Reversible Folding of Ada Protein (O^6-Methylguanine−DNA Methyltransferase) of *Escherichia coli*†

Debasish Bhattacharyya,‡,§ Tapas K. Hazra,|| W. David Behnke,⊥ Parkson L.-G. Chong,▽ Alexander Kurosky,|| J. Ching Lee,|| and Sankar Mitra*,‡,||

University of Tennessee Graduate School of Biomedical Sciences, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, Department of Biochemistry, University of Cincinnati, School of Medicine, Cincinnati, Ohio 45267, Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208, and Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Route 1079, Galveston, Texas 77555

Received July 29, 1997; Revised Manuscript Received November 10, 1997

ABSTRACT: The multifunctional 39 kDa *Escherichia coli* Ada protein (O^6-methylguanine−DNA methyltransferase) (EC 2.1.1.63), product of the *ada* gene, is a monomeric globular polypeptide with two distinct alkylacceptor activities located in two domains. The two domains are of nearly equal size and are connected by a hinge region. The Ada protein accepts stoichiometrically the alkyl group from O^6-alkylguanine in DNA at the Cys-321 residue and from alkyl phosphotriester at the Cys-69 residue. This protein functions in DNA repair by direct dealkylation of mutagenic O^6-alkylguanine. The protein methylated at Cys-69 becomes a transcriptional activator of the genes in the *ada* regulon, including its own. Each of the two domains functions independently as an alkyl acceptor. The purified homogeneous protein is unstable at 37 °C and spontaneously loses about 30% of its secondary structure in less than 30 min concomitant with a complete loss of activity. However, sedimentation equilibrium studies indicated that the inactive protein remains in the monomeric form without aggregation. Furthermore, electrospray mass spectroscopic analysis indicated the absence of oxidation of the inactive protein. This temperature-dependent inactivation of the Ada protein is inhibited by DNA. In the presence of increasing concentrations of urea or guanidine, the protein gradually loses more than 80% of its structure. The two alkyl acceptor activities appear to be differentially sensitive to unfolding and the phosphotriester methyltransferase activity is resistant to 7 M urea. The partial or complete unfolding induced by urea or guanidine is completely reversed within seconds by removal of the denaturant. The heat-coagulated protein can also be restored to full activity by cycling it through treatment with 8 M urea or 6 M guanidine. These results suggest that the nascent or unfolded Ada polypeptide folds to a metastable form which is active and that the thermodynamically stable structure is partially unfolded and inactive.

The 39 kDa, monomeric Ada protein (O^6-methylguanine−DNA methyltransferase, also abbreviated as MGMT[1]) (EC 2.1.1.63), the product of the *ada* gene, plays a central role in alkylation damage repair in *Escherichia coli*. The protein is also unique in acting as a stoichiometric alkyltransferase for two different substrates (*1*, *2*). These activities reside in two distinct functional domains. The Cys-69 residue in the N-terminal half of the protein as a phosphotriester−DNA methyltransferase (PMT) accepts alkyl groups from S_p diastereoisomers of alkylphosphotriester residues. Cys-321 in the C-terminal half with the O^6-methylguanine−DNA methyltransferase (MGMT) accepts alkyl groups from O^6-alkylguanine and O^4-alkylthymine in DNA (*3*). These alkyl adducts, as well as *N*-alkylpurines, are produced following exposure to simple alkylating mutagens and carcinogens such as *N*-methyl-*N*-nitrosourea (MNU). The Ada protein methylated at Cys-69 is a transcriptional activator of the *ada* regulon and acts by turning on transcription of its own gene and that of *alkA* and possibly several other alkylation repair genes by binding to the activator box named "ada box" (*4*−*7*). The distinct functional domains appear to function independently corresponding to the N- and C-terminal halves

† This work was supported in part by NIH Grants ES07572, ES06676, and CA31721 (to S.M.); by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-840R21400 with Martin Marietta Energy Systems, Inc., by NSF Grant RIMI-9014052 (to P.L.-G.C.); by Robert A. Welch Foundation Grants H-1238 and H0013 and NIH Grant RR8961 (to J.C.L.); and by NIH Grant NS29261 (to A.K.).

* To whom correspondence should be addressed at Sealy Center for Molecular Science, University of Texas Medical Branch, Route 1079, Galveston, Texas 77555.

‡ University of Tennessee Graduate School of Biomedical Sciences.

§ Present address: Indian Institute of Chemical Biology, Calcutta 700 032, India.

|| University of Texas Medical Branch.

⊥ University of Cincinnati School of Medicine.

▽ Meharry Medical College. Present address: Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140.

[1] Abbreviations: CD, circular dichroism; CT DNA, calf thymus DNA; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; MGMT, O^6-methylguanine−DNA methyltransferase; MNU, *N*-methyl-*N*-nitrosourea; PAGE, polyacrylamide gel electrophoresis; PMT, methylphosphotriester−DNA methyltransferase; SDS, sodium dodecyl sulfate.

S0006-2960(97)01852-7 CCC: $15.00
Published on Web 01/27/1998

of the protein linked by a protease-hypersensitive hinge region (*3, 8*). The tertiary structure of the full-length protein has not been elucidated although the X-ray crystallographic structure of the 19 kDa C-terminal half with MGMT activity was published (*9*). More recently, Verdine, Wagner, and their collaborators elucidated the solution structure of the 10 kDa N-terminal region of the Ada protein, which has the PMT activity but lacks the sequence-specific DNA binding domain (*10, 11*). Their studies have shown that this fragment has a tightly bound Zn^{2+} that is necessary for proper folding of the protein. Furthermore, functional switch from a methyltransferase to a transcriptional activator involves Zn^{2+} binding to the methylcysteine residue (*12*). Another unusual feature of the Ada protein is that, unlike many other known activators and repressors, it is present as a monomer in native state both with or without methylation (*8*).

In the course of physicochemical studies of the Ada protein, we observed that its methyltransferase activities were not completely inhibited by high concentrations of urea, which warranted a detailed investigation of its unfolding properties. Such a study is all the more important because, while biochemical evidence suggests that the Ada protein consists of two modules functioning independently, X-ray scattering analysis suggested that it has an overall globular shape (*8*). In this paper, we investigated the correlation of loss of MGMT and PMT activities with the overall unfolding of the protein.

The principles and mechanisms of folding of linear polypeptide chains into biologically active and native tertiary configuration have been extensively studied but are still not completely understood (*13*). Many of the proteins currently under investigation as model systems are small, secretory proteins containing disulfide bridges and usually contain a single domain. Emphasis has been focused on the role of disulfide bridges and proline isomerization in the proper folding of unfolded molecules (for reviews, see refs *14*−*16*). However, most of the intracellular monomeric proteins do not have S−S bonds and many of them have more than one domain. The correct folding of such molecules obviously involves a complex process. Several models have been proposed to explain the rapid folding of unfolded molecules to active form. Fersht proposed that small protein molecules may fold by a nucleation mechanism that is characterized by the lack of detectable intermediates (*17*). Panchenko et al. (*18*) suggested that foldons, quasi-independent folding units, can fold simultaneously as modules and thus reduce the time needed for correct folding. Until recently, it was generally believed that folding is under thermodynamic control and that the active protein conformation correlates to a global energy minimum. However, several studies have recently shown that some proteins fold to a metastable but active state; those are subsequently converted to a thermodynamically stable but inactive form (*19, 20*). The results described in this report suggest that the Ada protein also folds to a metastable active form. The protein is subsequently converted into a stable form that is inactive.

MATERIALS AND METHODS

Materials. [^{3}H-CH_3]-*N*-methyl-*N*-nitrosourea (MNU) (4 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. Urea (AR, Mallinckrodt)[2] was further recrystallized from hot ethanol to eliminate possible cyanate ion contaminations (*21*). Guanidine hydrochloride (Gdn-HCl) (8 M, sequence grade) was a gift from Pierce. Urea or Gdn-HCl even at the highest concentration did not show any absorption or emission spectra over that of the buffer solution during optical measurements.

Purification of Ada Protein. The construction and characterization of high-level *ada* expression plasmid pSM41 has been described by Tano et al. (*22*). The purification procedure of Ada protein from these *E. coli* cells was essentially that of Bhattacharyya et al. (*8*) except that the composition of the buffer used in the final gel-filtration step was 20 mM Tris-HCl (pH 8.0) containing 1 mM DTT, 0.1 mM EDTA, 1% glycerol, and 20 mM NaC1 (buffer A). The gel-filtration column fractions containing Ada corresponding to the band of 39 kDa in SDS−PAGE were concentrated 10-fold in a Speedvac vacuum centrifuge. The protein (10−15 mg/mL) produced by this treatment was highly active (>90%). This preparation was free from protease activity as judged by the integrity of the 39 kDa band in SDS−PAGE even after incubation at 37 °C for 6 h. Densitometric scanning of the gel containing purified Ada protein showed that the preparation was over 95% pure with a trace amount of high molecular weight contaminants. In the case of samples for sedimentation equilibrium and mass spectroscopic analysis, the protein was further purified by FPLC on a Mono S column (Pharmacia). The protein was eluted at 0.5 M NaCl. Concentration of the protein was measured either by using its extinction coefficient $E^{1\%}_{280nm} = 5.3$ (*8*) or using the Pierce BCA protein assay reagent with bovine serum albumin as standard (*23*).

Preparation of Substrate and Activity Assay Systems: (a) O^6-Methylguanine−DNA Methyltransferase. Synthetic poly(dC,dG,d[8-^{3}H]m^{6}dG) substrate was used for this purpose. Preparation of this substrate and related assay procedure have been described by Foote et al. (*1, 24*).

(b) Methylphosphotriester−DNA Methyltransferase. Calf thymus DNA (CT DNA) was methylated with [^{3}H]MNU and heated to remove *N*-alkylpurines. The resulting DNA, after alcohol precipitation and dialysis, contained ^{3}H-label in nearly equal proportion in O^6-methylguanine and methylphosphotriester residues and accounted for more than 90% of the total radioactivity (*2, 25*). This methylated DNA was then further treated with excess O^6-methylguanine−DNA methyltransferase purified from human placenta (*25*). The human methyltransferase is specific for O^6-methylguanine residues and does not react with methylphosphotriesters (*26*). Therefore, the residual radioactivity in CT DNA was primarily present in phosphotriesters. Assay of phosphotriester repair was carried out according to Karran et al. (*27*).

The compositions of both substrates were checked by acid hydrolysis followed by chromatography on an Aminex A-6 column (*24*).

Circular Dichroism Measurements. CD spectra were typically scanned at 25 °C between 205 and 260 nm using a Cary 61 spectropolarimeter, equipped with a thermostat, in a 3-mL cuvette with 1-cm path length. Protein concentration was 0.064 mg/mL. Samples were scanned immediately

[2] Urea obtained from other sources, even after recrystallization, irreversibly inactivated the Ada protein.

or after 16−24 h incubation with the denaturant (0−8 M urea or 0−6 M Gdn-HCl) in 0.05 M sodium phosphate buffer, pH 7.5. The ellipticity at 225 nm was used to measure the fraction of native structure remaining in each denaturing condition. Each spectrum was corrected for baseline. The molar mean residue ellipticity at wavelength λ, $[\theta]_\lambda^{25°C}$ in degrees centimeter^2 decimole^{-1}, was obtained from

$$[\theta]_\lambda^{25°C} = \frac{\theta\ (\mathrm{MRW})}{10lc}$$

where θ = experimentally observed ellipticity in degrees at wavelength λ, l = path length in centimeters, c = protein concentration in grams per milliliter, and MRW = mean residue weight of 112 calculated from the amino acid composition of the Ada protein (*28*).

Fluorescence Measurement. All fluorescence measurements were carried out in a ISS Greg PC photon counting spectrofluorometer (ISS, Champaign, IL) equipped with an AT&T PC6300 computer. Quartz cells of 1-cm path length was used in experiments with 0.3 mg/mL Ada protein in buffer A. The emission spectra were corrected for instrument response and for the background signals from the buffer. Fluorescence polarization of tryptophan residues was measured at 345 nm with excitation at 297 nm.

Sedimentation Equilibrium. The quaternary structure of native, inactivated, and renatured MGMT was monitored by sedimentation equilibrium in a Beckman XL-A analytical ultracentrifuge. The high-speed, meniscus-depletion procedure was employed (*29*). The loading protein concentrations were about 0.5 mg/mL. Data were acquired by averaging 10 scans after reaching equilibrium at the speed of 30 000 rpm and 4 °C. The equilibrium data were fit to a model of a single homogeneous sedimenting species. The partial specific volume of MGMT was calculated using the amino acid composition (*28*) and the procedure of Cohn and Edsall (*30*).

Denaturation and Renaturation. Ada protein was denatured by addition of stock solutions of 8.8 M urea or 7 M Gdn-HCl at pH 7.5 and at 25 °C for 1 min or between 16 and 20 h before any physical measurement was done. In typical renaturation studies, 10 μL of the Ada protein was denatured with 90 μL of 8.8 M urea or 7 M Gdn-HCl followed by 30-fold dilution with 0.05 M sodium phosphate buffer, pH 7.5. For reactivation studies the denaturant was further diluted 10-fold to prevent any possible inhibition of methyltransferase arising from the ionic strength of the solvent (*25, 31*).

Kinetics of Methyltransferase Reaction. The second-order rate constants of methyl group transfer of Ada protein using the synthetic poly(dC,dG,[8-^{3}H]m^{6}dG) was measured at 25 °C in the presence of 0−8 M urea. The concentrations of Ada protein (*a*) and substrate (*b*) were as follows: between 0 and 3 M urea, $a = 0.388 \times 10^{-9}$ M and $b = 0.124 \times 10^{-9}$ M; at 4 M urea, $a = 0.388 \times 10^{-9}$ M and $b = 0.264 \times 10^{-9}$ M; at 5 M urea, $a = 1.94 \times 10^{-9}$ M and $b = 0.94 \times 10^{-9}$ M; and between 6 and 8 M urea, $a = 1.94 \times 10^{-9}$ M and $b = 2.375 \times 10^{-9}$ M. Reactions were followed up to 1 and 60 min for sets 0−4 M and 5−8 M urea, respectively, and stopped by adding 1% SDS. Because of very fast reactions, experiments with samples containing up to 4 M urea were repeated three times. Demethylation of the substrate was measured as described earlier. It was shown that the Ada protein reacts extremely rapidly with poly(dC,dG[8-^{3}H]m^{6}dG) even at 0 °C, with a rate constant of 1.1×10^9 M^{-1} min^{-1} (*8*). Because of the low solubility of urea at low temperature, this rate was determined again at 25 °C and the rate constant was found to be 4.4×10^9 M^{-1} min^{-1} (Figure 3).

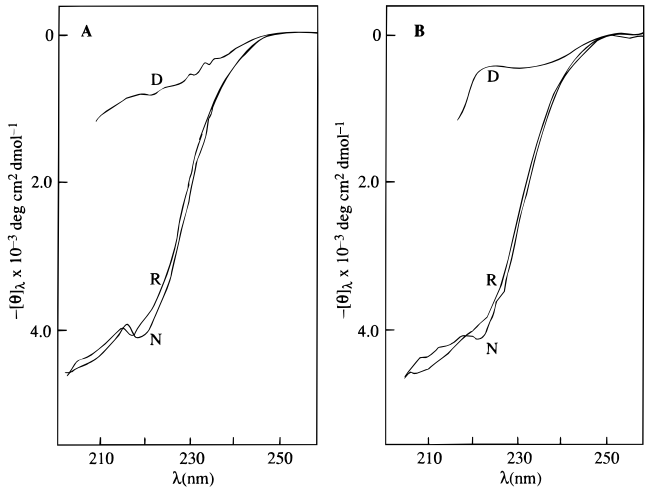

FIGURE 1: Circular dichroism spectra of native, denatured, and renatured Ada proteins. (A) Native Ada protein scanned immediately after dilution with 0.05 M sodium phosphate buffer, pH 7.5, or after incubation at room temperature for 4 h (N). D represents the scan of Ada protein after denaturation in 8 M urea in 0.05 M sodium phosphate, pH 7.5, immediately after denaturation or after 4 h incubation. R represents the scan of Ada protein after denaturation with 8 M urea followed by 30-fold dilution with the phosphate buffer both immediately and after 4 h of incubation. No significant difference was observed between the two measurements. (B) Spectra were obtained as described in panel A except that 6 M Gdn-HCl was the denaturant. Final protein concentration was 0.064 mg/mL in all experiments.

In some experiments, the fraction of active molecules, rather than the rate of reaction of the Ada protein, was determined after treatment with urea or Gdn-HCl. In these cases, reactions were carried out in the presence of excess poly(dC,dG,[8-^{3}H]m^{6}dG) or methylated CT DNA. The reactions were allowed to go to completion following incubation for 4 h at 37 °C.

RESULTS

Native State of Ada Protein. The circular dichroism spectrum of the Ada protein in the UV region (205−260 nm) is shown in Figure 1. The α-helix and β-sheet contents of the protein as calculated from this spectra were 19.8% and 20.8%, respectively. The fluorescence emission spectrum of the Ada protein following excitation at 297 nm is shown in Figure 2A. It has $\lambda_{max,em}$ at 337 nm. When excited at 280 and 295 nm, the $\lambda_{max,em}$ was shifted to 300 and 334 nm, respectively (results not shown). This suggests that, when excited at 297 nm, the fluorescence was mostly due to tryptophans rather than tyrosines. This was expected as Ada protein contains five tryptophan and six tyrosine residues (*4, 28*).

Denatured State of Ada Protein. The Ada protein was denatured by 8 M urea or 6 M Gdn-HCl at pH 7.5 either for 30 s or for 16−24 h and the circular dichroism spectra of

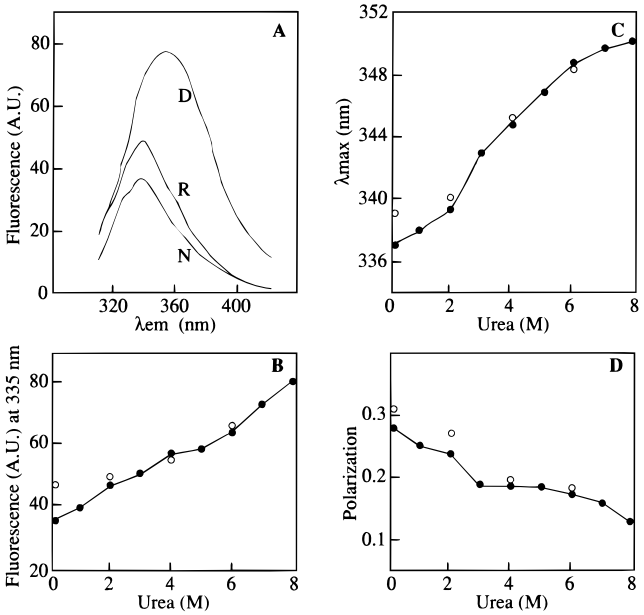

FIGURE 2: Fluorescence properties of native, denatured, and renatured Ada proteins at 22 °C. (A) Emission spectra of tryptophan fluorescence of Ada protein: native (N), denatured by 8 M urea (D), and renatured by 30-fold dilution with phosphate buffer (R). The spectra were corrected for baseline. (B) Fluorescence intensity at 335 nm as a function of urea concentration. (C) Emission maxima (nanometers) as a function of urea concentration. (D) Polarization at 345 nm in the presence of urea. AU, arbitrary units. In all experiments 0.06−0.12 mg/mL protein was used and the excitation wavelength was 297 nm. (●) Native protein; (○) Ada protein after denaturation and renaturation by dilution of urea.

the protein under these two denaturing conditions have been shown in Figure 1. Longer incubation did not further affect the profile observed after 30 s of incubation. In either case, the profiles resemble the character of random coils. In the case of treatment with urea, a 20% residual molar ellipticity at 225 nm was observed while with Gdn-HCl treatment it was reduced to 12%. This indicated an almost complete collapse of the secondary structure of the protein. The fluorescence properties of the Ada protein in 8 M urea are shown in Figure 2. When excited at 297 nm, the $\lambda_{max,em}$ was shifted from 337 to 350.4 nm with over 100% enhancement of emission intensity at 335 nm. This result suggests that the tryptophan residues are exposed from a more hydrophobic medium to a more polar medium as a result of urea addition. The polarization value of tryptophan fluorescence, measured at 345 nm when excited at 297 nm, also changed from 0.28 to 0.16. Interestingly, the Ada protein can carry out repair of O^6-methylguanine and methylphosphotriester even in the presence of 6 M urea. The rates were, however, 10^3- and 2-fold slower compared to that in the absence of denaturant for the two processes, respectively (Figures 3 and 4).

Renatured State of Ada Protein. Renaturation by dilution with buffer from 8 M urea or 6 M Gdn HCl instantly restores the secondary structure of the Ada protein to a state that is almost indistinguishable from the native state (Figure 1). A blue shift of $\lambda_{max,em}$ from 350.4 to 339 nm together with a 43% quenching of fluorescence intensity at 335 nm compared to that of the denatured state was observed upon exciting at 297 nm. This and the change of polarization from 0.16 to 0.31 indicate that the exposed tryptophan residues were once again shielded in a nonpolar environment (Figure 2). The

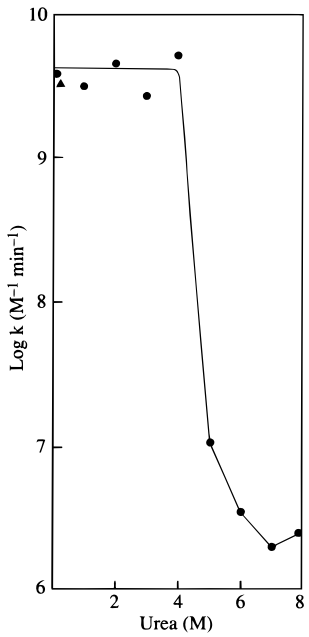

FIGURE 3: Effect of urea on the second-order rate constant of CH_3 group transfer from O^6-methylguanine to Ada protein. (●) Native protein; (▲) protein after denaturation/renaturation treatment. Details of experiments are described in Materials and Methods.

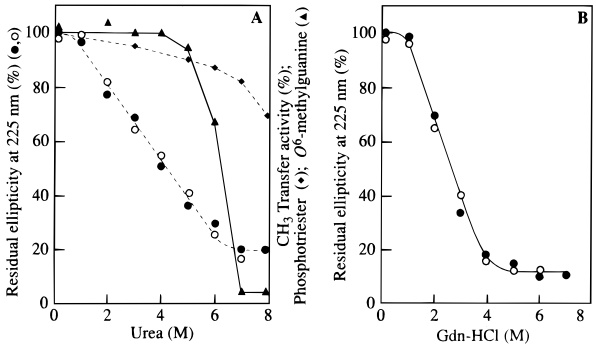

FIGURE 4: Reversible denaturation of Ada protein and its methyl-acceptor activity. (A) Methyl acceptor activities from O^6-methylguanine (▲) and phosphotriester (◆) were measured in the Ada protein in the presence of urea. Residual ellipticity of native protein (●) or urea denatured-renatured protein (○). (B) Denaturation of Ada with Gdn-HCl. Details of experimental procedures are given in Materials and Methods. All results were expressed as percentage of control values.

renatured enzyme reacts with the synthetic substrate with a second-order rate constant $k = 3.3 \times 10^9$ M^{-1} min^{-1} at 25 °C, which is, within limits of error, the same as that of the native protein (Figure 3). These results show the complete reversibility of the denaturation process. Thus, valid thermodynamic parameters can be derived from these denaturation studies.

Kinetics of Renaturation. The kinetics of renaturation was found to be fast. Even at 0 °C, the restoration of $[\theta]_{225nm}$ was quantitative in less than 30 s, the minimum time needed for CD spectral analysis (Figure 4). The fluorescence properties of the renatured enzyme are also changed to the full extent within that time interval. From the limit of detection, the first-order rate constant for conversion of the denatured state to the native state is estimated to be >0.2 s^{-1}. Determination of the kinetics of reactivation was not possible without a stopped-flow device. Nevertheless, a sample renatured for 30 s showed identical kinetics of

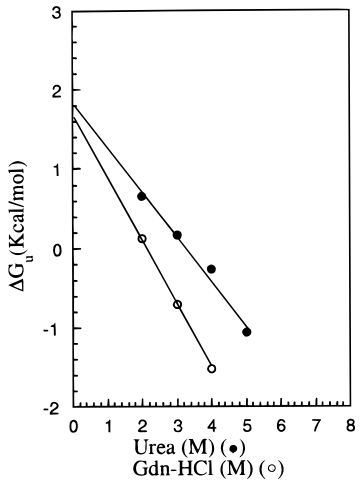

FIGURE 5: Estimation of ΔG of unfolding of Ada. ΔG_u was calculated from the fraction unfolded in various concentrations of urea (●) or Gdn-HCl (○) as described in the text.

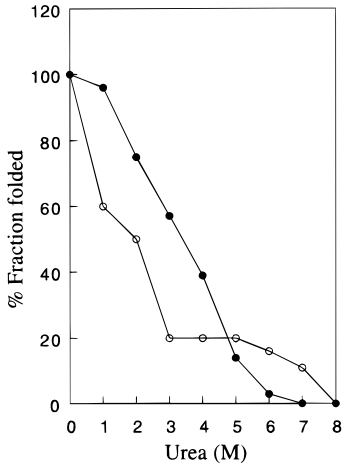

FIGURE 6: Unfolding of Ada by urea. The extent of folding of the Ada protein was calculated from fluorescence anisotropy (○) and CD data (●) as a function of urea concentration as described in the text.

methyltransfer as the native protein. Identical results were obtained with both urea- and guanidine-denatured proteins.

Equilibrium of the Chemical Denaturation and Renaturation Processes. Having established the reversibility of the denaturation process, we employed multiple physical techniques to define the mechanism of unfolding of this multidomain protein. Different physicochemical properties, e.g., $[\theta]_{225nm}$, tryptophan fluorescence intensity at emission maxima of 225 nm with excitation at 297 nm, polarization of tryptophans, and O^6-methylguanine−DNA and phosphotriester repair activities of the partially denatured states were used for their characterization (Figures 2 and 4). The partly unfolded states were generated either by exposing the native protein to varying concentrations of urea or Gdn-HCl (forward reaction) or by reducing the concentration of the denaturant after the protein was exposed to 8 M urea or 7 M Gdn-HCl (reverse reaction). As indicated by the change of $[\theta]_{225nm}$ as a function of denaturant concentration (Figure 4), the unfolding and refolding profiles were nearly identical and superimpose on each other. Thus, the unfolding pathway appears to be completely reversible, involving the same structural intermediates. The CD denaturation data were further analyzed to determine the equilibrium constant of unfolding at various denaturant concentrations within the transition zones. The plateau values at low and high denaturant concentrations were taken to reflect the folded and unfolded states, respectively. At each denaturant concentration, the equilibrium constant of unfolding, K_u, can be estimated by

$$K_u = \frac{1 - f}{f}$$

where f is the fraction in the folded state. K_u is converted to ΔG_u, free energy change of unfolding, by $\Delta G_u = -RT \ln K_u$. ΔG°_u, the free energy change of unfolding in the absence of denaturant, can be determined by the linear extrapolation procedure (*32*), which states that

$$\Delta G_u = \Delta G_u^\circ - m[D]$$

where m is the slope and [D] is the denaturant concentration. Results of such an analysis are shown in Figure 5. It is significant to note that, within experimental uncertainities, both sets of data extrapolate to a ΔG_u° of 1.7 ± 0.3 kcal/mol, although the slopes are significantly different. A steeper slope for the Gdn-HCl data reflects the more cooperative nature of unfolding induced by this denaturant. The convergence of both data sets to the same value of ΔG_u° indicates that the same denaturation process is induced by both denaturants.

Similar results on reversibility are obtained from the fluorescence measurements, except that a minor deviation was observed in samples in less than 2 M urea (data not shown). The intermediate unfolded states could be reached within seconds of denaturation or renaturation. The rationale of employing different physical techniques to monitor the chemical denaturation process of Ada, a multidomain protein, is to probe the cooperativity of unfolding in order to detect the presence of interdomain interaction. The fluorescence polarization data were, therefore, further processed to define the fraction folded at each denaturant concentration. Fluorescence polarization data accurately reflect the unfolding process only if the fluorescence intensities of the folded and unfolded forms are identical (*33*). However, it is evident from Figure 2A that this requirement is not met in this system. Thus, a correction factor, F, needs to be introduced to convert the fluorescence polarization data to fraction of Ada in folded state, f:

$$f = \frac{F(\epsilon - \epsilon_{unfolded})}{\epsilon_{folded} - \epsilon + F(\epsilon - \epsilon_{unfolded})}$$

where F is the ratio of fluorescence intinsities of the folded and unfolded states and ϵ, ϵ_{folded}, and $\epsilon_{unfolded}$ are the observed polarization at a denaturant concentration and the polarization for folded and unfolded states, respectively. The folded fraction of Ada as a function of urea concentration as determined by fluorescence anisotropy and CD is shown in Figure 6. In accordance to the fluorescence data, it appears that unfolding of Ada occurred with two transition states. The transition below 3 M urea may not be interpreted to reflect a 80% unfolding since the CD data only indicate a loss of about 30% residual ellipticity (Figure 4A). It might actually imply a dissociation of domain−domain interaction

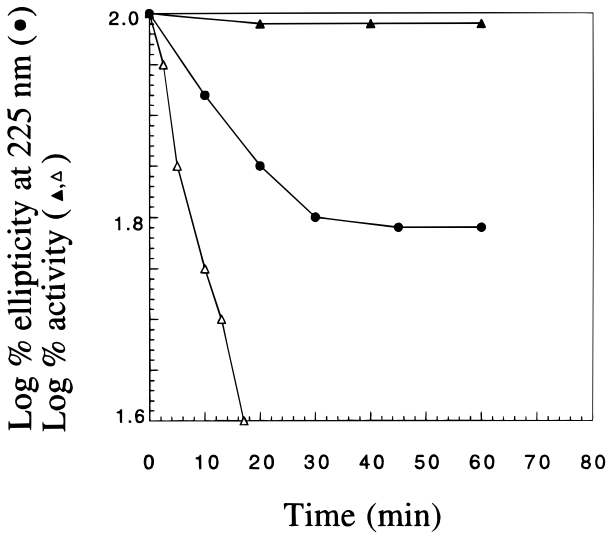

FIGURE 7: Inactivation of the Ada protein. Ada (0.064 mg/mL) was incubated at 37 °C in the presence of 0.05 M sodium phosphate buffer, pH 7.0, for various times before measuring circular dichroism signal. Aliquots of the samples were used for measuring the methyltransferase activity in buffer A as described in Materials and Methods. Biphasic kinetics of thermal denaturation as measured by the loss of ellipticity at 225 nm (●) and loss of methyltransferase activity for O^6-methylguanine in the absence (△) and in the presence of 100 μg/mL CT DNA (▲) are shown.

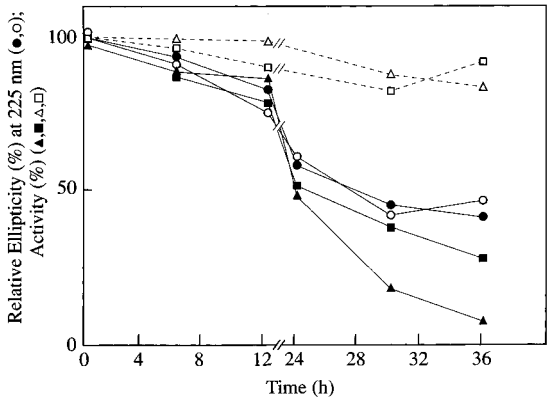

FIGURE 8: Stabilization of tertiary structure and O^6-methylguanine−DNA methyltransferase activity of Ada by DNA. Molar ellipticity (●, ○) and activity (▲, △, ■, □) were measured as percentage of control values in the presence (open symbols) or absence of DNA (solid symbols) (■, □, Native protein; (▲, △) renatured protein.

Table 1: Apparent Weight-average Molecular Weight of Ada[a]

sample	$\bar{M}_w \times 10^{-4}$
native	3.5_6 (3.5_2, 3.6_0)
inactivated	3.16 (3.1_2, 3.2_1)
renatured	3.4_8 (3.4_4, 3.5_1)

[a] Error in parentheses are expressed in terms of 95% confidence intervals.

that leads to an acquisition of higher local mobility around the tryptophan residues. The lack of superimposable data obtained by two different spectroscopic techniques indicate that the unfolding of Ada does not proceed as a two-state process.

Our studies with the partly unfolded molecules showed that the rate constant of methyltransferase was not significantly affected by up to 4 M urea. On the basis of circular dichroism measurement, the protein molecules retained 15.8% α-helix and 20.3% β-sheet structure in 4 M urea. A sharp drop in O^6-methylguanine−DNA methyltransferase activity was observed in the presence of more than 5 M urea. Surprisingly, methylphosphotriester methyltransferase activity was more resistant to urea-induced unfolding. It is interesting that the loss of PMT activity corresponded with the transition in high urea concentration (Figure 6). It should be noted here that while urea weakens the duplex structure of DNA, complete strand separation does not occur even in 8 M urea at room temperature. Furthermore, the data on the extent and not the rate of methyltransfer are shown in Figure 4 and these should not be affected by changes in the DNA duplex structure.

Stability of Ada Protein. At 37 °C, the purified Ada protein was much more unstable than at 25 °C in terms of both its activity and secondary structure. It was completely inactivated after 30 min, with a first-order rate constant k of 1.2×10^{-3} s^{-1}. Under identical conditions, the loss of secondary structure was biphasic, with a fast phase $k = 3.6 \times 10^{-4}$ s^{-1} followed by a slower phase (Figure 7). This inactivation was partially dependent on the protein concentration. Higher concentration of protein (~11 mg/mL) prevented inactivation to some extent (30% activity after 30 min). The inactive protein and the native protein were eluted from both an Ultrogel-A54 gel-filtration column and reverse-phase HPLC on a C_{18} reverse-phase column at identical positions, suggesting lack of aggregation of the inactive protein as described later.

We routinely added DTT to 1 mM to the stored enzyme and in reaction mixtures in order to prevent oxidation of cysteine, the active-site residue. However, DTT did not affect protein denaturation with urea or Gdn-HCl. If left overnight at 25 °C for 24−36 h, a considerable drop in activity (70−90%) was observed. This inactivation could not be prevented by including 5 mM β-mercaptoethanol in the incubation buffer or by flushing the solution with nitrogen or by adding bovine serum albumin (100 μg/mL), which is expected to protect dilute Ada from surface denaturation. The inactivation patterns were basically the same in the native and in the refolded protein. Thus this activation was not due to spontaneous oxidation of the protein. However, the inactivation could be prevented if Ada protein was incubated in the presence of excess CT DNA (Figure 8).

When heated at 70 °C the Ada protein immediately coagulated, and most of the protein was in the precipitate. A small fraction (10%) of the protein was recovered in the supernatant and was also inactive. The coagulated protein was dissolved in 6 M Gdn-HCl or 8 M urea. Dilution of the solution with buffer A completely restored the methyltransferase activities (results not shown).

Sedimentation Equilibrium Analysis of Ada Protein. A possible mechanism of spontaneous inactivation of the Ada protein is that a dynamic equilibrium exists between the native and partially unfolded, inactive molecules. Aggregation of the latter leads to complete transfer of the protein to the inactive state. We, therefore, tested the possibility of aggregation of spontaneously inactivated protein by equilibrium sedimentation analysis. Table 1 summarizes the molecular weight data of native, inactivated and refolded protein. The values of $\bar{M}_{w,app}$ indicate that MGMT exists as monomer under these experimental conditions. There is no indication of larger aggregates up to a protein concentration

of 3 mg/mL. It is interesting to note that the value of $\bar{M}_{w,app}$ for the inactivated sample is lower than that of either the native or renatured forms. The lower value is not a consequence of heterogeneity because there is no speed dependence in the observed $\bar{M}_{w,app}$. In calculating $\bar{M}$, it was assumed that all samples have the same value for partial specific volume; i.e., all protein species are folded into the same tight motif. This assumption is probably valid for the native and renatured sample. However, if the inactivated MGMT assumes a less tightly folded motif, then the value for partial specific volume should increase to reflect a more loosely folded structure. In that case, the value for $\bar{M}_{w,app}$ will be greater than 3.2×10^4 and approaches that of the native form.

Absence of Oxidation during Inactivation of Ada Protein. We subsequently addressed the question of whether reversible inactivation of the Ada protein results from its oxidation. Although we have shown by sedimentation analysis that the inactive protein remains as a monomer and that the presence of 1 mM DTT did not affect denaturation/renaturation of the protein, it is possible that the protein can be oxidized spontaneously at one or more of its cysteine and methionine residues in a situation analogous to that observed with the c-Jun protein (*34*, *35*). Oxidation of c-Jun was reversed or prevented only by high concentration of DTT. Reversible oxidation and reduction of cysteine can significantly affect the folded state (*36*). We tested this possibility directly by determining the molecular mass of the active and inactivated proteins by electrospray mass spectrometry. The recombinant protein was inactivated by incubation for 45 min at 37 °C. Both active and inactive preparations were purified by reverse-phase HPLC on a C_{18} column. The active and inactive species were eluted using acetonitrile gradient nearly identically at 46% acetonitrile/0.1% trifluoroacetic acid. After removal of the solvent in a vacuum centrifuge, the proteins were dissolved in 50% methanol and 0.25% acetic acid and subjected to mass analysis (M-Scan, Inc.), using myoglobin as calibration standard. The mass of the active and inactive proteins were calculated to be 39 377.6 and 39 356.3 Da, respectively. Although the molecular weight was somewhat different from the mass of 39 409 Da based on the predicted amino acid sequence of a cloned *ada* gene (*28*), the difference is likely due to polymorphism of the protein. Importantly, the fact that the mass of the inactive protein was actually lower by 21.3 mass units than that of the active protein precludes the possibility of oxidation of the Ada protein during inactivation. Such oxidation would add at least 16 mass units of an oxygen atom to the protein. We believe that the presence of a bound Na^+ in the active protein is the reason for its higher molecular weight.

The binding of Na^+ to native form of Ada was not entirely unexpected given that during the penultimate purification step the protein was eluted from an FPLC ion-exchange column with buffer containing 0.5 M NaCl. The Na^+ adduct on the native protein was not removed by subsequent C18 reverse-phase HPLC; however, in the case of the inactive protein the Na^+ was removed. The mass difference between the native and inactive forms of Ada was 21.3, approximating that of a Na^+ ion. The fact that both proteins were treated identically suggests that the differential Na^+ binding may be related to conformational differences between the native and inactive Ada forms that give rise to a high-affinity Na^+ binding site on the native enzyme. This was consistent with conformational differences demonstrated by other physicochemical observations obtained from CD and fluorescence spectroscopy. All containers for the proteins used in the final HPLC purification step and during ES-MS analysis avoided glass.

DISCUSSION

The 39 kDa *E. coli* MGMT, also called the Ada protein, is more complex than the mammalian MGMTs in that it is larger than the 22−23 kDa mammalian proteins and has a second alkyl acceptor activity for methylphosphotriesters in DNA, which are also induced by simple alkylating agents. The Ada protein has two distinct domains connected by a hinge region which is extremely susceptible to proteases (*3*, *8*). The domains are independently active. The methyl acceptor cysteine residues for methylphosphotriesters and O^6-methylguanine are located in the N-terminal and C-terminal halves, respectively (*3*, *11*). X-ray scattering data indicated that the protein is globular with an axial ratio of less than 2 (*8*).

We have shown in this study that, based on activity, the two halves of the Ada protein exhibit differential susceptibility to chemical denaturation. CD spectra as well as fluorescence data indicate that the unfolding is gradual and is a function of concentration of urea. In 4−5 M urea, the O^6-methylguanine acceptor activity dropped sharply while the methylphosphotriester acceptor activity remained unaffected. Although the Ada protein reacts stoichiometrically and the extent rather than the rate of its reaction was measured in the experiments described in Figure 4, the rate of reaction was not significantly affected by up to 4 M urea (Figure 3).

The fact that MGMT and PMT activities were differentially inactivated by urea is consistent with the previous observation on the association of these activities with distinct domains. It appears likely that the N-terminal half containing the active site (alkyl acceptor Cys-69) of PMT is more resistant to unfolding by urea and that the residual structure left in the Ada protein in the presence of 8 M urea may be located in the N-terminal half.

Elegant studies have been carried out on the cooperative folding of small proteins with a single domain, e.g., apomyoglobin. Partially folded states have been identified and their structures have been characterized (*37*). However, most of the folding data are available for only a few and largely single-domain proteins; the two-domain structure of the Ada protein adds to the complexity of the folding process with the possibility of additional intermediate states. Nevertheless, it appears reasonable that the core structure, resistant to denaturation by 8 M urea or 6 M Gdn-HCl, acts as the nucleation site for the fast and correct refolding of Ada molecules (*17*). Further studies are needed to characterize the structure of this core. The folding process may occur in a successive assembly of "subdomains" in both directions of the core structure (*37*, *38*). Thus the overall folding may not be a cooperative two-state process.

The folding of proteins is generally believed to be under thermodynamic control in that it leads to the production of active protein which has a stable conformation in the lowest energy state. The free energy of folding of average globular

protein is 5−15 kcal/mol (*39*, *40*). The calculated free energy of folding of the Ada protein of 1.7 kcal/mol, significantly smaller than the average value, appears to be reliable because of the closeness of numbers obtained from two distinct experiments using different denaturants and implies that the Ada protein is loosely folded. The denaturation and renaturation pathways of the protein in the presence of urea or Gdn-HCl are readily reversible. The rapid renaturation (with a first-order rate constant of >0.2 s^{-1}), presumably with the same rate of reactivation to the active state, yields a folded structure that is indistinguishable from that of the native molecules. The observation that these molecules lose a fraction of their structure at room temperature associated with their loss of biological activity, and with a faster rate of such loss at 37 °C, suggests partial unfolding of the molecule, although it is possible that a fraction of molecules lost their structure completely while others were inactivated by a different mechanism. Analysis of molecular weight distribution of the spontaneously unfolded and refolded molecules clearly shows that inactivation results from unfolding rather than aggregation of these molecules. However, aggregated and inactivated molecules can be generated by heating at 70 °C, causing coagulation of the protein to an insoluble form. Finally, full structure and activity can be restored to all forms of unfolded and inactivated Ada protein molecules by cycling them through a denaturation step in urea or Gdn-HCl and subsequent removal of the denaturant.

Our results are consistent with the model that the nascent Ada protein is initially folded rapidly into a metastable state that is active. However, the protein loses its activity concomitant with a partial loss of its structure and reaches a thermodynamically stable state that is inactive. However, the metastable state can be stabilized by DNA. Thus the initial folding is controlled kinetically, analogous to the situation of plasminogen activator inhibitor (PAI) (*41*, *42*). The structural alteration of PAI-1 for the transition of the metastable to the inactive form has recently been characterized (*43*). Similar studies for the Ada protein will require elucidation of its tertiary structure. In any case, kinetic control of protein folding has been observed with proteases and protease inhibitors that are extracellular and contain disulfide bridges (*44*). Our studies with the Ada protein provide the first evidence of kinetic control of folding of an intracellular protein without disulfide bonds.

Although the structures of the 19 kDa C-terminal domain and 10 kDa N-terminal domain of the Ada protein have been elucidated by X-ray crystallography (*9*) and multidimensional NMR spectroscopy (*10*), respectively, an obvious consequence of the unstable state of the free Ada protein is that it may be difficult to crystallize the full-length protein in the active form for X-ray diffraction studies. However, it may be possible to obtain crystals of the protein bound to DNA because of the large difference in stability of free and DNA-bound states. We have shown previously that the active-site mutant C145A of the human MGMT forms a stable complex with O^6-methylguanine-containing oligonucleotide (*45*). A similar substrate-bound Ada mutant will be a good candidate for X-ray crystallographic studies.

ACKNOWLEDGMENT

We thank W. C. Dunn for expert technical assistance and D. W. Bolen for suggesting critical experiments and a careful reading of the manuscript. Ms. Wanda Smith provided expert secretarial assistance. Mass spectrometry discussions with Dr. Mark Rogers of M-Scan, Inc., were also appreciated.

REFERENCES

1. Foote, R. S., Mitra, S., & Pal, B. C. (1980) *Biochem. Biophys. Res. Commun. 97*, 654−659.
2. Olsson, M., & Lindahl, T. (1980) *J. Biol. Chem. 255*, 10569−10571.
3. Lindahl, T., Sedgwick, B., Sekiguchi, M., & Nakabeppu, Y. (1988) *Annu. Rev. Biochem. 57*, 133−157.
4. Demple, B., Sedgwick, B., Robins, P., Waterfield, M. D., & Lindahl, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2688−2692.
5. Nakabeppu, Y., & Sekiguchi, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6297−6301.
6. Teo, I., Sedgwick, B., Kilpatrick, M. W., McCarthy, T. V., & Lindahl, T. (1986) *Cell 45*, 315−324.
7. Volkert, M. R. (1988) *Environ. Mol. Mutagen. 11*, 241−255.
8. Bhattacharyya, D., Tano, K., Bunick, G. J., Uberbacher, E. C., Behnke, W. D., & Mitra, S. (1988) *Nucleic Acids Res. 14*, 6397−6410.
9. Moore, M. H., Gulbus, J. H., Doson, E. J., Demple, B., & Moody, P. C. E. (1994) *EMBO J. 13*, 1495−1501.
10. Habazettl, J., Myers, L. C., Yuan, F., Verdine, G. L., and Wagner, G. (1996) *Biochemistry 35*, 9335−9348.
11. Myers, L. C., Jackow, F., and Verdine, G. L. (1995) *J. Biol. Chem. 270*, 6664−6670.
12. Ohkubo, T., Sakashita, H., Sakuma, T., Kainosho, M., Sekiguchi, M., & Morikawa, K. (1994) *J. Am. Chem. Soc. 116*, 6035−6036.
13. Lattman, E. E., and Rose, G. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 439−441.
14. Ghelis, C., and Yon, J. M. (1982) *Protein Folding*, Academic Press: New York.
15. Creighton, T. E. (1990) *Biochem. J. 270*, 1−16.
16. Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29*, 2205−2212.
17. Fersht, A. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10869−10873.
18. Panchenko, A. R., Luthey-Schulten, Z., and Wolynes, P. G. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2008−2013.
19. Wang, Z., Mottonen, J., and Goldsmith, E. J. (1996) *Biochemistry 35*, 16443−16448.
20. Zhang, H., Stöckel, J., Mehlhorn, I., Groth, D., Baldwin, M. A., Prusiner, S. B., James, T. L., and Cohen, F. E. (1997) *Biochemistry 36*, 3543−3553.
21. Fröhlich, O., and Jones, S. C. (1987) *J. Membr. Biol. 98*, 33−42.
22. Tano, K., Foote, R. S., and Mitra, S. (1988) *Gene 64*, 305−311.
23. Smith, P. K., Krohn, R. I., Hermanson, C. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Funimoto, E. K., Gocke, N. H., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
24. Foote, R. S., Pal, B. C., and Mitra, S. (1983) *Mutat. Res. 119*, 221−228.
25. Bhattacharyya, D., Foote, R. S., Boulden, A. M., and Mitra, S. (1990) *Eur. J. Biochem. 193*, 337−343.
26. Yarosh, D. B., Fornace, A. J., and Day, R. S., III (1985) *Carcinogenesis 6*, 949−953.
27. Karran, P., Lindahl, T., and Griffin, B. (1979) *Nature 280*, 76−77.
28. Nakabeppu, Y., Kondo, H., Kawabata, S., Iwanaga, S., and Sekiguchi, M. (1985) *J. Biol. Chem. 260*, 7281−7286.
29. Yphantis, D. A. (1964) *Biochemistry 3*, 297−317.
30. Cohn, E. J., and Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, p 372, Van Nostrand-Reinhold, Princeton, NJ.

31. Boulden, A. M., Foote, R. S., Fleming, G. S., and Mitra, S. (1987) *J. Biosci. 11*, 215−224.
32. Pace, C. N., Shirley, B. A., and Thomson, J. A. (1989) in *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) pp 311−330, IRL Press, Oxford, U.K.
33. Eftink, M. R. (1997) *Methods Enzymol. 278*, 221−257.
34. Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E., & Curran, T. (1992) *EMBO J. 11*, 3323−3335.
35. Yang, S. F. (1970) *Biochemistry 9*, 5008−5014.
36. Lu, J., Baase, W. A., Muchmore, D. C., and Dahlquist, F. W. (1992) *Biochemistry 31*, 7765−7772.
37. Hughson, F. M., Wright, P. E., and Baldwin, R. L. (1990) *Science 249*, 1544−1548.
38. Zehfus, M. H., and Rose, G. D. (1986) *Biochemistry 25*, 5759−5765.
39. Dill, K. A. (1989) *Biochemistry 29*, 133−155.
40. Kim, P. S., and Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
41. Hekman, C. M., and Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581−11587.
42. Katagiri, K., Okada, K., Hattori, H., and Yano, M. (1988) *Eur. J. Biochem. 176*, 81−87.
43. Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., and Goldsmith, E. J. (1992) *Nature 355*, 270−273.
44. Baker, D., Sohl, J. L., and Agard, D. A. (1992) *Nature* (*London*) *356*, 263−265.
45. Hazra, T. K., Roy, R., Biswas, T., Grabowski, D. T., Pegg, A. E., and Mitra, S. (1997) *Biochemistry 36*, 5769−5776.

BI971852N