by binding to the intact operator, which contains two subsites. Because Phd in solution is an equilibrium mixture of folded and unfolded monomers, the subsite DNA binding reaction involves both folding and dimerization of the protein.

Equilibrium Binding of Phd to Operator DNA—
Fig. 5, A and B, show a gel-mobility-shift assay of Phd binding to DNA fragments containing the left (L) operator subsite (A) or the right (R) operator subsite (B). C, Scatchard plot (bound DNA in arbitrary Phosphor Imager units) of Phd binding to the left and right operator subsites (~10 pM) at room temperature in 50 mM NaCl, 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μg/ml bovine serum albumin, and 0.02% Nonidet P-40. Designation: circles, left subsite; squares, right subsite.

plot of the binding data is concave downward as expected for a reaction displaying positive cooperativity (Fig. 5C). Fits of this binding data reveal that subsite DNA binding shows an approximate second-order dependence on Phd concentration as expected for the model,

\[
2P + S \rightleftharpoons P_S K_d = [P]^2/[P_S]
\]

MODEL 1

where P represents a mixture of folded and unfolded Phd monomers and S represents subsite DNA. At half-maximal binding, \(K_d = [P]^2\) or about 6×10^{-16} M^2 for the left subsite and 1×10^{-15} M^2 for the right subsite binding experiments shown in Fig. 5.

Gel-mobility-shift assays were also used to assay Phd binding to the intact operator site (Fig. 6). In these experiments, both Phd dimer-bound and tetramer-bound DNA species are observed in the gel. The dimer-bound species are observed at lower Phd concentrations than the tetramer-bound species but continue to persist as populated intermediates as the Phd concentration is raised. Moreover, binding to the full-site DNA is observed over the same general range of Phd concentrations up to 500 nM.
as binding to the left subsite DNA (compare Figs. 5A and 6A). Both of these observations suggest that there is little, if any, mutual stabilization between Phd dimers bound at adjacent operator subsites. Indeed, the full-site binding curve can be fit by independent binding of Phd to the isolated left and right operator subsites (Fig. 6B).

Using the gel-mobility-shift assay, we attempted to measure the dissociation kinetics of complexes of Phd with the left operator subsite and with intact operator DNA. In both cases, however, dissociation was complete by the first time point. These experiments allow us to place an upper limit of approximately 10 s on the half-life of these Phd-DNA complexes.

**DISCUSSION**

In the studies reported here, the Phd protein of the plasmid addiction module of phage P1 was purified to homogeneity and assays probing structural stability and DNA binding were performed. Phd has the CD spectrum of a predominantly denatured protein at 37 °C, and only folds at this temperature when bound to operator DNA, or when stabilized by high concentrations of osmolytes. In its native state, Phd has a CD spectrum of a protein that is roughly 45% α-helical. Because denatured proteins are generally subject to proteolytic destruction in E. coli (23), it seems likely that the thermal instability of free Phd contributes to its degradation in the cell (5). In a cell containing the phage P1 genome, free Phd should be unfolded and sensitive to degradation, whereas operator-bound Phd should be folded and less susceptible to degradation. Moreover, the continual synthesis of new Phd maintains sufficient steady-state levels of Phd to inhibit the toxicity of Doc. In a cell that has lost the P1 genome, there is no operator to bind, new Phd synthesis cannot occur, and the degradation of free Phd eventually leads to Doc-mediated cell death (5).

The phd operator overlaps the promoter for Phd and Doc transcription, and Phd represses this operon and its own synthesis (12). This ensures that when Phd levels in a P1 lysogen fall too low, transcription is derepressed and new synthesis of Phd can occur. Our studies show that two Phd monomers bind cooperatively to each left and right operator subsite. As a consequence, operator occupancy changes as a function of the second power of the Phd concentration, and the system is quite responsive to moderate changes in steady-state Phd levels. For example, a 5-fold drop in Phd concentration would cause a 25-fold increase in the ratio of free to bound operator. Although binding of Phd to operator occurs in the 10 nM range, the half-life of the protein-DNA complex appears to be less than 10 s. Such a rapid dissociation rate might ensure a dynamic system capable of responding rapidly to sudden decreases in Phd levels. If the half-life of the Phd-operator complex were too long, a precipitous drop in Phd levels might result in Doc toxicity before transcription could be derepressed to permit new synthesis.

Phd shares some sequence similarities with the phage P22 Arc repressor and additional members of the ribbon-helix-helix family of transcription factors and also has a DNA recognition site whose overall size and organization is similar to the operators of many of these ribbon-helix-helix proteins (12). Some of the findings here extend these similarities. For example, Phd binds to each operator subsite as a dimer and to the intact operator as a tetramer in a fashion analogous to Arc repressor (24, 14). However, our studies show that Phd differs, in one very important way, from Arc and its relatives. The intertwined nature of the native Arc dimer permits folding only as a
dimer and dissociation of Arc dimers leads to denaturation (25). Phd, by contrast, is capable of folding as a monomer. This difference between Phd and Arc indicates that the native folds of these two proteins must be significantly different.

Another difference between Arc and Phd involves the presence or absence of dimer-dimer cooperativity in binding to their respective intact operators. Phd dimers bound to adjacent operator subsites do not appear to be mutually stabilizing and still dissociate rapidly, whereas Arc dimers bind in a highly cooperative fashion and stabilize a complex with a half-life in excess of 1 h (26). As discussed above, however, the short half-life of the Phd-operator complex may be functionally important in allowing rapid transcriptional responses to decreases in Phd concentration.

The P1 plasmid addiction system raises a number of intriguing physiological issues about mechanism and bacterial cell death and also provides a model system to study the interplay of protein folding, protein degradation, protein-protein interactions, and protein-DNA interactions. The studies reported here should provide a basis for future biochemical, biophysical, and structural studies of many of these issues.

Acknowledgments—We thank Roy Magnuson and Michael Yarmolinsky for a gift of the pHAL20 plasmid and Carl Pabo and Tania Baker for use of equipment.

REFERENCES
1. Jensen, R. B. & Gerdes, K. (1995) Mol. Microbiol. 17, 205–210
2. Yarmolinsky, M. B. (1995) Science 267, 836–837
3. Rosner, J. L. (1972) Virology 49, 679–680
4. Lehnherr, H., Maguin, E., Jafri, S. & Yarmolinsky, M. B. (1993) J. Mol. Biol.

233, 414–428
5. Lehnherr, H. & Yarmolinsky, M. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3274–3277
6. Loh, S. M., Cram, D. S. & Skurray, R. A. (1988) Gene (Amst.) 66, 259–266
7. Young, C., Prince, A. S. & Figurski, D. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7374–7378
8. Gerdes, K., Rasmussen, P. B. & Molin, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3116–3120
9. Holčík, M. & Iyer, V. N. (1997) Microbiology 143, 3403–3416
10. Aizenman, E., Engelberg-Kulka, H. & Glaser, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6059–6063
11. Tyndall, C., Lehnherr, H., Sandmeier, U., Kulik, E. & Bickle, T. A. (1997) Mol. Microbiol. 23, 729–736
12. Magnuson, R., Lehnherr, H., Mukhopadhyay, G. & Yarmolinsky, M. B. (1996) J. Biol. Chem. 271, 18705–18710
13. Phillips, S. E., Manfield, I., Parsons, I., Davidson, B. E., Rafferty, J. B., Somers, W. S., Margarita, D., Cohen, G. N. I., S.-G. & Stockley, P. G. (1989) Nature 341, 711–715
14. Raumann, B. E., Roul, M. A., Pabo, C. O. & Sauer, R. T. (1994) Nature 367, 754–757
15. Brenstein, R. J. (1989) NONLIN for Macintosh, Robelko Software, Carbondale, IL
16. Fried, M. G. & Crothers, D. M. (1981) Nucleic Acids Res. 9, 6505–6525
17. Vershon, A. K., Bowie, J. U., Karplus, T. M. & Sauer, R. T. (1986) Proteins: Struct. Funct. Genet. 1, 302–311
18. Vershon, A. K., Liao, S. M., McClure, W. R. & Sauer, R. T. (1987) J. Mol. Biol. 195, 311–322
19. Wang, A. & Bolen, D. W. (1997) Biochemistry 36, 9101–9108
20. Andrade, M. A., Chacon, P., Merelo, J. J. & Morán, F. (1993) Protein Eng. 6, 383–390
21. Merelo, J. J., Andrade, M. A., Prieto, A. & Morán, F. (1994) Neurocomputing 6, 443–454
22. Vershon, A. K., Liao, S. M., McClure, W. R. & Sauer, R. T. (1987) J. Mol. Biol. 195, 323–331
23. Parsell, D. A. & Sauer, R. T. (1989) J. Biol. Chem. 264, 7590–7595
24. Brown, B. M., Bowie, J. U. & Sauer, R. T. (1989) Biochemistry 28, 11189–11195
25. Bowie, J. U. & Sauer, R. T. (1989) Biochemistry 28, 7139–7143
26. Brown, B. M. & Sauer, R. T. (1993) Biochemistry 32, 1354–1363