Evidence for an Equilibrium Intermediate in the Folding–Unfolding Pathway of a Transforming Growth Factor-α–*Pseudomonas* Exotoxin Hybrid Protein

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ABSTRACT: TP40 is a chimeric protein containing transforming growth factor-α (TGF-α) at the N-terminus and a Cys → Ala mutant (PE40δCys) of a 40 000-dalton segment (PE40) of *Pseudomonas* exotoxin (PE). The guanidine hydrochloride (Gdn·HCl)-induced unfolding of TP40 and PE40δCys has been studied by tryptophan fluorescence, circular dichroism (CD), and high-performance size exclusion chromatography (HPSEC). The equilibrium unfolding of both proteins involves at least one intermediate (I). In the I state(s), which may be induced by 1.3–2.0 M Gdn·HCl, the tertiary structure is fully or partially collapsed as detected by tryptophan fluorescence and near-UV CD, but the protein largely retains the native secondary structure and a semicompact shape as judged by far-UV CD and HPSEC, respectively. Soluble aggregates of TP40 and PE40δCys are observed in addition to monomers at these intermediate (but not at higher) Gdn·HCl concentrations, suggesting that self-association is possibly mediated by thermodynamically stable, partially unfolded I states. The kinetics of refolding of TP40 upon dilution of Gdn·HCl involve two or more phases. Re-formation of secondary structure occurs rapidly ($t_{1/2}$ < 10 s) as determined by CD and is followed by a biphasic refolding of the native tertiary structure as detected by changes in tryptophan fluorescence. The midpoint (T_m) of the thermal unfolding transition occurs at a lower temperature when measured by tryptophan fluorescence than when detected by DSC and CD. These data suggest that Gdn·HCl and temperature can induce conformation(s) of TP40 that are distinct from native (N) and unfolded (U) states. These I forms may be qualitatively similar to the low pH-induced conformation(s) implicated in membrane translocation [Sanyal et al. (1993) *Biochemistry 32,* 3488–3497] and have features in common with the now widely recognized molten globule states.

TGF-α-PE40,[1] a chimeric protein with potential antitumor activity, contains transforming growth factor-α (TGF-α) fused to a 40-kDa fragment (PE40) of *Pseudomonas* exotoxin (PE). The natural form of PE possesses three domains: (a) a cell binding region that is believed to bind to an α_2-macroglobulin/low-density lipoprotein receptor-related protein present on the surface of most eukaryotic cells (Kounnas et al., 1992); (b) a translocation domain that permits the protein to pass across endosomal membranes into the cytoplasm; and (c) an enzymatic portion which ADP-ribosylates elongation factor 2 (EF-2), thereby inhibiting protein synthesis (Pastan & FitzGerald, 1989). PE40 contains the membrane translocation and ADP-ribosylation domains of PE, but lacks the cell binding fragment. In designing TGF-α-PE40, the cell binding domain of PE was replaced with TGF-α to confer selective targeting toward certain carcinoma cells expressing high concentrations of epidermal growth factor receptor (EGFr) while retaining the other two domains required to inhibit protein synthesis in the cell cytoplasm once the toxin is internalized (Chaudhury et al., 1987; Pastan & FitzGerald, 1991). Selective cytotoxicity against several EGFr-rich cell lines was manifested by this chimeric protein (Edwards et al., 1989), suggesting that neither the high EGFr affinity of TGF-α nor the cytotoxic activity of PE has been significantly compromised in this hybrid protein. A modification of TGF-α-PE40 was made in which the four cysteines of the PE40 segment were changed to alanine, primarily to avoid possible inactivating disulfide scrambling with the disulfides in the TGF-α portion (Edwards et al., 1989). This modified TGF-α-PE40, which will be designated TP40 throughout this paper, has indeed manifested antitumor activity in a nude mice model (Heimbrook et al., 1990). The modified PE40 (Cys → Ala) will be referred to as PE40δCys.

We have recently shown that TP40 retains the conformational flexibility of PE that permits its translocation across endosomal membranes (Sanyal et al., 1993). At acidic pH values, intramolecular interactions in the tertiary structure of TP40 are altered, leading to exposure of hydrophobic surfaces, while the secondary structure of the protein is virtually unaltered. Qualitatively similar observations have been made for PE40δCys which contains the translocation domain and constitutes about 89% of TP40 by mass. Tryptophan fluorescence of PE was earlier reported to undergo low pH-induced changes that were suggestive of partial unfolding (Farahbakhsh et al., 1987; Jiang & London, 1990). Acidic pH also induces a non-native conformation in diphtheria toxin in which apolar regions become solvent-exposed (Blewitt et al., 1985; Collins & Collier, 1987). These and other studies have suggested that structural changes of the protein in the low-pH environment of endosomes is an important step in the

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[1] Abbreviations: PE, *Pseudomonas* exotoxin A; PE40, a 40 000-Da fragment of PE; TGF-α, transforming growth factor-α; TGF-α-PE40, a chimera of TGF-α and PE40; PE40δCys, a mutant of PE40 in which all four cysteine residues are replaced with alanine; TP40, a chimera of TGF-α and PE40δCys; ADP, adenosine 5′-diphosphate; EF-2, elongation factor 2; EGFr, the epidermal growth factor receptor; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; HPSEC, high-performance size exclusion chromatography; DSC, differential scanning calorimetry; N, native; U, unfolded; I, intermediate; τ, lifetime of singlet excited state measured by fluorescence decay; MHz, megahertz; ns, nanoseconds; nm, nanometers; μm, micrometers or microns; [θ], mean residue ellipticity; T_m, temperature at which the measured experimental parameter lies halfway along the thermal unfolding curve.

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translocation interaction of these toxins with the apolar membrane interior.

The stability of low pH-induced states raises the interesting possibility that related intermediates might be detectable in the folding pathway of these proteins. To explore this idea, we have studied the effect of the denaturant guanidine hydrochloride (Gdn·HCl) on the secondary and tertiary structure of TP40 and PE40δCys. Specifically, we ask if the unfolding–refolding of these proteins is a two-state process or if intermediate structures such as those found at low pH can be detected. We have therefore utilized changes in tryptophan fluorescence and near-ultraviolet (UV) circular dichroism (CD) to monitor perturbations in tertiary structure, and far-UV CD of the peptide backbone to study changes in secondary structure as a function of Gdn·HCl concentration. Furthermore, the average hydrodynamic size of the TP40 molecule at different Gdn·HCl concentrations has been estimated by high-performance size exclusion chromatography (HPSEC). The thermal unfolding of TP40 has been studied by monitoring the effect of temperature on tryptophan fluorescence and circular dichroism (CD), as well as by differential scanning calorimetry (DSC). These data provide complementary information on the mechanism of unfolding of this protein and suggest the presence of at least one folding intermediate that possesses properties like the forms seen at low pH and of molten globules generally.

MATERIALS AND METHODS

Proteins and Reagents. The purifications of TP40 and PE40δCys have been described earlier (Edwards et al., 1989; Heimbrook et al., 1990). The purities of these proteins were greater than 99% as determined by SDS–PAGE. Their identities were also confirmed by N-terminal amino acid sequencing (Sanyal et al., 1993). The following standard proteins of known Stokes radii were used as standards in HPSEC experiments: transferrin and β-lactoglobulin (Sigma), and bovine serum albumin, ribonuclease A, and ovalbumin (Pharmacia). High-purity Gdn·HCl was obtained from BRL (Gaithersburg, MD).

Equilibrium Unfolding. Both TP40 and PE40δCys were stored at approximately −60 °C at 1 mg/mL in a pH 7.25 buffer containing 6.2 mM sodium phosphate and 150 mM sodium chloride (buffer A). Protein concentrations were determined employing extinction coefficients of 45 460 and 43 980 M^{-1} cm^{-1} at 280 nm for TP40 and PE40δCys, respectively. For CD and fluorescence measurements, the samples were prepared as follows: The protein was mixed with a stock Gdn·HCl solution in buffer A to achieve a final protein concentration of 0.5 mg/mL in 4 M Gdn·HCl. Samples at lower concentrations of Gdn·HCl were prepared by diluting this solution with buffer A. Alternatively, TP40 samples were also prepared by direct dialysis into buffers containing desired concentrations of Gdn·HCl. In HPSEC measurements two stock solutions of protein were prepared, which contained 0.4 mg/mL protein in 6.2 mM sodium phosphate and 200 mM NaCl at pH 7.2 (buffer B), or protein at 0.4 mg/mL in buffer B containing 4 M Gdn·HCl. Final Gdn·HCl concentrations were prepared by combining appropriate amounts of these two solutions. Samples were incubated at ambient temperature for 24 h and then filtered through a 0.22-μm cellulose acetate filter prior to injection. The pH of all samples was maintained at 7.2 ± 0.1.

Fluorescence. An SLM 8000 photon-counting spectrofluorometer was used to monitor Gdn·HCl-induced changes in tryptophan fluorescence spectra of TP40 and PE40δCys. Thermal perturbation of fluorescence was measured in a SPEX Fluorolog fluorometer modified to obtain automated spectral measurements over a prescribed temperature range. Data were obtained at 2 °C temperature intervals with a 2-min incubation time between temperature shifts. Spectra were corrected for Raman scattering but not for the wavelength dependence of the monochromator. Fluorescence lifetimes were measured at ambient temperature by frequency domain phase fluorometry using an ISS K2 multiple-frequency instrument, and data were analyzed by employing a method of the sum of exponentials (Lakowicz et al., 1984).

CD. Far-UV CD spectra were measured at 8 and 25 °C using a JASCO J-720 spectropolarimeter. Protein concentrations of 0.1, 0.5, and 1.2 mg/mL were employed. Quartz cuvettes of 1- or 0.1-mm path length were used. Mean residue ellipticities at 222 nm, $[\theta]$ in deg·cm^2·$dmol^{-1}$, were calculated using a mean residue weight of 107 for both proteins. Thermal unfolding was monitored in an AVIV 62DS spectropolarimeter. The TMELT program provided by AVIV allows the monitoring of multiple wavelengths at preset temperature intervals. The sample was monitored at 2 °C intervals from 4 to 90 °C with a 2-min incubation at each measurement temperature, which is sufficient to obtain equilibrium. The data were base line-corrected by subtracting the signal at 320 nm from the signal at 206 nm. Near-UV CD of TP40 was measured using a protein concentration of 1.2 mg/mL and a quartz cuvette of 10-mm path length.

HPSEC. A flow rate of 0.5 mL/min through a Superose 12 column was maintained by a Rabbit HP pump (Rainin). Proteins with known Stokes radii in the absence and presence of 6 M Gdn·HCl were used to estimate the Stokes radii for TP40 and PE40δCys as described by Corbett and Roche (1984).

DSC. A Hart differential scanning calorimeter was used to monitor the thermal unfolding of TP40 at 1 mg/mL in buffer A. The temperature was increased from 5 to 100 °C at a rate of 60 °C/h, held at 100 °C for 5 min, and reduced to 5 °C at a rate of 65 °C/h. To determine the reversibility, the experiment was then immediately rerun on the same sample under the same conditions. Since no transition was observed in the second scan, it was used as a buffer blank for subtractions. All data manipulations were performed using the software provided with the instrument.

Dynamic light scattering (DLS) was measured with a Malvern System 4700C at right angles (Sanyal et al., 1993) employing a protein concentration of 1 mg/mL in buffer A.

Cell Kill Assays. The cell-killing activity of TP40 against A431 epidermoid carcinoma cells (American Tissue Type Collection) was determined as described (Sanyal et al., 1993).

Analysis of Unfolding Experiments. A two-state unfolding model (N ↔ U) was used to analyze the equilibrium unfolding data. To compare the transitions detected by different methods, each unfolding curve was normalized to the apparent fraction of the unfolded form, F_{app}, as follows:

$$F_{app} = (Y_{obs} - Y_{nat})/(Y_{unf} - Y_{nat}) \quad (1)$$

where Y_{obs} is the wavelength of maximum fluorescence intensity, molar ellipticity, or Stokes radius at a given Gdn·HCl concentration and Y_{nat} and Y_{unf} were the observed values of the native and unfolded forms. Y_{nat} and Y_{unf} are obtained by performing a linear regression of the plateau region observed at low and high Gdn·HCl concentrations, respectively (Pace, 1986).

Determination of Midpoints of Unfolding Transition Curves. F_{app} data for each protein were analyzed with the

4-parameter logistic curve fitting program ALLFIT.[2] For each protein, the curves which were obtained by the different methods were analyzed together so that the maximum and minimum (fully unfolded and fully folded, respectively) could be constrained. The slope of each curve and its midpoint were allowed to vary.

Kinetics. The kinetics of the changes in tryptophan fluorescence which occur in TP40 upon refolding were measured using a Hitachi F2000 fluorometer (in the seconds to minutes range) and an OLIS 4300S stopped-flow spectrofluorometer (in the milliseconds to seconds range). For stopped-flow experiments TP40 at 0.2 mg/mL in 2.5 M Gdn·HCl was diluted to a final volume of 0.06 mg/mL in 0.7 M Gdn·HCl using drive syringes that allow uneven mixing volumes. Samples were excited at 280 nm, and an interference filter centered at 340 nm with a bandpass of 15 nm was placed in the emission path. For measurements with the Hitachi 2000 fluorometer, TP40 at a concentration of 0.1 mg/mL in 2.5 M Gdn·HCl was diluted to 0.04 mg/mL in 1.0 M Gdn·HCl. The solution in the cuvette was stirred during measurement. The sample was excited at 280 nm, and the emission monochromator was set at 335 nm. All kinetic data were analyzed with SpectraCalc software (Galactic Industries). The kinetics of the refolding of secondary structure were monitored in a JASCO J-720 spectropolarimeter. A 1-cm path length cuvette was used, with spectral changes detected at 222 nm. An aliquot of unfolded TP40 was added to a volume of PBS buffer, the sample was mixed quickly, and data acquisition was initiated. There was a 10-s dead time between addition of the unfolded sample and the first recording of data. Two experiments were performed. The first employed a dilution from 4 M Gdn·HCl to 0.2 M. The second experiment recreated the fluorescence refolding conditions in which 2.5 M Gdn·HCl was diluted to 0.7 M.

RESULTS

Unfolding Detected by Fluorescence. TP40 contains four tryptophan residues all of which occur in the PE40δCys segment. The wavelength of maximum fluorescence (λ_{max}) of TP40 is independent of excitation wavelength (280–295 nm), suggesting that tyrosine residues do not make a major contribution to the protein's fluorescence. TP40 and PE40δCys manifest the same λ_{max} of 336 nm (uncorrected), suggesting that the presence of TGF-α in TP40 does not significantly alter the environment of the PE40δCys tryptophan residues. Both proteins' fluorescence spectra were progressively red-shifted (Figure 1A) and their intensity quenched (data not shown) as they were exposed to increasing concentrations of Gdn·HCl. The midpoints of the λ_{max} versus Gdn·HCl concentration curves were calculated to be 1.5 and 1.4 M for TP40 and PE40δCys (Table 1), respectively. The main transition of TP40 manifests a fairly sharp change in λ_{max} from 336 to 349 nm between Gdn·HCl concentrations of 1 and 2 M. Approximately 90% of the total fluorescence quenching occurs in this phase. This is followed by a broader transition which is accompanied by a further red shift of the fluorescence λ_{max} to 354 nm. The midpoint of this second transition occurs at approximately 2.1 M Gdn·HCl. This transition accounts for 33% of the total red shift in fluorescence λ_{max} and 10% of the total quenching of fluorescence intensity

[2] This software (version 2.7, Nov 1988) was written by A. D. Lean, P. J. Munson, V. Guardabasso, and D. Rodbard of the Laboratory of Theoretical and Physical Biology at the National Institute of Child Health and Human Development, NIH.

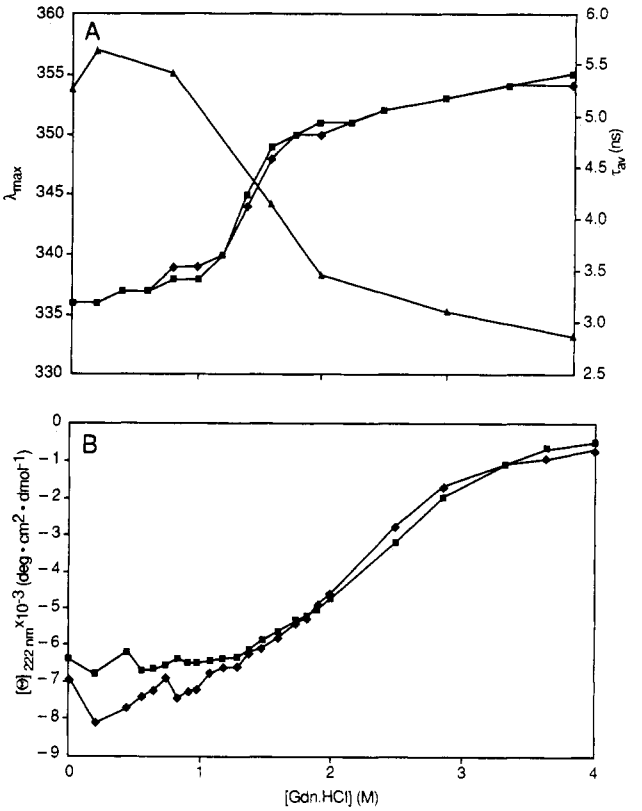

FIGURE 1: Unfolding of TP40 and PE40δCys as a function of Gdn·HCl concentration as monitored by intrinsic tryptophan fluorescence and far-UV CD spectra. (A) ♦, λ_{max} in nm of the fluorescence emission spectrum of TP40; ■, λ_{max} in nm of the fluorescence emission spectrum of PE40δCys; ▲, average tryptophan fluorescence lifetime (ns). (B) CD mean residue ellipticity at 222 nm of TP40 (■) and PE40δCys (♦). Experimental details are described in the Materials and Methods section.

Table 1: Gdn·HCl Denaturation Midpoints of TP40 and PE40δCys As Determined by Various Methods

	Gdn·HCl (M) for half-maximal change		
protein	fluorescence	CD	HPSEC
TP40	1.4	2.1	1.6
PE40δCys	1.5	2.0	1.7

produced by Gdn·HCl. It may reflect the disruption of residual tertiary structure (as detected by average tryptophan fluorescence) that remained in an intermediate state produced in the first phase. A similar second transition is also observed for PE40δCys.

The average tryptophan fluorescence lifetime (τ) of TP40 also exhibits a dependence on Gdn·HCl concentration similar to that observed for steady-state fluorescence. The lifetime decreases with increasing Gdn·HCl concentration, with the midpoint of this transition occurring at approximately 1.4 M (Figure 1A). Measurements of tryptophan alone over the same range of Gdn·HCl concentrations suggest that the decrease in fluorescence intensity or lifetime is not caused by a simple dynamic quenching of the protein's tryptophan by Gdn·HCl. The resolution of the lifetime measurements was insufficient to detect the presence or absence of any second transition.

Unfolding Detected by CD. The far-UV CD spectra of TP40 and PE40δCys have been previously described, and the secondary structure contents have been estimated (Sanyal et al., 1993). The spectrum of TP40 is dominated by the PE40δCys component, which constitutes 89% of TP40 by mass.

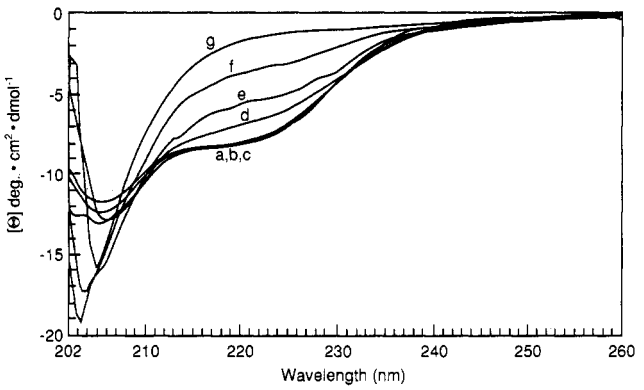

FIGURE 2: Far-UV (202–260 nm) spectra of TP40 in the (a) absence and presence of (b) 0.5 M, (c) 1.0 M, (d) 1.5 M, (e) 2.0 M, (f) 2.5 M, and (g) 4.0 M Gdn·HCl. The TP40 concentration was 1.2 mg/mL, and a cuvette of 0.1-mm path length was employed.

The contribution of TGF-α to the far-UV CD of TP40 is small (Sanyal et al., 1993), and at 222 nm it amounts to approximately 1%. Both TP40 and PE40δCys spectra manifest a minimum at 206 nm and a band of negative ellipticity around 220 nm. These features are characteristic of proteins containing quantitatively similar contributions from α-helix and β-sheet structure. CD spectra were measured as a function of Gdn·HCl concentration, and the decrease in the magnitude of CD ellipticity at 222 nm with increasing Gdn·HCl concentration was used to monitor the unfolding of secondary structure in TP40 and PE40δCys (Figure 1B). The approximate midpoints for these broad transitions were estimated to be 2.0 and 2.1 M for TP40 and PE40δCys, respectively (Table 1). Nearly identical transition curves were obtained at 8 and 25 °C (not illustrated). The effect of Gdn·HCl on the CD of TP40 in the wavelength range of 202–250 nm is shown with a few representative spectra in Figure 2. In 4 M Gdn·HCl an almost complete loss of secondary structure was apparent from the CD spectra. The convergence of all spectra to essentially zero ellipticity at 250 nm suggests that a differential light scattering artifact was absent or insignificant. A progressive decrease in the magnitude of the CD in the 215–230-nm range was produced as a function of increasing concentration of Gdn·HCl. It is apparent from these data (Figure 2) that Gdn·HCl-induced changes in CD occur less cooperatively than corresponding fluorescence transitions. Plots of $[\theta]_{214nm}$, $[\theta]_{218nm}$, and $[\theta]_{222nm}$ as a function of Gdn·HCl concentration yield very similar transition curves (not illustrated), with approximate midpoints falling between 2.0 and 2.5 M Gdn·HCl. This suggests that (a) CD is monitoring a global perturbation of secondary structure by Gdn·HCl and (b) wavelength-dependent absorption-flattening artifacts are absent or not significant. Furthermore, although formation of a soluble aggregate was indicated to a small extent for TP40 and to a greater degree for PE40δCys by HPSEC measurements (see below) at intermediate concentrations of Gdn·HCl up to 2.0 M, this did not produce detectable light scattering artifacts in CD (or fluorescence) measurements. The protein samples were completely soluble at the concentrations of protein and Gdn·HCl employed. Absence of any significant light scattering was also confirmed by examining base lines of UV absorbance spectra in the 310–350-nm wavelength region. To determine if there was any adsorption of protein onto the surface of the cuvette, measurements of the UV absorbance of TP40 samples in the absence and presence of varying Gdn·HCl concentrations were repeated following their removal from the cuvette. There was no detectable difference in the absorbance spectra between the two measurements, suggesting no adsorptive loss of protein from solution onto cuvette surfaces.

Unfolding Detected by HPSEC. The hydrodynamic radii (r) of TP40 and PE40δCys were measured as a function of Gdn·HCl concentration with HPSEC by comparison to proteins of known Stokes radii eluted in the presence (6 M) and absence of Gdn·HCl. Plots of retention times (r) versus

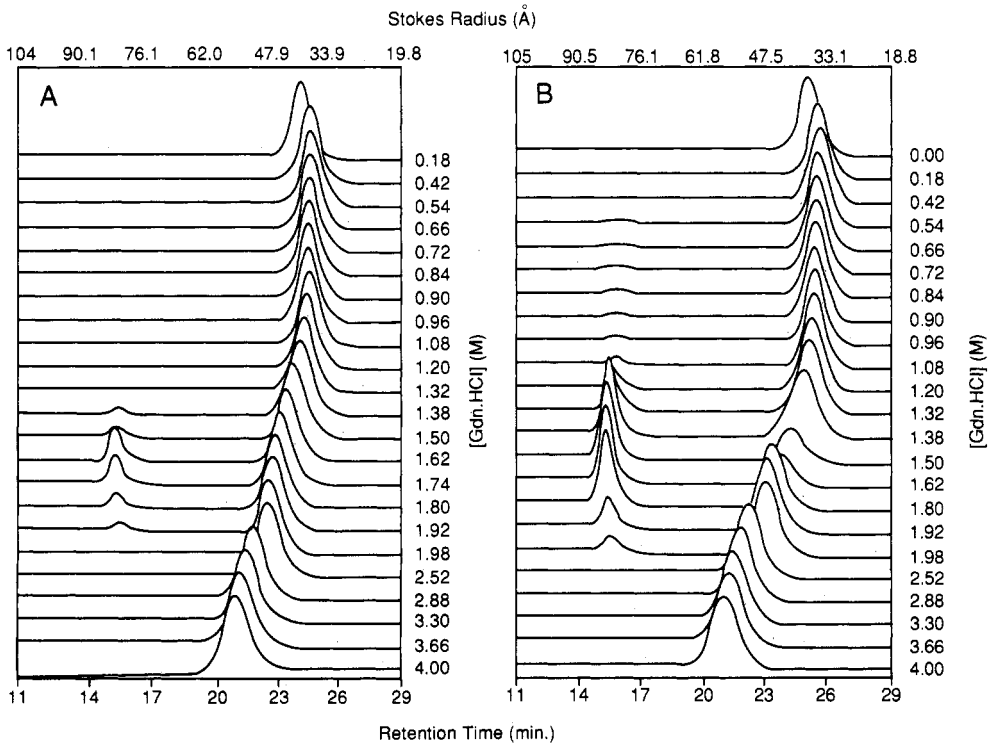

FIGURE 3: Determination of Stokes radii of (A) TP40 and (B) PE40δCys as a function of Gdn·HCl concentration. Each protein was chromatographed at a concentration of 0.4 mg/mL. The y-axis is proportional to absorbance at 280 nm. The column was calibrated by measuring retention times for proteins of known Stokes radii.

absorbance over a wide range of Gdn·HCl concentration are shown in Figure 3. The value of r for TP40 increased from 3.8 nm in the absence of Gdn·HCl [in agreement with DLS results reported in Sanyal et al. (1993)] to 5.7 nm in the presence of 4 M Gdn·HCl. For PE40δCys values of r were 3.7 and 5.7 nm at 0 and 4.0 M Gdn·HCl, respectively. At 1.3 M Gdn·HCl, where the major fluorescence transition is complete but the change in CD is approximately only 15% of the total change, the values of r for the smaller sized species were 4.3 and 4.5 nm for PE40δCys and TP40, respectively. Thus, both proteins maintain a semicompact state at denaturant concentrations that largely disrupt their tertiary structures (as detected by fluorescence and near-UV CD) but are insufficient to destroy secondary structure. At Gdn·HCl concentrations between 1.3 and 2.0 M, a relatively small population of an early eluting species corresponding to an r of 8.3 nm was observed for TP40 in addition to the monomer peak, suggesting self-association at these intermediate denaturant concentrations. The position of this peak (retention time) was independent of Gdn·HCl concentration within the above-mentioned range, but the amount of aggregated protein was maximal at 1.7 M Gdn·HCl as detected by UV absorbance. PE40δCys behaved similarly to TP40, manifesting a self-associated species with an r of 8.3 nm at intermediate Gdn·HCl concentrations. Small amounts of this species, however, began to appear at Gdn·HCl concentrations even below 1.0 M. These data suggest that partial unfolding of both proteins is accompanied by exposure of presumably hydrophobic surfaces that interact intermolecularly to form relatively small, soluble aggregates. HPSEC-detected aggregation of porcine growth hormone at Gdn·HCl concentrations in the range of 2.0–3.5 M, but not at lower or higher concentrations, has also been reported (Bastiras & Wallace, 1992).

Comparison of Unfolding Profiles Obtained by Fluorescence, CD, and HPSEC. The apparent fraction (f_{app}) of unfolded TP40 and PE40δCys as a function of Gdn·HCl concentration was calculated from individual unfolding curves detected by fluorescence, CD, and HPSEC. A plot of f_{app} as a function of Gdn·HCl concentration (Figure 4) dramatically illustrates the much greater Gdn·HCl sensitivity of the proteins' tertiary structure (fluorescence) compared to their secondary structure (CD). The f_{app} curve calculated from Stokes radii is intermediate between those derived from the fluorescence and CD data, but more closely follows the fluorescence data.

Near-UV CD. The CD spectrum of TP40 was measured in the 260–320-nm wavelength range to further probe the conformationally dependent environment of tyrosine and tryptophan side chains (Figure 5). In the absence of Gdn·HCl, this spectrum is dominated by a broad, positive peak near 282 nm with a shoulder at approximately 290 nm. A dramatic decrease in the magnitude of ellipticity at these wavelengths is observed upon increasing Gdn·HCl concentration from 1.0 to 1.3 M. This observation supports fluorescence data and again suggests that major perturbations in tertiary structure occur at Gdn·HCl concentrations that are insufficient to disrupt secondary structure. Another important observation derived from these spectra is that the average tyrosine environment appears to be more strongly affected than that of tryptophans. This conclusion is based on the greater effect of Gdn·HCl on the CD band around 280 nm compared to the indole ^1L_b type band in the 290–292-nm region. The ratio of mean residue ellipticity values between 282 and 290 nm is 1.30 in the absence of Gdn·HCl. In the presence of 1.3 M Gdn·HCl, this ratio changes to 0.74 and the λ_{max} shifts to 290 nm. In contrast, more complete unfolding of TP40 in 4 M

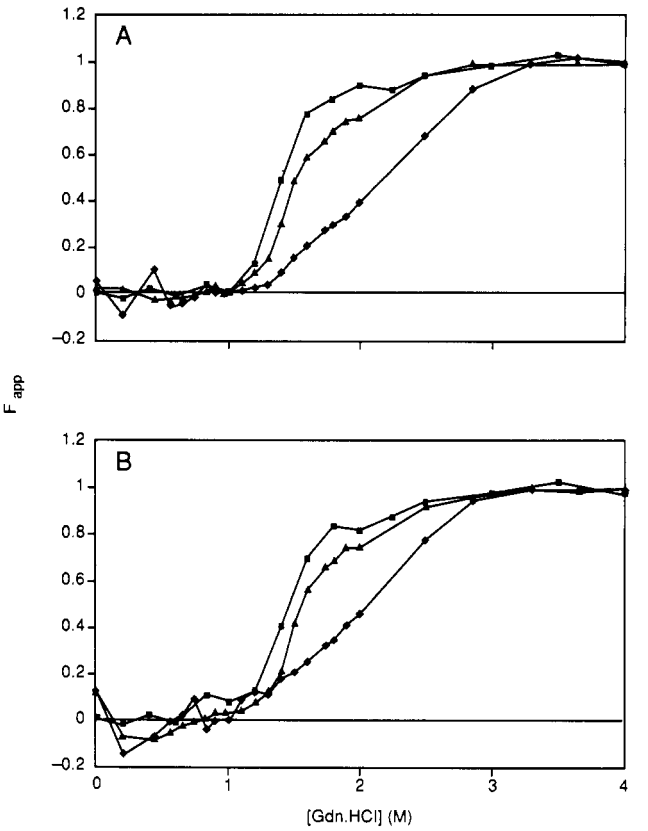

FIGURE 4: Fraction (F_{app}) of (A) unfolded TP40 and (B) PE40δCys as a function of Gdn·HCl concentration. $F_{app} = (Y_{obs} - Y_{nat})/(Y_{unf} - Y_{nat})$, where Y_{obs} is the observed CD (at 222 nm) or fluorescence (at λ_{max}) or Stokes radius at a given Gdn·HCl concentration, and Y_{nat} and Y_{unf} are the observed values for the native and completely unfolded forms, respectively. ■, f_{app} from fluorescence; ▲, f_{app} from CD; ♦, f_{app} from HPSEC.

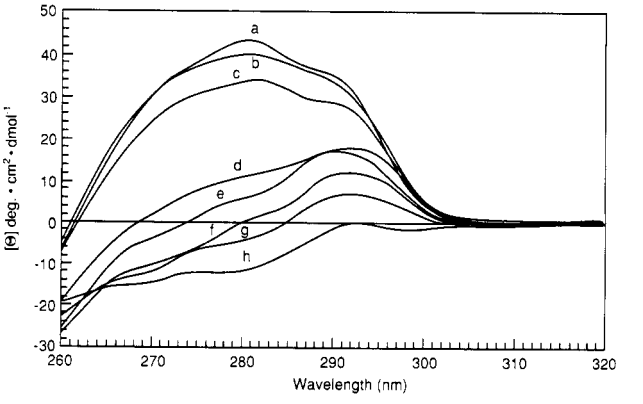

FIGURE 5: Near-UV (260–320 nm) CD spectrum of TP40 (a) in the absence of Gdn·HCl (—) and presence of (b) 0.5 M, (c) 1.0 M, (d) 1.3 M, (e) 1.5 M, (f) 2.0 M, (g) 2.5 M, and (h) 4.0 M Gdn·HCl. The samples contained 1.2 mg/mL of TP40, and a cuvette of 10-mm path length was used.

Gdn·HCl leads to a weak, negative, featureless spectrum. This suggests that the local structure around a fraction of the four tryptophan residues is maintained even in 1.3 M Gdn·HCl although a substantial loss in tertiary structure does appear to occur. This is qualitatively consistent with the two-stage unfolding curve indicated by tryptophan fluorescence measurements (Figure 1).

Kinetics of TP40 Refolding from the Gdn·HCl-Denatured State. The kinetics of the fluorescence change of TP40 upon dilution of Gdn·HCl from 2.5 M to 1.0 M were measured at ambient temperature employing steady-state fluorescence measurements (Figure 6). At 2.5 M Gdn·HCl, the tertiary

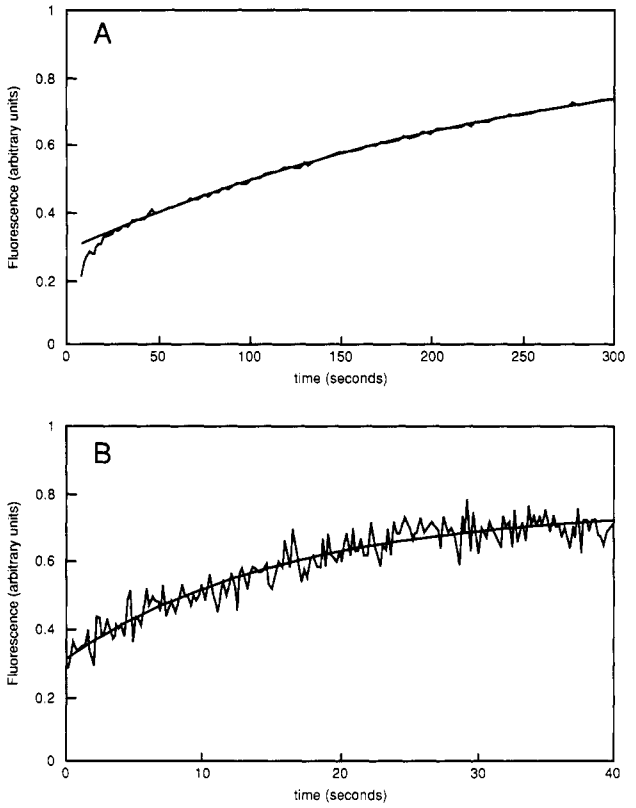

FIGURE 6: Kinetics of refolding of TP40, from a largely unfolded state in 2.5 M Gdn·HCl, as measured by changes in tryptophan fluorescence intensity (excited at 280 nm) produced upon dilution of Gdn·HCl. (A) Measurement was made in a Hitachi 2000 fluorometer. Final Gdn·HCl concentration was 1.0 M. The emission monochromator was set at 335 nm. (B) Measurement was made in an OLIS stopped-flow spectrofluorometer. Final Gdn·HCl concentration was 0.7 M. A 10-nm bandpass filter centered at 340 nm was placed in the emission path. Both panels show the actual data and the line calculated for a first-order reaction.

structure is about 90% unfolded and the secondary structure is approximately 65–75% disrupted (see Figure 1). Analysis of data over a time range of 40–300 s yielded a first-order rate constant of approximately 0.4 min^{-1} ($t_{1/2} \simeq 104$ s). An initial faster phase was apparent but could not be reliably analyzed because of the relatively long dead time (approximately 8 s) of this measurement. To examine this more rapid phase of refolding, stopped-flow kinetics were employed. The rate of fluorescence increase over the initial 40 s was measured upon dilution of Gdn·HCl from 2.5 to 0.7 M. A first-order rate constant of approximately 3.5 min^{-1} ($t_{1/2} \simeq 12$ s) was obtained, which is approximately 9 times faster than the rate constant for the slower phase.

The kinetics of changes in the secondary structure content of TP40 upon refolding were monitored by employing the CD signal at 222 nm (data not shown). When TP40 was equilibrated in 4 M Gdn·HCl and then diluted to 0.2 M Gdn·HCl, the total change in magnitude of the CD at 222 nm occurred in a time period shorter than the dead time of the measurement (10 s). There was no further detectable change of the CD signal with time. The same experiment was performed using a Gdn·HCl concentration of 2.5 M diluted to 0.7 M. In both cases, the samples at equilibrium exhibited quantitatively the same difference in CD (222 nm) between the high and low Gdn·HCl concentrations as that measured in the kinetics experiment. These data suggest that the rate of refolding of TP40 secondary structure is much faster than the two refolding processes detected by tryptophan fluorescence.

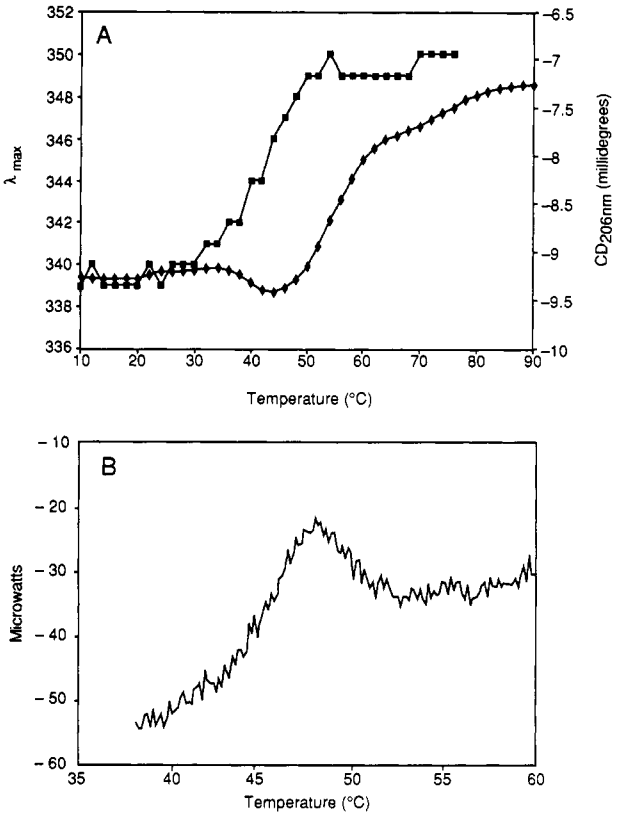

FIGURE 7: Effect of temperature on TP40 conformation as measured by (A) ■, tryptophan fluorescence [at λ_{max} (nm)], ♦, CD (206 nm), and (B) differential scanning calorimetry. TP40 concentrations were (A) 0.1 mg/mL and (B) 1.0 mg/mL.

Thermal Unfolding. The thermal unfolding of TP40 was studied by monitoring its intrinsic tryptophan fluorescence (λ_{max}) and CD (at 206 nm) as a function of temperature as well as by DSC, at a pH of approximately 7.2 in 100 mM sodium phosphate buffer (Figure 7). The midpoints for the thermal transition curves (T_m) as monitored by fluorescence and CD occur at approximately 42 and 55 °C, respectively (Figure 7A). This once again suggests that the tertiary structure as probed by the average environment of the tryptophan residues is more readily disrupted (in this case by temperature) than the secondary structure of TP40. DSC finds a T_m (48 °C) intermediate between these values (Figure 7B). Thermal unfolding is accompanied by protein aggregation as detected by DLS measurements at 50 °C, with the apparent hydrodynamic radius increasing from approximately 3.8 nm at 23 °C to 11.5 nm at 50 °C. Temperature-induced changes in TP40 are irreversible when thermally unfolded samples are returned to a lower temperature, as judged by DSC, HPSEC, CD, fluorescence, and a cell kill activity assay. Since aggregation accompanied unfolding at temperatures above 40 °C, it was not possible to determine whether this irreversibility was a direct result of unfolding, aggregation, or a combination of both processes. A sample of TP40 incubated at 37 °C for 18 h was fully bioactive and manifested less than 1% aggregation in HPSEC experiments. However, when incubated at 50 °C for the same length of time, TP40 was almost entirely converted to aggregated material which did not manifest detectable A431 cell kill activity. The characteristic negative CD band of TP40 around 220 nm was irreversibly lost at elevated temperatures, although the minimum at 206 nm remained. The changes in mean residue ellipticities across the 180–240-nm wavelength range were, however, much smaller than those induced by 4 M Gdn·HCl. Similarly, the thermally induced maximal change in λ_{max} of

the TP40 fluorescence spectrum is only 11 nm, whereas 4 M Gdn·HCl produces an 18-nm shift.

DISCUSSION

TP40 is a multidomain chimeric protein. The folding pathway of TP40 is of particular interest because a partially unfolded conformation has been implicated in the translocation of this protein across the endosomal membrane (Sanyal et al., 1993). The translocation domain of TP40 occurs in the PE40δCys portion which is, in turn, derived from PE. It has been suggested that membrane translocation of PE involves a partially denatured state (Farahbakhsh et al., 1987; Jiang & London, 1990). In these membrane-interacting states, presumably induced by the low pH of endosomes, the hydrophobic surfaces of the protein are proposed to be partially exposed while the secondary structure remains nearly intact. These characteristics are reminiscent of the "molten globule" states that are thought to form as intermediates in the folding pathway of many, if not most, proteins (Christensen & Pain, 1991; Kim & Baldwin, 1982; Kuwajima, 1989). Such intermediates have generally been observed at low pH and high ionic strength as well as at concentrations of structure-perturbing agents that are insufficient to completely unfold a protein. The most well-studied protein in this regard is α-lactalbumin (Kuwajima et al., 1976; Baum et al., 1989). The formation of a thermodynamically stable, partially folded intermediate should produce deviations from two-state folding–unfolding behavior. This is commonly reflected in noncoincident transition curves when equilibrium unfolding is measured by different techniques that probe different structural levels of the protein (Kim & Baldwin, 1982). TP40 and PE40δCys both manifest a greater susceptibility to the Gdn·HCl- or temperature-induced disruption of tertiary than secondary structure as detected by tryptophan fluorescence or near-UV CD and far-UV CD, respectively. At relatively low (1.3–1.8 M) Gdn·HCl concentrations one or more partially unfolded states of TP40 appear to exist which fit most if not all of the criteria for a molten globule state, i.e., a semicompact form with near-native secondary structure but substantially reduced tertiary structure. Thus, one or more unfolding intermediates can be invoked to explain these data. The observation of a second component showing a relatively large Stokes radius at these low denaturant concentrations suggests the presence of self-associating, thermodynamically stable, partially folded intermediates. Aggregation at low pH, possibly induced by similar mechanisms, was previously observed for TP40 and PE40δCys (Sanyal et al., 1993). Thus, on the basis of spectroscopic, kinetic, and size data, TP40 appears to be an example of a protein in which a partially folded state is responsible for aggregation rather than a more completely unfolded form as suggested for apomyoglobin (Young et al., 1993). Self-association via partially folded intermediates has been recently reported for human growth hormone (DeFelippis et al., 1993) and acidic fibroblast growth factor (Mach et al., 1993). Furthermore, the presence of molten globule intermediates in apomyoglobin has been proposed on the basis of extensive NMR and kinetic data (Barrick & Baldwin, 1993).

The refolding of TP40 upon dilution of Gdn·HCl manifests three kinetic phases: (1) a fast phase ($t_{1/2} < 10$ s) in which secondary structure forms, as detected but too fast to be quantitated by CD; (2) a second phase in which tryptophan fluorescence is increased with a $t_{1/2}$ of approximately 12 s; and (3) a slower phase in which tryptophan fluorescence is further increased with a $t_{1/2}$ of approximately 1.7 min. These last two phases are not accompanied by a change in CD. Thus, in the refolding process the native secondary structure of the protein is formed prior to its folding into the final native state. The formation of the fully folded structure then appears to occur in two phases, the precise origin of which is unknown.

The noncoincidence of thermal transition curves detected by fluorescence, CD, and DSC suggests that thermal denaturation of TP40 also proceeds via one or more intermediates. Among the three techniques, fluorescence yields the lowest T_m. This is consistent with large changes in tryptophan environment preceding the more global structural perturbation reflected in CD and DSC measurements. The reason for the difference between T_m values obtained from CD and DSC is unclear but could reflect the kinetic nature of the DSC measurements or the higher protein concentrations required for DSC. It is also possible that this thermodynamic technique which detects the sum of all the endo- and exothermic processes in the unfolding reaction actually reflects both secondary and tertiary structure disruption thereby falling intermediate between the fluorescence and CD results. Both of these transitions are rather broad, however, and estimates of midpoints are, at best, approximations.

The degree of similarity of the intermediate(s) formed by low pH, Gdn·HCl,, and temperature is unclear. Interestingly, both low pH-induced and thermal unfolding are accompanied by aggregation. Furthermore, at 50 °C where the fluorescence change has reached its maximal level, CD changes are small. At low pH where the hydrophobic surfaces of TP40 are exposed, changes in CD are also minimal compared to those seen at neutral pH (Sanyal et al., 1993). Although these effects are not quantitatively comparable, they suggest that qualitatively similar changes in tertiary structure can be induced by these different perturbations. Flexibility in TP40 and PE40δCys is also suggested by changes in tertiary structure induced by relatively low concentrations of Gdn·HCl as detected by tryptophan fluorescence and near-UV CD. Exposure of apolar surfaces and induction of aggregation are also found at these (<2 M) Gdn·HCl concentrations.

The data presented in this report suggest the existence of one or more thermodynamically stable intermediate(s) in the unfolding or refolding pathway of PE40δCys and TP40. The important question is, however, whether such intermediates are physiologically relevant. Low pH-induced partial unfolding of PE is thought to facilitate its interaction with and translocation across the endosomal membrane (Farahbakhsh et al., 1987; Jiang & London, 1990). A similar low-pH induced conformational change of TP40 (and PE40δCys), in which apolar regions are exposed without any major alteration of secondary structure, has been observed and implicated in its membrane translocation mechanism (Sanyal et al., 1993). We have now shown that qualitatively similar intermediate conformation(s), with native secondary structure but disrupted tertiary structure, can also be induced by temperature and Gdn·HCl. These results contribute to the emerging view that such intermediates, structurally distinct from native and unfolded states of proteins, may indeed be playing important functions. In the case of TP40 (and perhaps other proteins requiring translocation), this function is probably to allow membrane translocation events of which the fully folded protein is incapable. It should be noted, however, that translocation itself may be facilitated by additional proteins.

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REFERENCES

Barrick, D., & Baldwin, R. L. (1993) *Protein Sci. 2*, 869–876.
Bastiras, S., & Wallace, J. C. (1992) *Biochemistry 31*, 9304–9307.
Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) *Biochemistry 28*, 7–13.
Blewitt, M. G., Chung, L. A., & London, E. (1985) *Biochemistry 24*, 5458–5464.
Chaudhury, V. K., FitzGerald, D. J., Adhya, S., & Pastan, I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4538–4542.
Christensen, H., & Pain, R. H. (1991) *Eur. Biophys. J. 19*, 221–229.
Collins, C. M., & Collier, R. J. (1987) in *Membrane-Mediated Cytotoxicity* (Bonavide, B., & Collier, R. J., Eds.) Liss, New York.
Corbett, R.-J. T., & Roche, R. S. (1984) *Biochemistry 23*, 1888–1894.
DeFelippis, M. R., Alter, L. A., Pekar, A. H., Havel, H. A., & Brems, D. N. (1993) *Biochemistry 32*, 1555–1562.
Edwards, G. M., DeFeo-Jones, D., Tai, J. Y., Vuocolo, G. A., Patrick, D. R., Heimbrook, D. C., & Oliff, A. (1989) *Mol. Cell Biol. 9*, 2860–2867.
Farahbakhsh, Z. T., Baldwin, R. L., & Wisnieski, B. J. (1987) *J. Biol. Chem. 262*, 2256–2261.
Heimbrook, D. C., Stirdivant, S. M., Ahern, J. D., Balishin, N. L., Patrick, D. R., Edwards, G. M., DeFeo-Jones, D., FitzGerald, D. J., Pastan, I., & Oliff, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4697–4701.
Jiang, J. X., & London, E. (1990) *J. Biol. Chem. 265*, 8636–8641.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.
Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., & Saelinger, C. B. (1992) *J. Biol. Chem. 267*, 12420–12423.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
Lakowicz, J. R., Laczko, G., Cherek, H., Gratton, E., & Limkeman, M. (1984) *Biophys. J. 46*, 463–477.
Mach, H., Ryan, J. A., Burke, C. J., Volkin, D. B., & Middaugh, C. R. (1993) *Biochemistry 32*, 7703–7711.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.
Pastan, I., & FitzGerald, D. (1989) *J. Biol. Chem. 264*, 15157–15160.
Pastan, I., & FitzGerald, D. (1991) *Science 254*, 1173–1177.
Sanyal, G., Marquis-Omer, D., Gress, J. O., & Middaugh, C. R. (1993) *Biochemistry 32*, 3488–3497.
Young, L. R. D., Dill, K. A., & Fink, A. L. (1993) *Biochemistry 32*, 3877–3886.