Kinetics of Folding of the IgG Binding Domain of Peptostreptoccocal Protein L

Michelle L. Scalley, Qian Yi, Hongdi Gu, Ashley McCormack,‡ John R. Yates III,‡ and David Baker*

Department of Biochemistry, University of Washington, Seattle, Washington 98195

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ABSTRACT: The kinetics of folding of a tryptophan containing mutant of the IgG binding domain of protein L were characterized using stopped-flow circular dichroism, stopped-flow fluorescence, and HD exchange coupled with high-resolution mass spectrometry. Both the thermodynamics and kinetics of folding fit well to a simple two-state model: (1) Guanidine induced equilibrium denaturation transitions measured by fluorescence and circular dichroism were virtually superimposable. (2) The kinetics of folding/unfolding were single exponential under all conditions examined, and the rate constants obtained using all probes were similar. (3) Mass spectra from pulsed HD exchange refolding experiments showed that a species with very little protection from exchange is converted to a fully protected species (the native state) at a rate very similar to that of the overall change in tryptophan fluorescence; no intervening partially protected species were observed. (4) Rate constants (in H_2O) and m values for folding and unfolding determined by fitting observed relaxation rates obtained over a broad range of denaturant concentrations to a two-state model were consistent with the equilibrium parameters ΔG and m: $-RT \ln(k_u/k_f)/\Delta G_U^{H_2O} = 1.02$; $(m_u + m_f)/m = 1.08$. In contrast to results with a number of other proteins, there was no deviation from linearity in plots of ln k_{obs} versus guanidine at low guanidine concentrations, both in the presence and absence of 0.4 M Na_2S0_4, suggesting that significantly stabilized intermediates do not accumulate during folding. Although all of the change in fluorescence signal during folding in phosphate buffer was accounted for by the simple exponential describing the overall folding reaction, fluorescence-quenching experiments using sodium iodide revealed a small reduction in the extent of quenching of the protein within the first two milliseconds after initiation of refolding in low concentrations of guanidine, suggesting a partial collapse of the unfolded chain may occur under these conditions. Comparison with results on the structurally and functionally similar IgG binding domain of streptococcal protein G show intriguing differences in the folding of the two proteins.

We have chosen the 62 residue IgG binding domain of peptostreptococcal protein L (Wikstrom et al., 1993) as a model system for studying protein folding: the small size of the protein reduces the complexity of the folding problem, and the lack of disulfide bonds and proline residues simplifies data analysis. The solution structure of protein L, determined by high-resolution NMR (Wikstrom et al., 1994, 1993), consists of a single helix packed against a four-stranded β sheet. The structure is similar to that of the IgG binding domain of protein G (Achari et al., 1992), despite the absence of significant sequence similarity.

In this study, the kinetics of folding of a tryptophan containing mutant protein L (wt(trp)) are characterized using a variety of spectroscopic probes and hydrogen deuterium exchange coupled with mass spectrometry. Both equilibrium and kinetic data fit well to a simple two-state model of folding, which is confirmed by the observation of two dominant populations in mass spectra of deuterated protein allowed to refold for short periods of time in H_2O. The results are compared to data on the kinetics of folding of protein G and other small proteins.

EXPERIMENTAL PROCEDURES

Chemicals. Ultrapure Guanidine-HCl (guanidine) was purchased from USB. Guanidine stock solutions were made gravimetrically in volumetric flasks with the concentration determined from the refractive index. All solutions containing guanidine were adjusted to pH 7.0 with sodium hydroxide. Solutions used in equilibrium and kinetic measurements were sodium phosphate and sodium sulfate from JT Baker and sodium iodide from Sigma (St. Louis, MO). All solutions were made using double-distilled, deionized water.

Cloning, Expression, and Purification. The wild-type protein L was cloned into a modified version of the expression plasmid pET 15b (Novagen) as described previously (Gu et al., 1995). Wt(trp) was generated by PCR on the wild-type protein L construct using primers with sequences (1) 5′-cggataacaatttcacacag-3′ and (2) 5′-gggggggtcgacagtccattctccattg-3′ (the Y43W mutation is underlined). The product was purified and digested with *Xba*I and *Sal*I and ligated into the wild-type expression vector digested with the same enzymes. All constructs were verified by DNA sequencing.

Expression and purification of the histidine-tagged protein were carried out as previously described (Gu et al., 1995). The histidine residues preceding the N-terminal methionine were cleaved using cyanogen bromide, and the completely cleaved protein was purified from the reaction mixture as described by Yi and Baker (1996). The molecular weight was confirmed using mass spectrometry; the final protein preparation was >95% pure. Protein concentrations were determined using absorbance at 280 nm with an extinction coefficient of 5200 M^{-1} cm^{-1} for wt and 9970 M^{-1} cm^{-1} for wt(trp) as calculated using the method of Gill and von

* Corresponding author.
‡ Department of Molecular Biotechnology, University of Washington, Seattle, WA 98195.
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Hippel (1989). No differences were detectable between the folding rates of protein L with and without the His-tag; protein L with the His-tag removed was used for all experiments described here except for the stopped-flow circular dichroism experiments.

Equilibrium Fluorescence and Circular Dichroism Measurements. All equilibrium fluorescence measurements were made using a Spex Fluorolog2 spectrofluoremeter with a 10 mm cuvette. The excitation wavelength was 280 nm and 0.7 mm and 1.0 mm slits were used for the excitation and emission monochrometers, respectively. Equilibrium fluorescence emission spectra for wild type and wt(trp) samples were measured in varying concentrations of guanidine buffered with 50 mM sodium phosphate, pH 7.0. Protein samples used for equilibrium measurements of sodium iodide fluorescence quenching were prepared similarly with the addition of 1 M sodium iodide. All measurements were taken at 295 ± 1 °K.

Circular dichroism measurements were made using an Aviv circular dichroism spectrometer 16A DS. CD spectra of wt and wt(trp) in 50 mM sodium phosphate, pH 7.0, 5 M guanidine, and 50 mM sodium phosphate, pH 7.0, were measured with a 2 mm cuvette. Guanidine denaturation curves of both wt and wt(trp) were obtained using a 10 mm cuvette. The observation cell was thermostated at 295 ± 0.2 K using a Peltier device.

NMR Spectroscopy. ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled wt and wt(trp) were carried out as described previously (Yi & Baker, 1996). The assignments of wt(trp) were based on those of wt (Wikstrom et al., 1993) and confirmed using HSQC-TOCSY (100 ms) experiments.

Quench Flow HD Exchange. Refolding and pulse labeling HD exchange experiments were carried out in the quench mode of a BioLogic SFM4/QFM4. ^{15}N-labeled wt(trp) (10 mg mL^{-1}) was first denatured and fully deuterated in 4.5 M guanidine (GuDCl/D_2O), pH 3.0 (uncorrected for the isotope effect). Refolding was initiated by diluting 1 part of the unfolded protein into 5 parts of 20 mM sodium acetate pH 4.4 (in H_2O). The refolding time varied from 4.7 ms to 1 s. After the refolding reaction, the samples were pulse labeled with 160 mM glycine, pH 10.4, for approximately 16 ms (the mixing ratio was 1:5) and then immediately mixed with 500 mM acetic acid, pH 2.7, in a 1:5 ratio to quench further HD exchange. Finally, all samples were washed with H_2O adjusted with formic acid to pH 3.7 to reduce the salt concentration and stored at −80 °C prior to MS analysis. The actual pH values during the refolding, labeling, and quenching reactions were 4.0, 9.3, and 4.1, respectively, as determined separately by mixing the reaction solutions in the appropriate ratios. Measurements were taken at 295 K.

Mass spectra were recorded on a TSQ700 (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source (Griffen et al., 1991). The capillary temperature was held at 150 °C with a potential of 24 V. Sample was infused through an entrance line of fused silica capillary (360 micron OD × 50 micron ID) connected to a syringe pump to drive the liquid. A fused silica microspray needle (4 cm of 375 mm OD × 20 mm ID) was stripped of its polyimide coating near the exit. The microspray needle was butted up against the entrance line inside a 26 gauge stainless steel hypodermic needle, and both ends of the tube were epoxied shut. A voltage (900−2000 V) was placed on the stainless steel junction to transfer the potential to the liquid and form the electrospray. The exit end of the electrospray needle was placed close (0.1−0.5 mm) to the entrance of the heated capillary. The sample solution flowed through the fused silica capillary at flow rates of 50−100 nL/min.

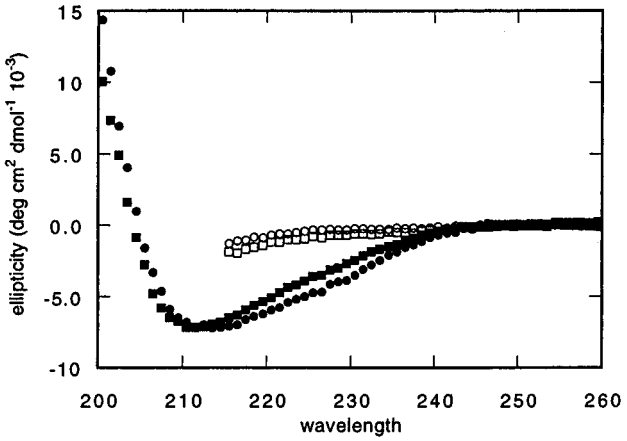

FIGURE 1: Far UV CD spectra of wt and wt(trp). Wt and wt(trp) are represented by circles and squares, respectively. Closed symbols denote folded conditions (50 mM sodium phosphate, pH 7.0) and open symbols denote unfolded conditions (5 M guanidine, 50 mM sodium phosphate, pH 7.0). Each spectrum is the average over 3 scans. Protein concentrations were 24.8 ± 1.8 μM.

Stopped-Flow Kinetic Measurements. All stopped-flow kinetics were obtained with a BioLogic SFM4/QFM4, and data were analyzed using the Biokine analysis software. All solutions and the cuvette holder were maintained at 295 K using a circulating water bath. Fluorescence measurements were made with an excitation wavelength of 280 nm using a 0.8 mm cuvette (FC-08) and a 309 nm cutoff filter. Refolding reactions were initiated by mixing 1 part of denatured protein solution, generally 50 μM wt(trp) in 3 M guanidine, 50 mM sodium phosphate, pH 7.0, into 9 parts of refolding buffer consisting of 50 mM sodium phosphate, pH 7.0, and low concentrations of guanidine. The final guanidine concentrations ranged between 0.3 and 2.75 M guanidine. The fluorescence signal of the unfolded protein was measured by diluting the unfolded protein solution 1:10 into 3 M guanidine, 50 mM sodium phosphate, pH 7.0. For refolding of wt(trp) in the presence of sodium sulfate, 1 part of denatured protein was diluted into 9 parts of refolding buffer supplemented with 440 mM sodium sulfate. Buffer baselines were subtracted from the measured signals in all experiments.

Unfolding was initiated by diluting 1 part of folded protein solution, generally 50 μM wt(trp) in 50 mM sodium phosphate, pH 7.0, into 9 parts of unfolding buffer containing 50 mM sodium phosphate, pH 7.0, and high concentrations of guanidine. The final concentration of guanidine ranged between 2.25 and 6.4 M guanidine. We were unable to monitor the unfolding of wt(trp) in the presence of sodium sulfate due to its low solubility in the presence of guanidine. The dead time for both the refolding and unfolding experiments calculated by oxidation of tryptophan by *N*-bromosuccinimide (Peterman, 1979) was 1.7 ms. The final concentrations of denaturant in all experiments were determined from the refractive index of the samples after the reactions were complete.

Fluorescence quenching with sodium iodide was examined under the same conditions as the other stopped-flow fluorescence measurements. Refolding was initiated by diluting 1 part of unfolded protein solution, 100 μM wt(trp) in 2 or 3 M guanidine, 1 M sodium iodide, 50 mM sodium

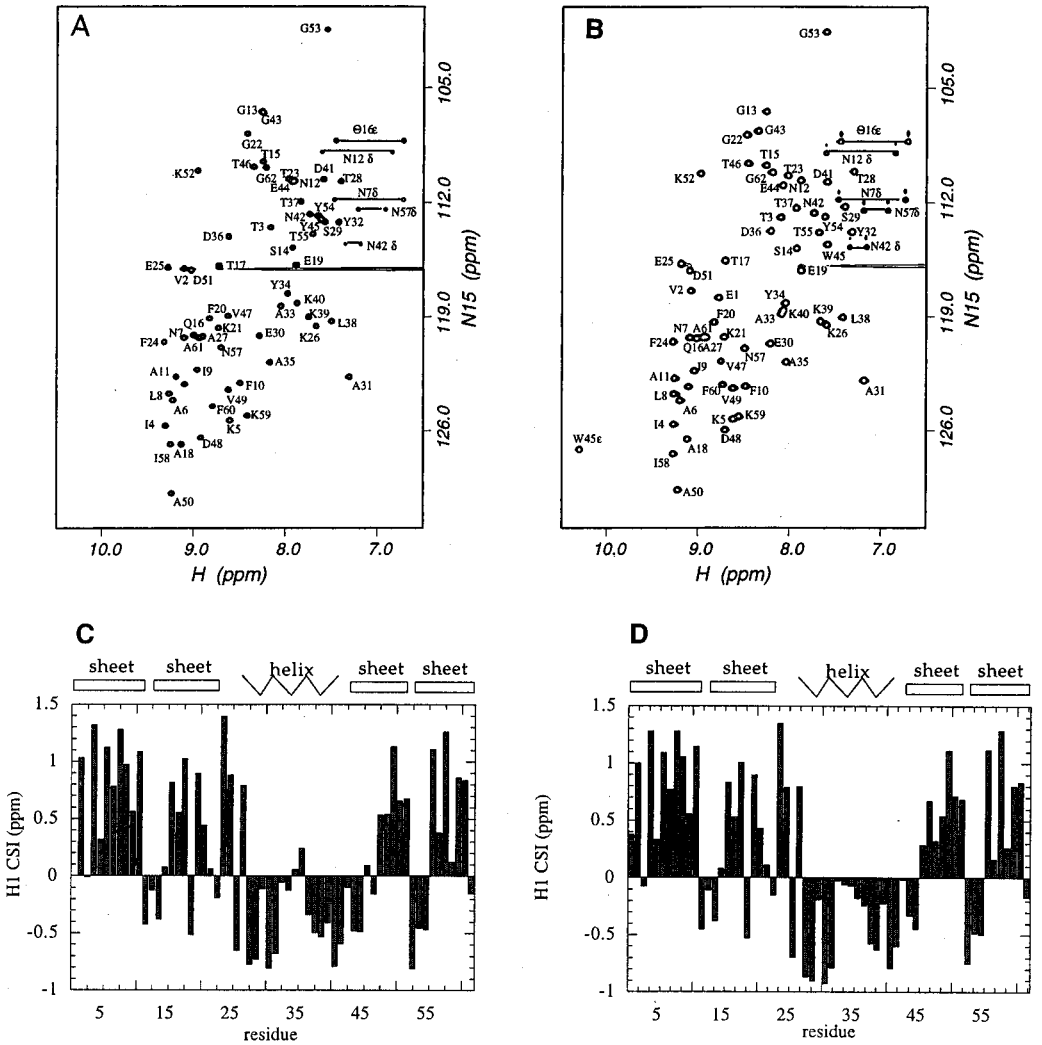

FIGURE 2: 1H-^{15}N HSQC spectra of wt and wt(trp). The spectra for wt and wt(trp) are shown in A and B, respectively. The chemical shift difference plots for main-chain amide protons for wt and wt(trp) are shown in C and D, respectively. Samples were in 50 mM sodium phosphate, pH 7.0.

phosphate, pH 7.0, into 9 parts of refolding buffer containing 1 M sodium iodide, 50 mM sodium phosphate, pH 7.0, and low concentrations of guanidine. The final concentration of guanidine varied between 0.2 and 2.1 M guanidine. The calculated dead time of the experiment was 1.7 ms. The signal corresponding to the unfolded protein was obtained by diluting 1 part of unfolded protein mixture into 9 parts of 2 or 3 M guanidine, 1 M sodium iodide, and 50 mM sodium phosphate, pH 7. Sodium iodide stock solutions containing 1 mM DTT were made fresh for each experiment to prevent oxidation.

Stopped-flow CD measurements were made with a 2 mm cuvette (FC-20) at 225 nm. Refolding was initiated by rapidly diluting 1 part of 5.0 mg mL^{-1} wt(trp) in 3 M guanidine, 50 mM sodium phosphate, pH 7.0, into 9 parts of 50 mM sodium phosphate, pH 7.0. The dead time under these conditions was determined to be 7.4 ms using the oxidation of tryptophan test reaction. The CD signal of the unfolded protein was measured by diluting the unfolded protein solution 1:10 into 3 M guanidine, 50 mM sodium phosphate, pH 7.0.

Kinetic Data Analysis. All kinetic data were fit to a single exponential using an implementation of the Pade−Laplace algorithm in the Biokine software. The refolding and unfolding data were fit to a two-state model:

$$\ln k_{obs} = \ln[k_f^{H_2O} \exp(-m_f[\text{guanidine}]) + k_u^{H_2O} \exp(m_u[\text{guanidine}])] \quad (1)$$

The observed relaxation rate, k_{obs}, at any given guanidine concentration is the sum of the folding and unfolding rates (k_f and k_u); the logarithms of these rate constants are assumed to be linear functions of the denaturant concentration with coefficients m_f and m_u respectively. $k_f^{H_2O}$ and $k_u^{H_2O}$ are the rates of folding and unfolding in the absence of denaturant. Standard deviations were obtained from the best fit of the data.

RESULTS

Wt(trp) Mutation. Wild type protein L does not contain tryptophan residues. To provide a sensitive probe for the folding of the protein, tyrosine 43 was changed to a tryptophan residue using site directed mutagenesis (see Experimental Procedures; the mutant protein is referred to as wt(trp) throughout the text). The structure of wt(trp) was compared to that of the wild type protein using circular dichroism (CD) and NMR spectroscopy. CD spectra for wt (Figure 1, closed circles) and wt(trp) (Figure 1, closed squares) in 0 M guanidine were similar with single minima at 214 and 211 nm, respectively, consistent with their α/β structure. The small differences in the spectra probably

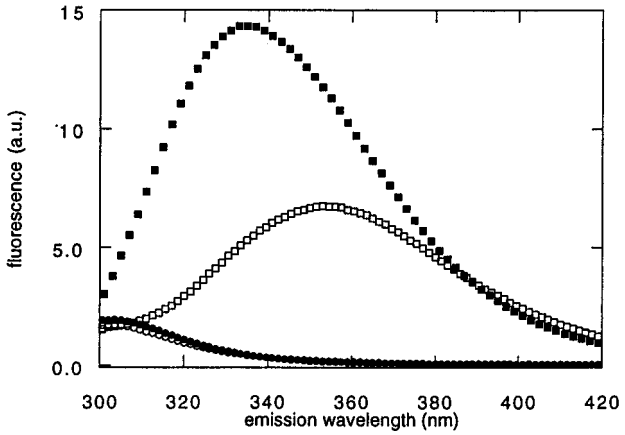

FIGURE 3: Fluorescence emission spectra of wt and wt(trp). Wt and wt(trp) are represented by circles and squares, respectively. Closed symbols denote folded conditions (50 mM sodium phosphate, pH 7.0) and open symbols denote unfolded conditions (5 M guanidine, 50 mM sodium phosphate, pH 7.0). Each spectrum is the average over 3 scans. Protein concentrations were 21.2 ± 0.5 μM.

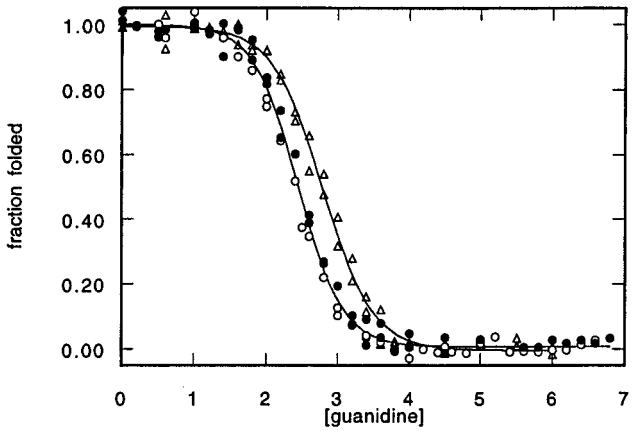

FIGURE 4: Equilibrium denaturation of wt and wt(trp). Guanidine induced denaturation of wt followed by (open triangles) CD at 220 nm and of wt(trp) followed by (closed circles) CD at 220 nm and (open circles) fluorescence emission at 328 nm. The solid line represents the average of the best fits of the data from independent experiments. The data were fit using a two-state model with a linear dependence of the free energy of unfolding on denaturant concentration. Wt(trp) denaturation curves were fit allowing for sloping baselines of the folded and unfolded protein; the data were corrected for sloping baselines during conversion to the fraction folded scale. The folded and unfolded baselines of wt protein did not slope significantly. Protein concentrations were 10.7 ± 0.4 μM.

reflect the contribution of the tryptophan in the far-UV region. NMR HSQC spectra of wt and wt(trp) are shown side by side in Figure 2A and B. The ^{1}H-^{15}N cross peak patterns in the two proteins are similar except for residues in the immediate vicinity of the mutation. The deviations of the amide proton chemical shifts from the random coil values (Figure 2C and D) suggest the proteins have the same $\beta-\beta-\alpha-\beta-\beta$ secondary structure elements. The HD exchange patterns of the two proteins are also very similar (data not shown). Taken together, these data suggest that the Y43W mutation in wt(trp) does not cause large-scale perturbations of the structure.

To be useful for monitoring folding, the fluorescence spectrum of the introduced tryptophan residue must be sensitive to the overall structure of the protein. The fluorescence emission spectra of folded wt(trp) in phosphate buffer (closed squares) and unfolded wt(trp) in 5 M guanidine (open squares) are compared in Figure 3. There is an approximately 2-fold decrease in fluorescence intensity upon unfolding, and a red shift of roughly 20 nm. The dominant contribution to the fluorescence spectra of both folded and unfolded wt(trp) is from the single tryptophan residue; the fluorescence signal of the wt protein is relatively weak and does not change significantly upon unfolding (Figure 3, circles). The large change in fluorescence upon folding greatly facilitates the characterization of the folding of wt-(trp).

Equilibrium Denaturation. There is a substantial change in CD signal upon unfolding of both wt and wt(trp) with 5 M guanidine (Figure 1). Equilibrium unfolding of wt can thus be monitored by CD, while the unfolding of wt(trp) can be monitored by both CD and fluorescence. The stability of wt and wt(trp) was examined through guanidine denaturation experiments (Figure 4). Unfolding of wt protein with increasing guanidine concentration was followed by the decrease in negative CD signal at 220 nm (open triangles). Denaturation of wt(trp) was monitored both by the decrease in fluorescence emission at 328 nm and by the decrease in CD signal at 220 nm. The unfolding transitions for wt(trp) measured by fluorescence (open circles) and CD (closed circles) were superimposable upon one another. This suggests that the loss of secondary structure, as measured by CD, and the loss of tertiary structure, as measured by fluorescence, occur concertedly as expected for a two-state transition.

Free energies of folding can be derived from the data in Figure 4 if a two-state model of folding is assumed. In such a model, the protein is in one of two states (folded or unfolded) at all denaturant concentrations, and the fraction of the protein that is folded at a given denaturant concentration is determined directly from the CD or fluorescence signal, allowing for a linear dependence of the CD or fluorescence signal of the individual states on the guanidine concentration (Pace, 1986). The free energy of unfolding, ΔG_U, was calculated at specific guanidine concentrations within the transition region of the melting curves from the fraction of unfolded protein. The resulting ΔG_U values were plotted against the guanidine concentration; a linear relationship was observed, and the free energy of folding in the absence of guanidine, $\Delta G_U^{H_2O}$, was estimated by extrapolation (Pace, 1986). The slope of the curve is commonly referred to as the *m* value and is thought to be proportional to the change in solvent exposed surface area upon unfolding (Myers et al., 1995). Standard deviations were calculated from values given by independent experiments. Both the free energy of unfolding and the *m* value for wt(trp) (4.6 ± 0.2 kcal mol^{-1} and 1.9 ± 0.07 kcal mol^{-1} M^{-1}, respectively) are nearly identical to those of the wt protein (4.7 ± 0.2 kcal mol^{-1} and 1.7 ± 0.04 kcal mol^{-1} M^{-1}, respectively).

Stopped-Flow Fluorescence and Circular Dichroism. In earlier studies of the IgG binding domain of protein G, refolding kinetics were monitored using a stopped-flow absorbance method at high pH through the coupling of folding to a change in the ionization state of a tyrosine residue with a subsequent large change in absorbance of the protein at 295 nm (Alexander et al., 1992). A similar stopped-flow absorbance method was used to compare the kinetics of folding of protein L wt and wt(trp) at high pH. Refolding of protein denatured at pH 12.0 was initiated by a jump to pH 11.0 as described by Alexander et al. (1992). The rates of refolding for wt and wt(trp) at pH 11.0 were 4.5 and 4.6 s^{-1}, respectively. Thus, the Y43W substitution

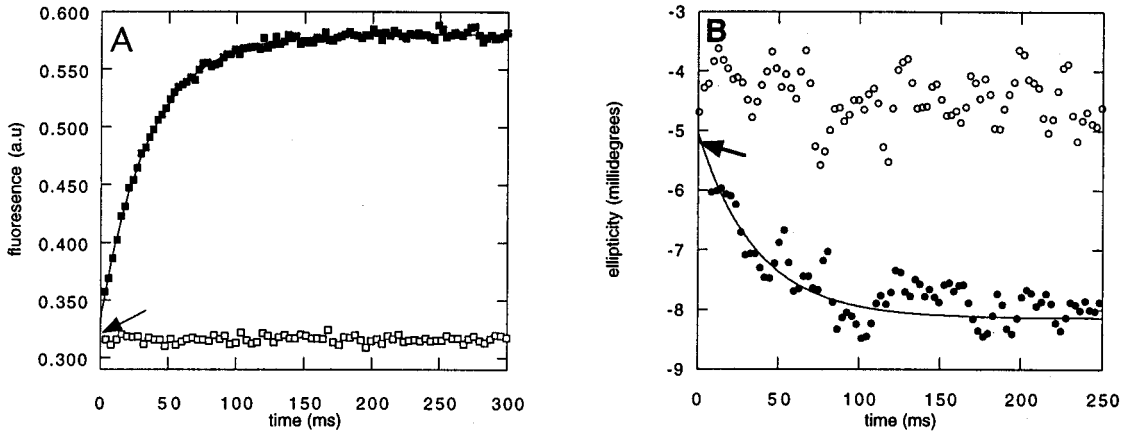

FIGURE 5: Stopped-flow fluorescence (A) and CD (B). The final refolding conditions were 0.3 M guanidine, 50 mM sodium phosphate, pH 7.0. The solid lines are the best fits for a single exponential reaction (k_{obs} = 27.8 s^{-1} for fluorescence measurements and 27 s^{-1} for CD measurements). The unfolded signals in 0.3 M guanidine (arrow), which can not be measured directly, was obtained through extrapolation using the linear dependence of the unfolded signal on guanidine concentration observed in equilibrium unfolding experiments where either the total fluorescence emission between 300−420 nm or the CD signal at 220 nm was measured. Because of the large amount of protein required, the stopped-flow CD experiments were carried out on protein containing the his-tag; in control experiments the presence of the his-tag had no detectable affect on folding kinetics as measured by fluorescence. Closed symbols, refolding reaction; open symbols, unfolded protein in 3.0 M guanidine (with guanidine containing buffer baseline subtracted).

in wt(trp) has little effect on the refolding rate. Interestingly, the protein L IgG binding domain folds considerably slower than the protein G IgG binding domain: the rates of folding of two protein G variants under these conditions were 20.3 and 60.7 s^{-1} (Alexander et al., 1992). Further studies at high pH were not undertaken due to chemical damage (probably deamidation) to the proteins after prolonged incubation at high pH (data not shown).

The change in fluorescence upon folding of wt(trp) made possible the characterization of the kinetics of folding of the protein at neutral pH using stopped-flow fluorescence. A typical wt(trp) refolding experiment in which denatured protein in 3 M guanidine was rapidly diluted 10-fold into phosphate buffer is shown in Figure 5A (closed squares). The data fit well to a simple exponential with a rate constant of 27.8 s^{-1}, considerably faster than the rate at pH 11.0. Both the measured signal for the unfolded protein in 3.0 M guanidine (open squares) and the signal extrapolated for the unfolded protein in 0.3 M guanidine (arrow) using the denaturant dependence of the signal of the unfolded protein observed in equilibrium unfolding experiments are shown in Figure 5A for comparison; we show the measured signal as well as the extrapolated signal in this and the similar experiments below because the extrapolation rests on an assumption of linearity that we cannot confirm directly. Accounting for the 1.7 ms dead time of the instrument, the extrapolated time zero value is very close to the signal of the unfolded protein. Thus, there are no submillisecond conformational changes early in the folding of protein L that produce substantial changes in the fluorescence emission intensity.

The folding of wt(trp) at pH 7.0 was also investigated by stopped-flow CD at 225 nm, but the data were of significantly poorer quality due to the relatively small change in CD signal upon folding and the inherent difficulties of stopped-flow CD experiments. Figure 5B shows the average of 60 individual wt(trp) refolding reactions as monitored by CD along with the measured CD signal for the unfolded protein in 3.0 M guanidine (open circles) and the unfolded protein signal extrapolated to 0.3 M guanidine (arrow). The unfolded baseline values differ because the CD signal of the unfolded protein becomes less negative as the guanidine concentration is increased from 3 to 6 M. Despite the poor quality of the data, the folding rate constant derived from a simple exponential fit (∼27 s^{-1}) was close to that obtained by fluorescence (27.8 s^{-1}). This simple exponential process appears to account for all of the signal change: the extrapolated signal at the start of the reaction is close to the extrapolated signal of the unfolded protein in 0.3 M guanidine. Some caution is necessary in comparing the initial signal in refolding reactions to the extrapolated signal of the unfolded protein under the same conditions because the extrapolation, which is based on the denaturant dependence of the CD signal of the unfolded protein in higher concentrations of denaturant, may in part incorporate denaturant dependent structural changes in the unfolded state. However, in this case the signal observed after the mixing period was also fairly close to that of the unfolded protein in 3 M guanidine, further suggesting that large changes in secondary structure do not occur within the mixing dead time. Thus, it appears that there is no rapid burst phase increase in secondary structure early in folding, as has been observed for lysozyme (Chaffotte et al., 1992), dihydrofolate reductase (Kuwajima et al., 1991), and several other larger proteins.

Denaturant Dependence of Folding Rates. The rate of wt(trp) folding and unfolding was determined over a broad range of guanidine concentrations. Figure 6 shows the natural logarithm of the observed rate constant, k_{obs}, as a function of guanidine concentration. The overall v shape of the curve is typical of such experiments: at low guanidine concentrations, ln k_{obs}, the sum of the folding and unfolding rates, is dominated by the rate of folding and thus decreases with increasing denaturant, whereas at high guanidine concentrations, the unfolding rate dominates and k_{obs} increases with increasing denaturant. Within the transition region (2−3 M guanidine), the rates obtained from the unfolding (closed circles) and refolding (closed squares) experiments were indistinguishable from one another.

The kinetic data were fit to a two-state model as described in the Experimental Procedures (solid line in Figure 6). The model assumes that the logarithms of the folding and unfolding rate constants are linear functions of the denaturant concentration. The extrapolated rate constants for folding and unfolding in the absence of denaturant and the corre-

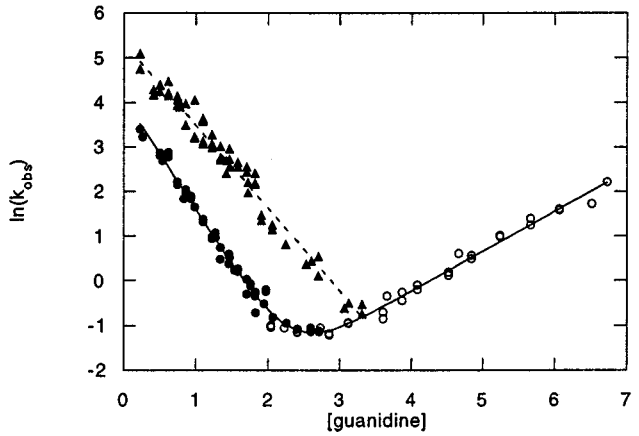

FIGURE 6: Denaturant dependence of ln k_{obs}. Relaxation rates were determined by stopped-flow fluorescence experiments in the (triangles) presence and (circles) absence of 0.4 M Na_2SO_4. Data from refolding and unfolding experiments are indicated by closed and open symbols, respectively. The solid line represents the best fit of the kinetic data obtained in the absence of 0.4 M Na_2SO_4 using a two-state model (eq 1). The dotted line is a linear fit of the kinetic data obtained in the presence of 0.4 M Na_2SO_4.

sponding m values were calculated according to eq 1; $k_f^{H_2O} = 60.6 \pm 3.6\ s^{-1}$, $k_u^{H_2O} = 0.02 \pm 0.002\ s^{-1}$, $m_f = 1.5 \pm 0.01$ kcal mol^{-1} M^{-1}, and $m_u = 0.5 \pm 0.03$ kcal mol^{-1} M^{-1}. In the two-state model, the kinetic and equilibrium parameters are not independent:

$$m = m_u + m_f \quad (2)$$

$$\Delta G_U^{H_2O} = -RT \ln(k_u/k_f) \quad (3)$$

The estimates of the $\Delta G_U^{H_2O}$ and the m value obtained from the protein L kinetic data (4.7 ± 0.1 kcal mol^{-1} and 2.0 ± 0.02 kcal mol^{-1} M^{-1}) are reasonably close to the values obtained from the equilibrium experiments (4.6 ± 0.2 kcal^{-1} mol^{-1} and 1.9 ± 0.07 kcal mol^{-1} M^{-1}).

For other proteins, such as hen lysozyme and ubiquitin, the v curves deviate from linearity at low denaturant concentrations (Khorasanikadeh et al., 1996; Keifhaber, 1995). This "rollover" is thought to reflect the population of an intermediate species at low guanidine concentrations. Whether the species is an on- or off-pathway intermediate is a subject of debate (Baldwin, 1996). The v curve for wt(trp) shows no rollover in phosphate buffer (Figure 6). For ubiquitin mutants, v curve rollover was observed when refolding was carried out in the presence of sodium sulfate, which may stabilize partially folded species (Khorasanizadeh et al., 1996). Prompted by this observation, wt(trp) refolding experiments were conducted in the presence of 400 mM sodium sulfate (Figure 6; closed triangles). Sodium sulfate increased the rate of folding and shifted the v curve so that the transition occurred at higher guanidine concentration. However, ln k_{obs}, did not deviate from linearity at low guanidine concentrations.

Fluorescence Quenching by Iodide. Fluorescence quenching by iodide may be a more sensitive probe of structure than intrinsic fluorescence since the iodide ion may be more readily excluded from hydrophobic clusters due to steric and electrostatic effects. The fluorescence emission spectra of unfolded wt(trp) (Figure 7A, open symbols) was quenched significantly more than that of folded wt(trp) (Figure 7A; closed symbols) in the presence of 1 M sodium iodide. The fluorescence (300−420 nm) of the unfolded protein is reduced 6.2-fold in the presence of 1 M NaI, while the fluorescence of the folded protein is reduced only 1.3-fold. The local environment of the tryptophan residue during folding can thus be probed by fluorescence quenching.

Wt(trp) refolding reactions in 1 M sodium iodide were monitored at low final concentrations of guanidine. The first 20 ms of refolding reactions in the presence and absence of 1 M NaI are shown in Figure 7 (B and C), along with the measured signal of denatured protein in 3 M guanidine (open symbols) and the extrapolated signal of the unfolded protein in 0.3 M guanidine (arrow; the fluorescence of the unfolded state in both the presence and absence of 1 M NaI varies only slightly with guanidine concentration). The initial signal in the sodium iodide experiments adjusted for the 1.7 ms dead time of the instrument is larger than the signal for the completely unfolded protein (Figure 7B). This deviation is not observed in the absence of sodium iodide (Figure 7C). The protection from quenching suggests that a submillisecond conformational change, perhaps chain collapse, partially excludes sodium iodide from the local environment of the tryptophan residue. Control experiments (see figure legend) suggest that the decrease in quenching is not due to solvent or ionic effects, but because of the relatively small changes involved we cannot entirely rule out the possibility that other factors beside conformational changes contribute to the loss of fluorescence quenching.

The reduction in the amount of fluorescence quenching during the dead time was measured for increasing final concentrations of guanidine (Figure 7D; the percentage increase in signal over that of the unfolded protein in high concentrations of guanidine is plotted). The reduction in quenching was greatest at low guanidine concentrations and decreased with increasing guanidine concentrations. These observations suggest that the conformation which partially shields the tryptophan from NaI is destabilized by denaturant. Similar results were obtained when 0.3 M instead of 1.0 M sodium iodide was used.

HD Exchange and Mass Spectrometry. The data from the spectroscopic experiments described above fit well to a two-state model, but since the signal monitored is the average over all species in solution, the two states cannot be resolved directly. As first illustrated in studies of lysozyme (Miranker et al., 1993), HD exchange in conjunction with mass spectrometry provides a method for resolving conformations that differ in the extent of protection of their amide protons. We have used such a technique to show that the major contribution to HD exchange in protein L at high pH and temperature is from transient global unfolding events which expose all protons to exchange (Yi & Baker, 1996). To probe the acquisition of protection from HD exchange during refolding we used a standard quench flow pulsed labeling method (see Experimental Procedures). Deuterated protein in 4.5 M guanidine was diluted into pH 4.2 buffer and allowed to refold for a variable period. The protein was then subjected to a 16 ms labeling pulse at pH 9.3 to allow exchange of exposed deuterons for protons. Exchange was then quenched by rapid reduction of the pH, and folding was allowed to continue to completion.

Figure 8 shows mass spectra of wt(trp) allowed to refold for times ranging between 0 and 200 ms prior to the labeling pulse. At very short times, the bulk of the molecules have molecular weights very close to that of the fully protonated protein. As the folding time increases, molecules are transferred from this population to a second population in

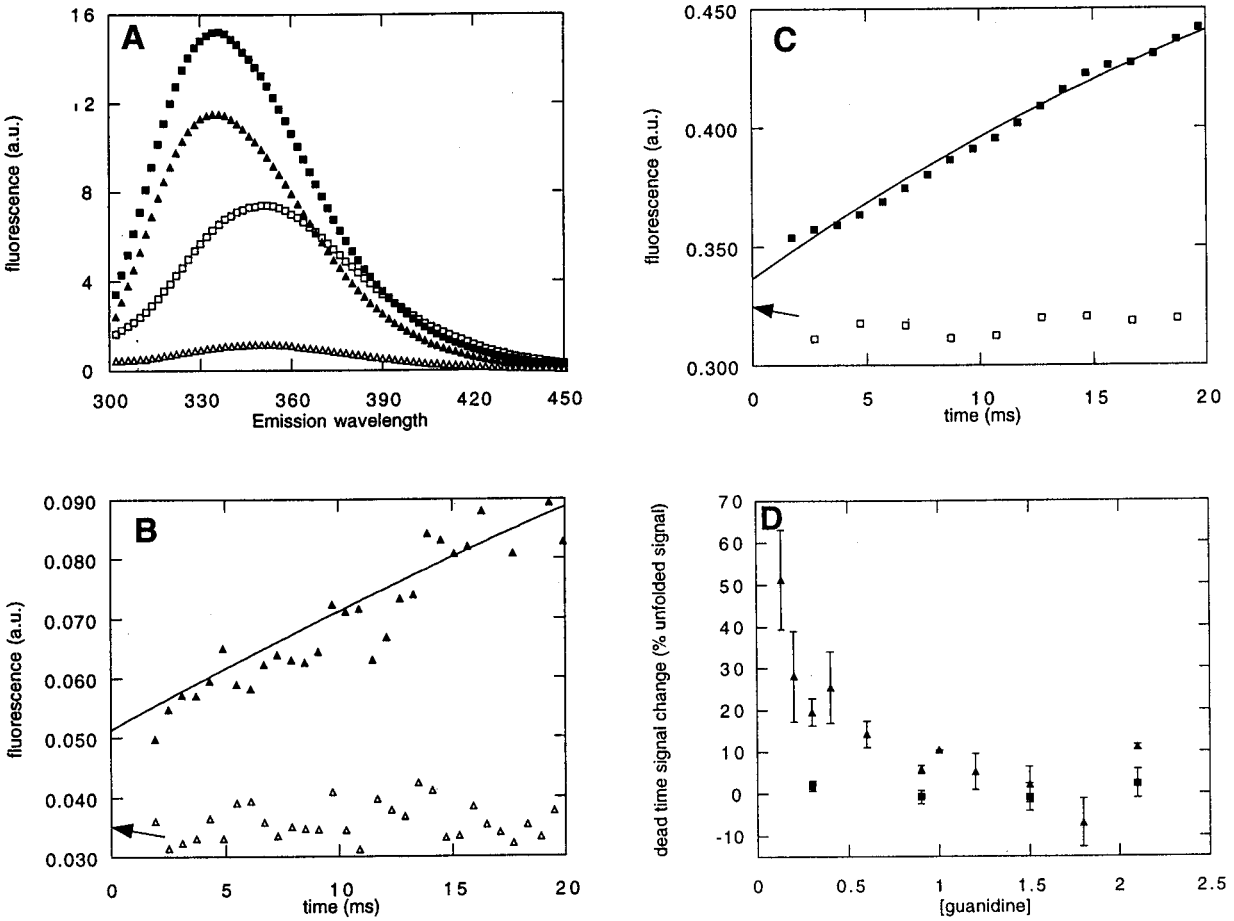

FIGURE 7: Fluorescence quenching experiments with 1 M sodium iodide. A; equilibrium fluorescence quenching measurements. The fluorescence emission spectra of wt(trp) in the (squares) absence and (triangles) presence of 1 M NaI. The protein concentration was approximately 24.2 ± 0.6 μM. Closed symbols, folded conditions (50 mM sodium phosphate, pH 7.0); open symbols, unfolded conditions (5 M guanidine, 50 mM sodium phosphate, pH 7.0). B and C; early time points of wt(trp) refolding in the presence and absence of 1 M NaI, respectively. The final guanidine concentrations for the experiments in the presence and absence of 1 M NaI were 0.2 M and 0.3M, respectively. The closed symbols represent the refolding data and the solid line is the best fit for a single exponential reaction. The open symbols represent the unfolded signal in high guanidine conditions (2 or 3M) and the arrows represent the extrapolated signal for the unfolded protein in low guanidine conditions (0.2 or 0.3M). The observed rates from the iodide experiments were consistently slower than the rates in the absence of sodium iodide at a given guanidine concentration. To confirm that the deviations were real and not an artifact of solvent effects on tryptophan fluorescence the experiment was repeated using free tryptophan (data not shown); with free tryptophan and 1 M NaI, no change in fluorescence signal occurred upon dilution into low final guanidine buffers compared to dilution into high guanidine buffers. The refolding experiments were also done in the presence of 1 M NaCl in place of NaI to determine whether the deviations were caused by ionic strength effects on fluorescence; no dead time gain of signal was observed. D; denaturant dependence of dead time changes in fluorescence quenching. Refolding was carried out in the (closed triangles) presence and (closed squares) absence of 1 M NaI. The magnitude of the signal gain is shown as a percentage of the signal of the unfolded protein in high concentrations of guanidine. Standard deviations were obtained from independent kinetic measurements.

which a substantial number of amides are protected from exchange. The molecular weight of this second population is close to that of folded deuterated protein incubated in H_2O (~6990 Da); NMR experiments (data not shown) indicate protons at surface amides and deuterons in the interior. The absence of populations with intermediate molecular weights suggests that the formation of hydrogen bonds and the burial of amide protons away from solvent occur in a highly concerted reaction. The experiment provides a direct view of the properties of the unfolded protein under folding conditions; future experiments will examine the protection of this species from HD exchange under stabilizing solvent conditions. The broad line shape of the folded population at short refolding times probably reflects the small number of molecules which initiate folding near the beginning of the labeling period but after a subset of the core deuterons have been exchanged for protons.

The relative sizes of the folded and unfolded populations were estimated from the peak heights of the mass spectra and plotted as a function of refolding time in Figure 9. For comparison, the rate of folding at pH 4.0 was determined by stopped-flow fluorescence to be 16.0 s^{-1} (Figure 9, solid line). The data points, which represent the percentage of molecules in the unfolded population in the mass spectra, are in good agreement with the simple exponential describing the overall fluorescence change during folding. The deviations may be in part due to errors in estimation of the peak heights caused by small contaminating side peaks and small inaccuracies in the delay times within the quench flow instrument. A simple exponential fit of the data in Figure 9 yielded a rate constant of 20 s^{-1}.

DISCUSSION

The Y43W mutation in wt(trp) provides a sensitive probe of folding without significantly perturbing the protein L IgG binding domain. The changes in NMR chemical shifts primarily involve residues in the immediate vicinity of the mutation; the chemical shifts of more distant protons are not significantly altered (Figure 2A and 2B). The stability of both wt and wt(trp) are comparable. The Y43W mutation

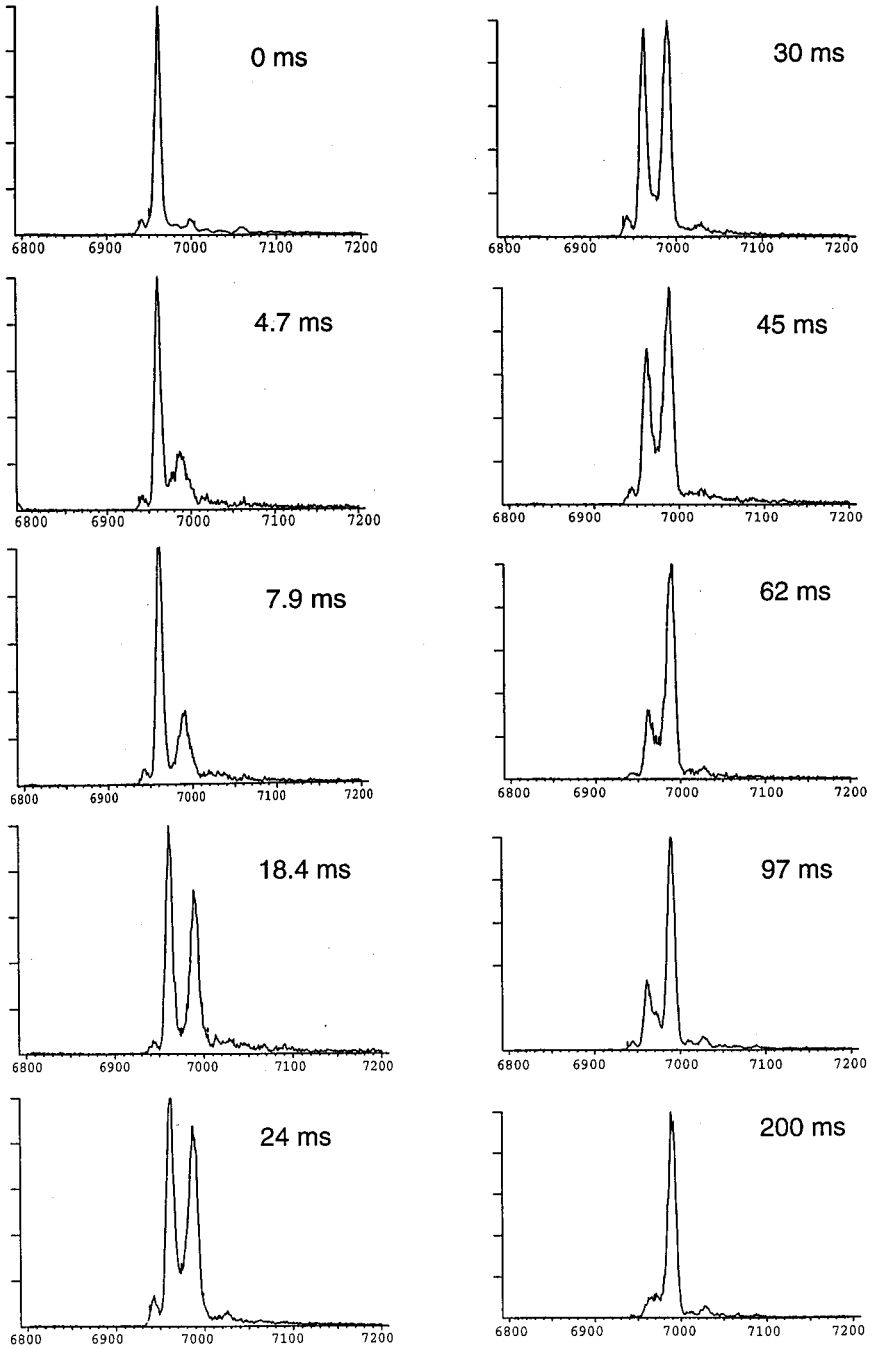

FIGURE 8: Wt(trp) refolding monitored by quenched flow HD exchange coupled to mass spectrometry. Shown are mass spectra of wt(trp) allowed to refold in 0.75 M guanidine, 20 mM sodium acetate, pH 4.0 for the times indicated.

does not appear to significantly perturb the refolding of the protein: the wt and mutant proteins fold at approximately the same rate at pH 11.0. The major difference between the two proteins is the striking change in fluorescence accompanying folding of wt(trp) (Figure 3), which greatly facilitates the characterization of the kinetics of folding.

The kinetics and thermodynamics of folding of wt(trp) fit well to a two-state model. Equilibrium denaturation data from CD and fluorescence experiments were superimposable (Figure 4). In low guanidine ln k_{obs} was a linear function of denaturant concentration (Figure 6), suggesting that intermediates do not accumulate during folding. A more stringent test for two-state folding is a comparison of kinetic and equilibrium folding parameters which in the two-state model are related by eqs 2 and 3. For wt(trp) the kinetic and equilibrium measurements yield values for the overall *m* value and the $\Delta G_U^{H_2O}$ that are in excellent agreement with one another.

In earlier studies using HD exchange and mass spectrometry, it was found that under conditions in which protein L is marginally stable, a highly concerted opening reaction simultaneously exposes all of the amide protons to solvent (Yi & Baker, 1996). In complementary experiments on refolding in this paper, we find that two species dominate the population at all refolding times: a relatively unfolded species in which there is little more protection from exchange than in the unfolded protein and a species with exchange behavior similar to that of the folded protein. The rate of transfer from the unfolded to the folded population, determined directly from the mass spectra, was very close to the rate of change of the fluorescence signal (an average over the two populations).

Table 1: Kinetic and Thermodynamic Parameters for Protein L wt(trp) and Several Other Small Proteins

protein name	$\Delta G_U^{H_2}$ (kcal mol^{-1})	$k_f^{H_2O}$ (s^{-1})	$k_u^{H_2O}$	m (kcal mol^{-1} M^{-1})	m_f (kcal mol^{-1} M^{-1})	m_u (kcal mol^{-1} M^{-1})	m_u/m	fold	size (# aa)
protein L, wt(trp)[a]	4.6	60.6	0.02	1.85	1.5	0.5	0.27	α/β	62
GCN4-p1[c,d]	10.3	4.2e5	0.003	1.9	1.00	0.9	0.47	α	2 × 33
Arc-repressor[b,c,e]	9.6	9.1e6	0.2	1.4	1.0	0.4	0.29	α/β	2 × 53
cytochrome C[a,f]	8.2			3.0	1.4	1.9	0.63	α	104
AcoAbp[a,g]	6.1	280.0	0.0004	2.7	2.1	1.4	0.52	α	86
ubiquitin[a,h]	7.2	264.0	0.0005	1.9	1.4	0.8	0.42	α/β	76
Cl2[a,i]	7.0	52.0	0.0001	1.8	1.1	0.8	0.44	α/β	64
ADA(2h)[b,j]	4.1	897	0.65	1.0	1.3	0.4	0.40	α/β	80
λ-repressor[b,k]	3.3	3600	27.0	1.2	0.5	0.6	0.50	α	80
spectrin-SH3[b,l]	2.9	8.2	0.05	0.7	1.0	0.3	0.42	β	62
CspB[b,m]	2.6	1070	12.0	0.8	0.7	0.1	0.13	β	67

[a] Guanidine was used as the denaturant and proteins were studied between pH 4.9 and 7.0 and 5 and 25 °C. [b] Urea was used as the denaturant, and the proteins were studied between pH 3.5 and 7.5 and 20 and 37 °C. [c] Refolding reaction is bimolecular (s^{-1} M^{-1}). Thermodynamic parameters are defined in eqs 1, 2, and 3 with m and $\Delta G_U^{H_2O}$ calculated from equilibrium experiments. [d] Zitzewitz et al., 1995. [e] Milla & Sauer, 1994. [f] Sosnick et al., 1996. [g] Kragelund et al., 1995. [h] Khorasanizadeh et al., 1993. [i] Jackson et al., 1993; Jackson & Fersht, 1991. [j] Villegas et al., 1995; Huang & Oas 1995a, 1995b. [l] Viguera et al., 1994. [m] Schindler et al., 1995.

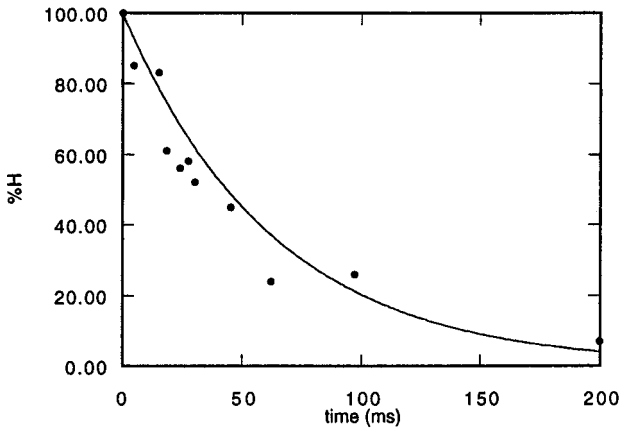

FIGURE 9: Kinetics of wt(trp) refolding measured by proton occupancy. The percentage of wt(trp) in the unfolded (fully protonated) population (closed circles) was estimated from the relative heights of the two peaks. The solid line is the single exponential fit (k_{obs} = 16.1 s^{-1}) of stopped-flow fluorescence data obtained under the same conditions.

In studies of the refolding of mutants of ubiquitin, it was found that addition of sodium sulfate changed the kinetic behavior from an apparent two-state process to a three-state process, probably by stabilizing an otherwise unstable intermediate (Khorasanizadeh et al., 1996). On the basis of this observation, it was suggested that under sufficiently stabilizing conditions intermediates might become apparent even for small proteins showing two state kinetics. To explore this possibility we measured the rate of folding in the presence of sodium sulfate as a function of guanidine concentration; although there was a significant increase in the rate of folding, the dependence of the logarithm of the folding rate did not deviate from linearity at low guanidine concentrations, suggesting that substantially stabilized intermediates do not accumulate under these conditions.

To investigate possible folding events on the submillisecond time scale, the kinetic data were closely examined for the presence of a burst phase change in signal during the mixing dead time. In all experiments, the signal obtained immediately after mixing of denatured protein with refolding buffer was compared to that of the denatured protein after correcting for the experimentally measured dead time of the stopped-flow instrument and the denaturant dependence of the unfolded signal. For intrinsic tryptophan fluorescence, the initial signal was very close to that of the unfolded state (Figure 7C). There also appeared to be little change in CD signal during the dead time, but the quality of the data was considerably poorer. However, in fluorescence-quenching experiments carried out in the presence of iodide, there was a small change in signal during the dead time (Figure 7B). The dead time change in the signal may reflect a rapid partial collapse of the chain following the change in solvent conditions; the decrease in the signal change with increasing guanidine concentration (figuRe 7D) could result from destabilization of such a collapsed state.

HD exchange experiments on the structurally related IgG binding domain of protein G (Kuszewski et al., 1994) suggested a rapid (<1 ms) collapse at low denaturant concentrations to a semicompact state in which protons in the second beta hairpin were partially protected from exchange. Stopped-flow fluorescence experiments suggested that Trp 43 might be associated with this rapid collapse since native-like fluorescence was attained during the dead time. The tryptophan in the wt(trp) protein L mutant is at a position equivalent to that of the single tryptophan in protein G; the lack of dead time changes in the intrinsic fluorescence of the tryptophan and the only modest changes in fluorescence quenching suggest that, if there is such a collapse, it does not involve this portion of the molecule. Interestingly, the most slowly exchanging amide protons in protein G are in the second beta hairpin (Orban et al., 1995), while the most slowly exchanging amide protons in protein L are in the first beta hairpin (Wikstrom et al., 1996; Yi, Qian, unpublished observations), consistent with differential protection of the two beta hairpins in the unfolded states of the two proteins under refolding conditions. The dramatic differences in the rate of change in fluorescence of the tryptophan residues during folding of the two proteins and the apparent differences in the initial collapse in low guanidine concentration for protein L and protein G are intriguing given the similarity in structure of the two proteins.

More experiments are clearly required to investigate possible structure in the unfolded state of protein L under refolding conditions. Unlike the collapsed states formed rapidly during folding for several larger proteins (Agashe et al., 1995; Jones et al., 1994; Itzhaki et al., 1994), no 1-anilinonapthalene-8-sulfonate (ANS) binding was detected during folding of protein L under any of the conditions examined (data not shown); hydrophobic clusters, if formed early in the folding, may be inaccessible or simply too small to bind ANS. Furthermore, the linear dependence of ln k_{obs}

on denaturant suggests that the solvent exposure of the nonpolar side chains does not change substantially as the guanidine concentration is reduced from 2 to 0.2 M; a collapsed state populated at low guanidine concentrations would have to be substantially hydrated.

It is worth noting that the linear denaturant dependence of ln k_{obs} over a wide range of denaturant concentrations differs from the significantly nonlinear denaturant dependence of the transfer free energies of the amino acids to guanidine containing solutions (Nozaki & Tanford, 1971) often used to model protein stability (Pace, 1986; Alonso & Dill, 1991). Because deviations from linearity resulting from a reduction in the exposed surface area of the unfolded state in low concentrations of denaturant (which could produce the v curve rollover seen with proteins such as ubiquitin (Khorasanizadeh et al., 1996)) would be opposite in sign to the deviations observed in the model compound studies, it is possible that the observed linearity reflects cancellation of the two effects. Alternatively, the effects of guanidine on the solubility of the amino acid side chains may not properly model the effects of guanidine on protein stability; denaturants are likely to also interact with the peptide backbone.

The folding and unfolding rates and their denaturant dependencies (m values) of protein L are compared to those of the other small proteins which have been subjected to similar studies in Table 1. Of particular interest is the ratio m_u/m which is thought to reflect the extent of exposure of hydrophobic residues in the transition state relative to the folded state. For protein L m_u/m is 0.27, suggesting that the hydrophobic residues are relatively buried in the transition state. There is considerable variation in m_u/m in the small proteins listed in Table 1; we have been unable to find significant correlations between m_u/m and protein structural features such as the compactness of the hydrophobic core. The all α proteins have slightly larger than average values of m_u/m (Huang and Oas, 1995a), but the amount of data is obviously extremely limited. An important question that remains open is whether the free energy barrier to folding is due entirely to loss of configurational entropy (prior to the formation of sufficient compensating favorable interactions) or whether other entropic or enthalpic effects such as desolvation (Waldburger et al., 1996; Rank & Baker, in press) and side chain freezing (Shakhnovich & Finkelstein, 1989) also block folding.

In summary, both the kinetics and thermodynamics of folding of protein L at neutral pH fit well to a simple two-state model. The kinetic and thermodynamic parameters are similar to those of other small proteins. There are indications that a rapid collapse occurs shortly after dilution of denatured protein into buffer containing very low concentrations of denaturant. These results form the basis for future studies of the folding mechanism of protein L using a combination of molecular biological (Gu et al., 1995) and biophysical methods.

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