a UV method for disappearance of the anhydride. At the time, we had no knowledge of the ester formation from anhydride. It now appears that the results for Phe AMP anhydride in that report involved mostly ester formation. The rate constant for anhydride disappearance at pH 5 was 1.78×10^{-3} min^{-1} and in the present report is 1.54×10^{-3} min^{-1}. The present report must be regarded as the more thorough and definitive one. Consequently, we can now conclude that the rate of hydrolysis of these anhydrides in the absence of CO_2 is really surprisingly slow.

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Reversible Denaturation of the Gene V Protein of Bacteriophage f1†

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ABSTRACT: The guanidine hydrochloride (GuHCl)-induced denaturation of the gene V protein of bacteriophage f1 has been studied, using the chemical reactivity of a cysteine residue that is buried in the folded protein and the circular dichroism (CD) at 211 and 229 nm as measures of the fraction of polypeptide chains in the folded form. It is found that this dimeric protein unfolds in a single cooperative transition from a folded dimer to two unfolded monomers. A folded, monomeric form of the gene V protein was not detected at equilibrium. The kinetics of unfolding of the gene V protein in 3 M GuHCl and the refolding in 2 M GuHCl are also consistent with a transition between a folded dimer and two unfolded monomers. The GuHCl concentration dependence of the rates of folding and unfolding suggests that the transition state for folding is near the folded conformation.

It has been known for many years that denatured proteins can spontaneously fold into their native conformations (Anfinsen, 1973). Despite intensive study, however, neither the mechanism of protein folding nor the details of the interactions that determine the conformations and stabilities of folded proteins are well understood (Ghelis & Yon, 1982; Creighton, 1984). Moreover, only a small number of proteins with known three-dimensional structures have been extensively analyzed in folding and stability studies. Most of these proteins are small monomeric proteins, and they have either largely helical or mixed α-helix and β-sheet secondary structures (Matthews, B. W., 1987; Alber, 1989). To be certain of the generality of observations on protein folding, studies on proteins in different structural classes and with varying states of oligomerization would be useful. We have therefore set out to develop a new model system for studying the process of protein folding

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and the effects of amino acid substitutions on stability and folding based on a dimeric, all β-sheet protein.

The gene V protein of bacteriophage f1 (Salstrom & Pratt, 1971; Alberts et al., 1972) is a small dimer containing 87 amino acids in each of its two identical subunits. The three-dimensional structure of this protein has been examined by X-ray crystallography (Brayer & McPherson, 1983) and nuclear magnetic resonance techniques (de Jong et al., 1989; van Duynhoven et al., 1990). The secondary structure of the gene V protein consists entirely of strands of β structure connected by turns. The protein contains no disulfide bonds, and a single cysteine is buried within each subunit (Nakashima et al., 1974; Brayer & McPherson, 1983). The protein is known to nonspecifically bind single-stranded DNA and RNA. Its biological function is thought to be to shift the synthesis of phage DNA from double-stranded form to single-stranded form in the late stage of phage infection, by binding to single-stranded DNA and thus preventing the synthesis of the complementary strand DNA (Mazur & Model, 1973). The gene V protein also binds specifically to the operator sequence of gene II mRNA of phage f1 and represses the translation of the gene II protein (Yen & Webster, 1982; Michel & Zinder, 1989).

A large collection of single amino acid substitution mutants of the gene V protein has recently been constructed (Terwilliger, 1988; Zabin et al., unpublished results). Genetic selections enriching for active and inactive variants of the gene V protein have been devised (Terwilliger et al., 1988) and used to isolate temperature-sensitive mutants of gene V protein and their intragenic suppressors (Zabin & Terwilliger, 1991). Directed mutagenesis of the gene V protein hydrophobic core has been used as a means of studying the contributions of side chain packing and hydrophobicity to protein stability (Sandberg & Terwilliger, 1989, 1991). Here we describe biochemical and biophysical methods for studying the effects of amino acid substitutions on folding and stability of this protein.

We have used three probes, the reactivity of a buried cysteine in the gene V protein to chemical modification of the sulfhydryl group, the circular dichroism (CD)[1] at 229 nm due to conformations of tyrosine residues, and the CD at 211 nm due to peptide backbone conformations, to differentiate gene V protein in the folded and unfolded forms and follow the folding and unfolding of the protein. Using these probes, we have characterized the equilibrium and the kinetics of the folding and unfolding of this dimeric protein, examining whether intermediates exist either at equilibrium or during the kinetic processes of folding and unfolding.

Materials and Methods

Purification of Gene V Protein. A procedure for purifying the gene V protein of bacteriophage f1 was developed based on the method of Garssen et al. (1977). *Escherichia coli* cells, strain K561 (Davis et al., 1985) containing plasmid pTT18, which confers resistance to ampicillin and encodes the wild-type gene V protein of bacteriophage f1 (Terwilliger et al., 1988), were grown in a LabLine SMS high-density fermenter at 30 °C. The media contained 22.5 g/L yeast extract, 10.8 g/L tryptone, 5 mL/L glycerol, 30 μL/L antifoam emulsion B (Sigma, St. Louis, MO), 72 μM K_2HPO_4, 23 μM KH_2PO_4, and 150 mg/L ampicillin. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of gene V protein when the bacterial culture had been grown to a density of OD_{650nm} = 3. Cells were harvested at a density of OD_{650nm} = 14, approximately 3 h after IPTG induction, by centrifuging at 1500*g* for 60 min at 4 °C. The cell pellets were resuspended in 1 volume of a buffer containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM β-mercaptoethanol, 1 mM EDTA, 10% glycerol, and 0.02 M Tris-HCl, pH 8.1. Cells were frozen in a −80 °C dry ice/ethanol bath and stored at −80 °C for up to several weeks before purification. All the following purification steps were carried out at 4 °C except where otherwise indicated. DNase I was added to a thawed *E. coli* cell suspension to a final concentration of 20 μg/mL, and the mixture was passed through a French press cell disrupter to break the *E. coli* cells. The broken cell suspension was incubated for 1 h at 15 °C to allow the DNase I to digest DNA. NaCl was then added to a final concentration of 1 M to dissociate any remaining single-stranded DNA from gene V protein complexes, and the solution was centrifuged at 150000*g* for 60 min to remove cell debris. A solution of ammonium sulfate saturated at 25 °C but cooled rapidly to 4 °C was added to the supernatant at a ratio of 0.69:1 (v/v) to precipitate proteins. The mixture was stirred for 30 min before centrifugation for 30 min at 16000*g*. Pellets were resuspended in 20 mL of 0.05 M NaCl, 5 mM EDTA, 1 mM β-mercaptoethanol, and 0.02 M Tris-HCl, pH 8.1 (buffer A), and dialyzed against two changes of 2 L of the same buffer. After centrifugation at 150000*g* for 30 min, 0.1 volume of glycerol was added to the supernatant. The mixture was applied to a 200-mL single-stranded DNA–agarose column (Schaller et al., 1972) at a flow rate of 40 mL/h. The column was washed at the same flow rate with about 600 mL of buffer A until the absorbance at 276 nm became steady, and eluted with 600 mL of buffer A containing a gradient of NaCl from 50 mM to 2 M NaCl. Electrophoresis on polyacrylamide slab gels in the presence of 0.1% SDS and 6 M urea followed by Coomassie blue staining (Ito et al., 1980) was used to identify the fractions containing gene V protein. Pooled gene V protein fractions were precipitated as described above by ammonium sulfate, and the proteins were resuspended in about 3 mL of a buffer containing 0.05 M 2-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 M NaCl, and 0.001 M EDTA, pH 7.0 (buffer B). The suspension was loaded onto a 500-mL Sephadex G-75 column and eluted with buffer B. Fractions containing the gene V protein were detected by the absorbance at 276 nm and pooled. The purified gene V protein was more than 99.5% pure as judged by SDS–polyacrylamide gel electrophoresis with either Coomassie staining or silver staining. The protein was free of DNA as determined by a diphenylamine assay (Giles & Myers, 1965) and an ethidium bromide fluorescence assay (Nelson & Kaufman, 1987). The yield of the purification was 90 mg from 4 L of culture (1.4 mg of gene V protein/g of cells). The purified gene V protein was frozen in a −80 °C dry ice/ethanol bath and stored at −80 °C.

Denaturation of Gene V Protein by GuHCl. Studies of GuHCl-induced denaturation of the gene V protein were carried out in buffer B at 25.0 °C. Thawed gene V protein solutions were centrifuged for 3 min in a microcentrifuge or filtered through a 0.22-μm filter to remove aggregates before use. Concentrations of gene V protein were measured by their absorbance at 276 nm using an extinction coefficient of 7100 (M monomer)$^{-1}$ cm^{-1} (Day, 1973). All concentrations of gene V protein reported in this paper are in M of monomers. Concentrations of GuHCl solutions were determined by their refractive indexes according to Nozaki (1972). Temperatures in all experiments were maintained at 25.0 ± 0.1 °C with a

[1] Abbreviations: CD, circular dichroism; CD_{229nm}, circular dichroism at 229 nm; GuHCl, guanidine hydrochloride; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 2-(*N*-morpholino)propanesulfonic acid; PDS, 4,4′-dithiodipyridine or pyridine disulfide.

water-jacketed cuvette. The changes of the apparent pH of buffer B in the presence of GuHCl, due to the influence of the increased ionic strength on the acid dissociation constant K_a of MOPS, were less than 0.2 pH unit from 0 to 5 M GuHCl.

To estimate the change of free energy upon unfolding ($\Delta G_u°$) of the gene V protein, the CD of the gene V protein at 229 nm (CD_{229nm}) was measured as a function of concentration of GuHCl. Gene V protein in buffer B was mixed with stock GuHCl solutions in buffer B to a final gene V protein concentration of 10 μM and final GuHCl concentrations from 0 to 5 M. Samples were equilibrated in a 25.0 °C water bath for at least an hour, and then their CD_{229nm} values were measured for 8 min on a JASCO J-600 spectropolarimeter.

For the estimation of the change of free energy upon unfolding, several assumptions were made. The fraction (f) of gene V protein that is in the folded state was assumed to be proportional to its exhibited CD:

$$f = (\theta - \theta_{unfolded})/(\theta_{folded} - \theta_{unfolded}) \quad (1)$$

where θ represents the measured molar CD of the protein sample (degrees per molar per centimeter) and θ_{folded} and $\theta_{unfolded}$ represent the molar CD values of fully folded and fully unfolded gene V protein at a given GuHCl concentration. The CD values of both folded and unfolded gene V proteins were assumed to depend linearly on GuHCl concentration, that is

$$\theta_{folded} = \theta_{folded,0M} + \alpha C \quad (2)$$

and

$$\theta_{unfolded} = \theta_{unfolded,5M} + \beta(C - 5) \quad (3)$$

where $\theta_{folded,0M}$ is the molar CD of folded gene V protein in 0 M GuHCl, $\theta_{unfolded,5M}$ is the molar CD of unfolded gene V protein in 5 M GuHCl, C is the molar concentration of GuHCl, and α and β are the dependence of the CD of folded and unfolded gene V protein on the GuHCl concentration.

We used a two-state model for describing the denaturation equilibrium:

$$F \rightleftharpoons 2U \quad (4)$$

in which it is assumed that the gene V protein exists either as a folded dimer (F) or as an unfolded monomer (U). The equilibrium constant (K_{eq}) and $\Delta G_u°$ were related to the fraction (f) of gene V protein in the native state in eq 1 and the gene V protein concentration (P_0) in M of monomers by

$$K_{eq} = 2P_0(1 - f)^2/f \quad (5)$$

and

$$\Delta G_u° = -RT \ln (K_{eq}/1\ M) \quad (6)$$

where R is the gas constant and T is the absolute temperature. The normalization to the concentration of 1 M gene V protein was explicitly included to emphasize that the transition is between a dimer and two unfolded monomers. The free energy change upon folding, $\Delta G_u°$, was assumed to be linearly proportional to GuHCl concentration (Pace, 1975), i.e.

$$\Delta G_u° = \Delta G_u°_{(C_m)} + m(C - C_m) \quad (7)$$

where C is the concentration of GuHCl, C_m is the concentration of GuHCl at the midpoint of the denaturation transition (where half of the gene V protein chains are in the folded form and half are denatured), $\Delta G_u°_{(C_m)}$ is the change of free energy upon unfolding when the concentration of GuHCl is C_m, and m is the dependence of the change of free energy upon unfolding on denaturant concentration. According to eq 5 and 6:

$$\Delta G_u°_{(C_m)} = -RT \ln P_0 \quad (8)$$

The six unknown parameters, $\theta_{folded,0M}$, $\theta_{unfolded,5M}$, α, β, C_m, and m, in eq 2, 3, and 7 were optimized by a nonlinear least-square regression method (Bevington, 1969) to fit the experimental data.

Measuring and Modeling the Dependence of the Denaturation Equilibrium on Gene V Protein Concentration. To determine the dependence of the denaturation of the gene V protein on the concentration of the protein, the CD_{229nm} of the gene V protein at different concentrations in buffer B containing 2.70 M GuHCl was measured. The fractions of gene V protein in the native state (f) were calculated by using eq 1. Measurements of the CD_{229nm} of gene V protein at 2.5 and 10 μM in the presence of varying concentrations of GuHCl were used to estimate the values of θ_{folded} and $\theta_{unfolded}$ (eq 1) at 2.70 M. These values were linearly extrapolated from experimental points at either low or high GuHCl concentrations outside the denaturation transition region, where gene V protein was nearly completely folded or unfolded.

In modeling the dependence of the fraction of gene V protein in the native state on gene V protein concentration, the following three-state model was considered:

$$F \overset{K_1}{\rightleftharpoons} 2M \quad (9)$$

$$M \overset{K_2}{\rightleftharpoons} U \quad (10)$$

where F is the folded dimer, M is the folded monomer, U is the unfolded monomer, and K_1 and K_2 are the equilibrium constants of the respective reactions. The equilibrium constant of the overall unfolding reaction, K_{eq}, is

$$K_{eq} = K_1K_2^2 \quad (11)$$

In this model, a folded monomer gene V protein (M) was assumed to exhibit the same CD as one of the two monomer subunits in a folded dimer. The two-state equilibrium model (eq 4), in which folded monomer does not exist at a high level, was considered as a special case of this three-state model with a large monomer unfolding equilibrium constant K_2 and a small dissociation constant K_1. The equilibrium constant (K_{eq}) for the overall reaction and the dimer dissociation constant K_1 were varied to fit the three-state model to the measurement of the molar CD (θ) as a function of gene V protein concentration. In considering models in which the folded monomer intermediate exists at different levels, K_2 was given fixed values, and K_{eq} was allowed to vary to give the best possible fit to the experimental data.

Cysteine Reactivity Assay and Determination of Unfolding and Folding Rate Constants. The accessibility of the single cysteine residue (Cys-33) in each monomer of the wild-type gene V protein to the chemical modification reagent 4,4'-dithiodipyridine (PDS; Grassetti & Murray, 1967) was used as a probe to monitor the unfolding of the protein in GuHCl solutions. Cysteine reactivity assays were carried out by reacting the gene V protein with PDS in buffer B, with or without GuHCl added prior to the reaction (see below). The reactions were followed by monitoring the formation of the product 4-thiopyridone using absorbance at 324 nm.

On the basis of an experiment in which measured amounts of the amino acid cysteine were reacted with PDS at various GuHCl concentrations, the following empirical formula was obtained and was used to relate the measured absorbance at 324 nm and the amount of reacted gene V protein in GuHCl solutions:

$$\epsilon_{324nm}\ (cm^{-1}\ M^{-1}) = (2.03 \times 10^4) - C(3.21 \times 10^2) \quad (12)$$

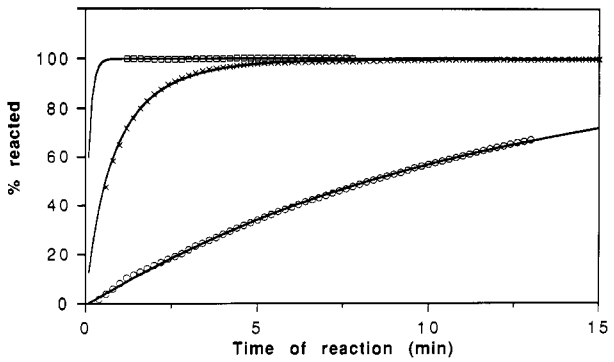

FIGURE 1: Use of cysteine reactivity to determine the unfolding rate constant and the unfolding equilibrium constant. The reactions of 10 μM gene V protein in 3.61 M GuHCl with PDS as followed by the absorbance at 324 nm are shown as examples of these experiments. The experiments represented by different symbols are (□) gene V protein equilibrated with 3.61 M GuHCl was reacted with 100 μM PDS, (×) gene V protein equilibrated with 3.61 M GuHCl was reacted with 15 μM PDS, and (○) a mixture of PDS and GuHCl was added to gene V protein equilibrated in buffer B in the absence of GuHCl, to final concentrations of 100 μM PDS and 3.61 M GuHCl. The solid lines are modeling results based on unfolding, folding, and modification reaction rate constants of $k_1^{app} = 0.095$ min^{-1}, $k_2^{app} = 2.7$ min^{-1} M^{-1}, and $k_3^{app} = 9.4 \times 10^4$ min^{-1} M^{-1}, according to eq 13 and 14 (see Materials and Methods).

where ϵ_{324nm} is the extinction coefficient of the reaction product at 324 nm and C is the molar concentration of GuHCl. The extinction coefficient measured in the solution containing no GuHCl, $\epsilon_{324nm} = 2.03 \times 10^4$ cm^{-1} M^{-1}, was in good agreement with the result of Grassetti and Murray, $\epsilon_{324nm} = 1.98 \times 10^4$ cm^{-1} M^{-1} (Grassetti & Murray, 1967).

Reactivity of the gene V protein to the cysteine modification reagent was measured at various concentrations of GuHCl from 0 to 5 M. At each GuHCl concentration, three experiments with different starting conditions were carried out. As shown in Figure 1, the reaction starting conditions differed in that two of the experiments were started when the gene V protein had been equilibrated with GuHCl ("equilibrium start"), while in the third experiment the protein was native at start, and GuHCl and PDS were added simultaneously ("folded start"). The ratio of PDS concentration to that of the protein was also varied in the two "equilibrium start" experiments. The protein, reactant, and denaturant solutions in all these experiments were prepared as described in the legend of Figure 1 except that the GuHCl concentration was varied from 0 to 5 M. For each experiment, a protein sample and blank sample were prepared. They had identical contents except that one set contained gene V protein and the other set had no protein. Absorbances at 324 nm of the blank samples were measured and subtracted from the reaction time courses of the protein samples. Small but reproducible differences were observed between the absorbance of the blank samples and the initial absorbance of the samples in which gene V protein was expected to be totally folded at the beginning of the reactions (e.g., "folded start" experiments and "equilibrium start" experiments at low GuHCl concentrations). These differences constituted 0–10% of the total absorbance resulting from the modification reaction and did not correlate with the concentration of GuHCl. They were also observed in experiments carried out using a mutant gene V protein in which cysteine-33 is substituted by serine (Zabin and Terwilliger, unpublished results). We assumed that this offset was due to causes other than the unfolding and modification reactions of the gene V protein, and subtracted it from the reaction time course. For such subtractions, differences between the absorbance of blank samples and that extrapolated to the beginning of reactions were used when such an extrapolation was possible, i.e., for the reactions in which gene V protein was folded when the reactions were started. When such extrapolations were not possible, an average of offsets in other experiments was used.

The kinetics of the unfolding and modification reactions were analyzed according to the reaction schemes:

$$F \underset{k_2^{app}}{\overset{k_1^{app}}{\rightleftharpoons}} 2U \quad (13)$$

$$U + PDS \xrightarrow{k_3^{app}} U\text{–}TP + 4\text{-}TP \quad (14)$$

where F is folded dimeric gene V protein, U is the unfolded monomer, U–TP is the unfolded and chemically modified gene V protein monomer, 4-TP is the reaction product 4-thiopyridone, k_1^{app} is the apparent unfolding rate constant, k_2^{app} is the apparent folding rate constant, and k_3^{app} is the apparent modification rate constant. For these experiments, the equilibrium constant of unfolding, K_{eq}, was assumed to be related to the apparent kinetic unfolding and folding rate constants by

$$K_{eq} = k_1^{app}/k_2^{app} \quad (15)$$

It was assumed in the modeling that the single cysteine residue in each gene V protein monomer is totally inaccessible to modification by PDS when the protein is in its native folded state. For each set of three experiments at a fixed GuHCl concentration, six independent parameters, k_1^{app}, K_{eq}, k_3^{app} and the gene V protein concentrations in each of the three experiments, were refined (Bevington, 1969) to give the best fit to the observed reaction time courses using numerical integration to evaluate eq 13–15. The total gene V protein in each experiment was calculated in this modeling procedure by using the independently determined extinction coefficient of PDS (eq 12). The gene V protein concentrations estimated in this way were on average about 75% of those expected from UV absorbance measurement using the extinction coefficient of Day (1973). We have not yet determined the origin of this difference. We concluded from separated modeling experiments that an error in the protein concentration would have no effect on the determination of the unfolding rate constant (k_1^{app}) as the unfolding reaction is independent of protein concentration, and that the determinations of fractions of folded gene V protein in GuHCl denaturation equilibria from this are also not affected by assuming a different gene V protein concentration. However, an error of 25% in protein concentration would have lead to a systematic error of 25% in the estimation of the folding rate constant (k_2^{app}).

Kinetics of Folding and Unfolding followed by CD at 229 nm. The unfolding reactions of gene V protein in the presence of 3 M GuHCl and refolding reactions in 2 M GuHCl were followed by CD changes at 25.0 °C for three gene V protein concentrations: 2.5, 5, and 10 μM. The unfolding reactions were initiated by increasing the GuHCl concentration in gene V protein solutions from 2 to 3 M. Gene V protein was first equilibrated in buffer B with 2 M GuHCl for at least an hour; then $^3/_{11}$ volume of buffer B containing 6.67 M GuHCl was added to bring the final GuHCl concentration to 3 M. The reaction was followed by measuring the CD of the solution at 229 nm with a time constant of 2 s. The experiment for determining the refolding rate constant was carried out in a similar fashion except that the GuHCl concentration was decreased from 3 to 2 M to initiate the refolding reaction. The gene V protein was equilibrated in buffer B containing 3 M GuHCl for at least an hour; then the concentration of GuHCl

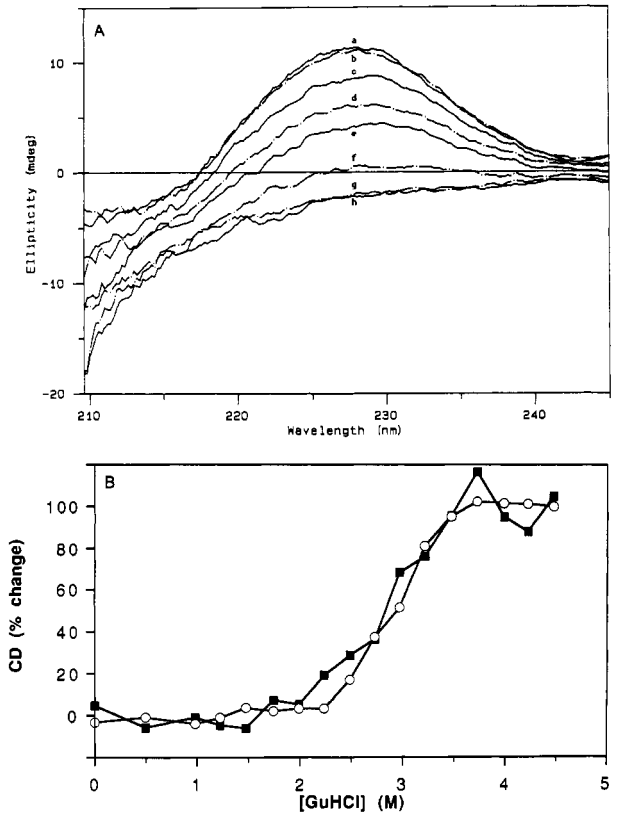

FIGURE 2: (A) CD spectra of gene V protein at various concentrations of GuHCl. At 25.0 °C, CD spectra of 68 μM of gene V protein in buffer B in the presence of various concentrations of GuHCl were measured with a 0.5-mm-path cuvette. The concentrations of GuHCl for each spectrum were (a) 0.00, (b) 1.23, (c) 2.48, (d) 2.73, (e) 2.97, (f) 3.22, (g) 3.72, and (h) 4.48 M. Spectra a, c, e, and g are represented by continuous lines, and spectra b, d, f, and h are represented by dotted lines. (B) Comparison of changes in CD of gene V protein at two wavelengths in different concentrations of GuHCl. CD spectra of 68 μM gene V protein in various concentrations of GuHCl were measured as described in (A). Plotted are percent changes in CD at 211 nm (■) and at 229 nm (○) as a function of GuHCl concentration.

was decreased to 2 M by adding 0.5 volume of buffer B. The time courses of the reactions were analyzed according to the kinetic scheme shown in eq 13. The fractions of folded or unfolded gene V protein in 2 or 3 M GuHCl were calculated by using the results of equilibrium denaturation experiments (Figure 5) and eq 4–8. For the unfolding experiments, the CD values at the beginning and the end of the reactions were refined to fit the observed curves. For the refolding reactions, the initial CD values for unfolded gene V protein in 2 M GuHCl could not be extrapolated from the observable portions of the reaction time courses, so only the final CD values were refined. The initial CD values for the refolding reactions were given values extrapolated from the measured CD of gene V protein in 3 M GuHCl and slopes of the CD of unfolded gene V protein as a function of concentration of GuHCl (determined from equilibrium denaturation experiments). The initial and final CD values extrapolated from the reaction time courses in the kinetic parameter refinement were in reasonable agreement (see legend to Figure 7) with values determined in or extrapolated from the equilibrium denaturation experiments.

RESULTS

Reversible Denaturation of the Gene V Protein. Nearly all proteins can be denatured by GuHCl. This salt is thought to decrease the contribution of the hydrophobic effect to the

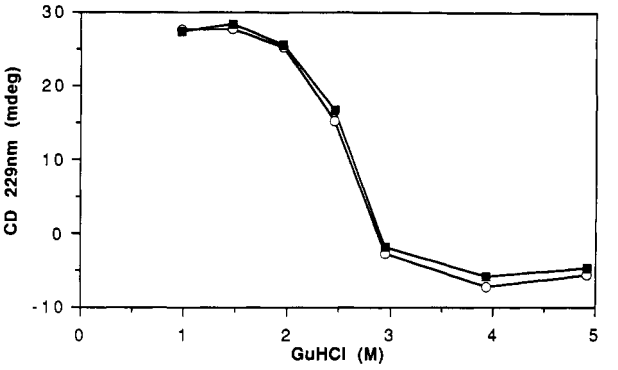

FIGURE 3: Reversibility of GuHCl denaturation of the gene V protein. CD_{229nm} of 10 μM gene V protein was measured for samples prepared under different starting conditions: (○) measured after GuHCl was added to native gene V protein to the indicated denaturant concentrations and equilibrated for at least an hour; (■) measured after gene V protein was first equilibrated in buffer B containing 5 M GuHCl for at least an hour and then GuHCl was diluted to the indicated concentrations and equilibrated for at least another hour.

stabilities of folded proteins by increasing the solubility of apolar side chains in aqueous solutions (Tanford, 1968). Figure 2A shows the effect of GuHCl on the CD spectrum of gene V protein from 210 to 245 nm. The native gene V protein shows a slightly negative CD at around 211 nm and a strong positive CD at 229 nm (Figure 2A). The backbone peptide CD at 211 nm reflects the secondary structure of the gene V protein (van Holde, 1971), while the CD at 229 nm was assigned as a tyrosine CD band, due to the conformations of the five tyrosines in each subunit of the protein (Day, 1973). In 4.48 M GuHCl, the CD spectrum of the gene V protein is similar to that of an extended chain (van Holde, 1971), with strongly negative CD at 211 nm and weak CD at 229 nm. In intermediate concentrations of GuHCl, the spectra are intermediate between these two extremes, with the greatest change in the spectrum occurring between 2.5 and 3.5 M GuHCl for a solution of 68 μM gene V protein.

The GuHCl-induced denaturation of the gene V protein is highly cooperative. Figure 2B shows the relative changes in the CD of 68 μM gene V protein at 211 nm and at 229 nm as functions of denaturant concentration. The CD at 229 nm can be measured much more accurately than at the CD at 211 nm under the conditions of our measurements, but both show a cooperative transition, centered at 2.9 M GuHCl, with only small changes in CD between 0 and 2 M GuHCl and between 3.5 and 4.5 M GuHCl, where the protein is expected to be largely folded or unfolded, respectively.

Figure 3 shows a test of the reversibility of the GuHCl-induced denaturation of the gene V protein. As shown in Figure 3, the CD of gene V protein is independent of the starting conditions, indicating that the gene V protein unfolded in 5 M GuHCl can be reversibly refolded when diluted to lower concentrations of the denaturant.

Dependence of the Denaturation on pH. The dependence of the stability of the gene V protein on pH was examined by measuring the CD of the protein as a function of pH in the presence of a moderate concentration of the denaturant. Figure 4 shows that in 2.6 M GuHCl and at a concentration of 10 μM gene V protein, the CD at 229 nm drops sharply as the pH of the solution is decreased from 8 to 6 or increased from 8 to 10. The CD of the protein in the absence of denaturant was unaffected by pH in the range of pH 6–9. Each gene V protein monomer subunit contains a single histidine residue, histidine-64, which is located at the monomer interface. We suspected that the titration of histidine-64 may be responsible for the observed structural transition near neutral

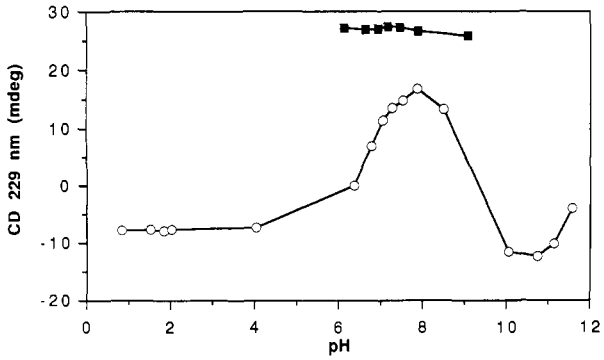

FIGURE 4: Influence of pH on CD_{229nm} of gene V protein. The CD of 10 μM gene V protein in buffer B containing either no GuHCl (■) or 2.6 M GuHCl (○) was measured by using a 1-cm-path cuvette for 2 min at various pH, at 25.0 °C. The gene V protein samples at different pH values were prepared by adding various amounts of HCl or NaOH to gene V protein solutions in buffer B to the indicated final pH.

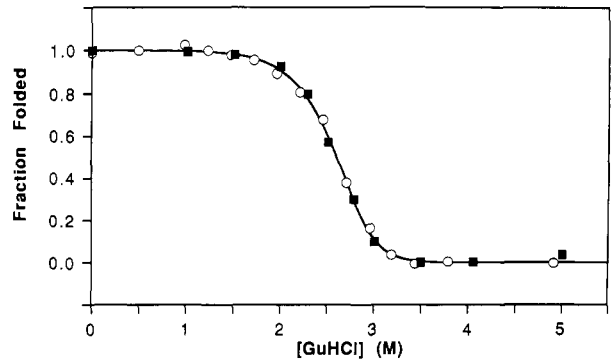

FIGURE 5: Denaturation of gene V protein by GuHCl at 25.0 °C as determined by two independent assays. Estimates of fractions of chains in the folded state were determined by using CD_{229nm} (○) and reactivity of gene V protein to cysteine modification (■). As described under Materials and Methods, the fractions of native gene V protein were calculated from the observed CD_{229nm} by using eq 1–3 with the following parameters: $\theta_{folded,0M}$ = 3000 deg M^{-1} cm^{-1}, $\theta_{unfolded,5M}$ = −400 deg M^{-1} cm^{-1}, α = 10 deg M^{-1} cm^{-1} (M GuHCl)$^{-1}$, β = 120 deg M^{-1} cm^{-1} (M GuHCl)$^{-1}$. The determination of fractions of gene V protein in the native state by the cysteine modification method is described under Materials and Methods. The solid line is a fit to both sets of measurements based on the two-state model and the assumption of a linear relationship between the change of free energy upon unfolding and the concentration of the denaturant (eq 5–8), in which C_m = 2.6 M GuHCl and m = −3.6 kcal mol^{-1} (M GuHCl)$^{-1}$.

pH. However, a mutant gene V protein mutant with histidine at position 64 replaced by cysteine exhibited a pH dependence of CD_{229nm} in 2.6 M GuHCl nearly identical with that of the wild-type protein (Zabin and Terwilliger, unpublished results), indicating that the observed pH-induced unfolding near neutral pH is not due to the titration of histidine-64.

Two-State Denaturation of Gene V Protein. Most small proteins denature cooperatively, with only two forms, the folded and unfolded proteins, present at substantial levels at equilibrium (Ghelis & Yon, 1982). Partially folded forms of proteins can exist at equilibrium in some cases, however (Ghelis & Yon, 1982), so it was important to determine whether any such states are highly populated during the denaturation of gene V protein. Additionally, as the gene V protein is a dimer, it seemed possible that the protein might dissociate into monomers at low concentrations of denaturant and then unfold at higher concentrations of denaturant.

One method of detecting the presence of partially folded forms of a protein at equilibrium is to compare the relative changes in several different types of measurements which depend on the structures of the folded, unfolded, and, possibly, partially folded protein in different ways. If no partially folded intermediates are present, these relative changes will all coincide. As was shown in Figure 2B, the relative changes in CD at 211 and 229 nm as functions of GuHCl concentration are similar. This suggests that secondary and tertiary structures of the gene V protein, as reflected in its backbone and tyrosine conformations, are disrupted in a single cooperative transition during the denaturation.

We have also used another, very different, measure of the fraction of gene V protein chains in the folded state: chemical reactivity of cysteine-33. There is only a single cysteine residue in each monomer of the gene V protein, and it is buried within the core of the monomer. It is inaccessible to chemical modification in the folded form of the protein but is highly reactive if the protein is denatured with GuHCl (Pretorius et al., 1975). We reasoned that if conditions could be found such that chemical modification of cysteine-33 in an unfolded chain were much more rapid than unfolding of the gene V protein, then the fraction of cysteine residues which could be rapidly modified would be an accurate measure of the fraction of chains in the unfolded state. Figure 1 demonstrates that these conditions can readily be obtained by using the sulfhydryl-reactive reagent 4,4'-dithiodipyridine (PDS) as a probe of cysteine exposure. Gene V protein, unfolded in 3.6 M GuHCl, reacts rapidly with PDS. When 10 μM protein and 100 μM PDS are used, the reaction is more than 99% complete in less than 1 min (Figure 1, squares). The unfolding of the gene V protein is much slower. Native gene V protein, exposed simultaneously to PDS and 3.6 M GuHCl, unfolds and reacts with PDS with a time constant of about 15 min (Figure 1, circles). Therefore, the chemical modification is much more rapid than unfolding, and the assay is a good measure of the accessibility of the cysteine residue at equilibrium in GuHCl.

Figure 5 illustrates the relationship between the fraction of gene V protein chains in the folded state estimated on the basis of the accessibility of cysteine-33 and that estimated from CD_{229nm} as functions of denaturant concentration. The two measurements yield nearly identical estimates of unfolding of the gene V protein, suggesting that the gene V protein unfolds in a single cooperative transition and that, at equilibrium, partially folded forms are not present at high levels. These experiments do not, however, rule out the possibility of small amounts of intermediate forms or even large amounts of a form for which both circular dichroism and cysteine exposure are similar in the folded or the unfolded form.

Although partially folded forms of the gene V protein appear not to be present at high levels at equilibrium, it was possible that the cooperative transition we observed was between folded and unfolded monomers and that the dimer had dissociated, without a change in CD or exposure of buried cysteines, at a lower concentration of denaturant. To explore this possibility, we measured the dependence of the folding transition on protein concentration. We then compared this with the protein concentration dependence expected of either a transition between folded dimers and unfolded monomers or a transition between folded and unfolded monomers. Figure 6A shows that the denaturation of the gene V protein by GuHCl is dependent on the concentration of the protein, as expected for a transition from a dimer to two monomers. It is inconsistent with the unfolding of a monomeric form, which should have no dependence on protein concentration. The shifts in the midpoint of the denaturation of different gene V protein concentrations are those expected by a two-state model (eq 4–8). Figure 6B shows that at 2.7 M GuHCl, in the denaturation transition region of gene V protein, the dependence of CD_{229nm} on gene V protein concentration is well described

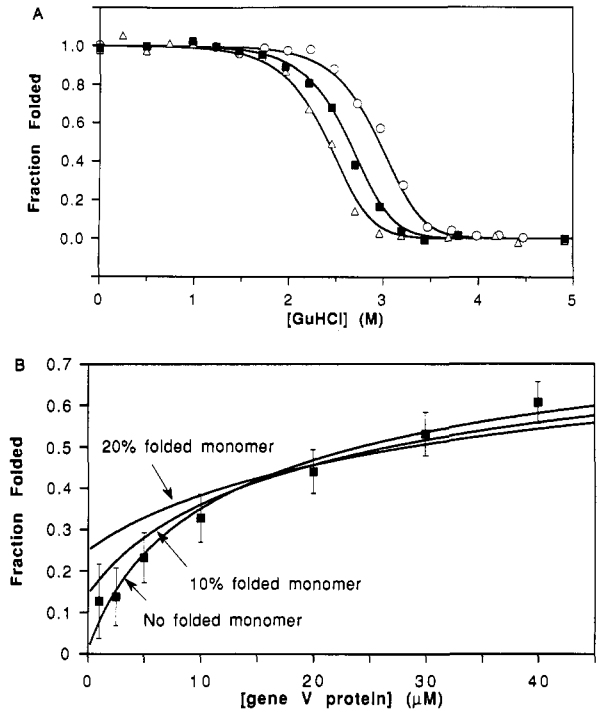

FIGURE 6: (A) Denaturation of gene V protein by GuHCl at three different protein concentrations. CD_{229nm} values of three concentrations of gene V protein at 2.5 (△), 10 (■), and 68 μM (○) were measured as functions of GuHCl concentration. The denaturation transitions of gene V protein at the three protein concentrations were analyzed separately according to eq 1–8 as described under Materials and Methods, and the fractions of gene V protein in native state were calculated and plotted. Then one set of the two parameters C_m and m in eq 4–7 were simultaneously fitted to the estimated fractions of native protein at all three protein concentrations. The solid lines represent the best fit in which C_m = 2.6 M GuHCl at 10 μM gene V protein and m = −3.6 kcal mol^{-1} (M GuHCl)$^{-1}$. (B) Dependence of the denaturation equilibrium in 2.70 M GuHCl on gene V protein concentration. The fractions of gene V protein in the native state (■) in 2.70 M GuHCl at different concentrations of gene V protein were determined as described under Materials and Methods. Uncertainties (1 standard deviation) represented by the error bars include the uncertainties in both the original experimental data and the extrapolations. Several models for describing the denaturation equilibrium were considered (see text), and the best fits of these models to the experimental data are represented by the continuous lines. The prediction of the two-state model (eq 4), with an equilibrium constant for the unfolding reaction (K_{eq}) of 2.4 × 10^{-5} M, is indicated by "No folded monomer". Modeling results of two three-state models (eq 9 and 10) which assume the folded monomer exists as an intermediate in the equilibrium are also plotted. In the model indicated by "10% folded monomer", it was assumed that 10% of total gene V protein molecules exist in the equilibrium in the form of a folded monomer when the concentration of gene V protein is 10 μM. The parameters used were K_1 = 9.0 × 10^{-7} M, K_2 = 6.0, and K_{eq} = 3.2 × 10^{-5} M. In the model indicated by "20% folded monomer", it was assumed that 20% of total gene V protein molecules exist in the equilibrium in the form of folded monomer when the concentration of gene V protein is 10 μM. The parameters used were K_1 = 4.8 × 10^{-6} M, K_2 = 3.0, and K_{eq} = 4.3 × 10^{-5} M.

by an equilibrium transition between a folded dimer and two unfolded monomers. To estimate the maximum amount of folded monomers which could be present under these conditions, we have considered a three-state model (eqs 9 and 10), consisting of a folded dimer, a folded monomer, and an unfolded monomer, where the folded forms (monomer or dimer) are assumed to exhibit equal CD_{229nm}. We compared the observed protein dependence of CD_{229nm} for gene V protein in 2.70 M GuHCl with the modeled dependence, using several values of the equilibrium constant between the folded and unfolded monomers. Figure 6B illustrates the predictions for the cases in which the ratio of unfolded to folded monomers is 6:1 or 3:1, so that at 10 μM gene V protein folded monomers account for about 10% or 20% of the polypeptide chains, respectively. These relatively small fractions of folded monomers would produce a change in the concentration dependence of CD. The prediction given by the model that 10% of gene V protein molecules exist in the form of folded monomers is just consistent with our experimental observations, and the model in which 20% of gene V protein exists as folded monomers is clearly inconsistent with the data. To a good approximation, in 2.7 M GuHCl, the gene V protein exists in just one of two states at equilibrium, the folded dimer and the unfolded monomer. At other concentrations of GuHCl, however, other forms may exist.

Conformation Stability of the Gene V Protein. By use of the two-state model, the change of free energy upon unfolding ($\Delta G_u°$) of the gene V protein can directly be measured in the denaturation transition region in the presence of GuHCl, and by assuming a linear relationship between $\Delta G_u°$ and denaturant concentration, $\Delta G_u°$ in the absence of denaturant can be roughly estimated (Pace, 1975). For gene V protein, $\Delta G_u°$ in the absence of denaturant is about 16.3 ± 0.7 kcal/mol for the unfolding reaction from a gene V protein dimer to two monomers at 25.0 °C, as determined from 10 CD-monitored GuHCl denaturation experiments (Figure 5).

The value of $\Delta G_u°$ obtained by using the two-state model is probably a good estimate of the actual free energy difference between the folded dimer and unfolded monomers, but the presence of some folded monomers or other intermediates will introduce some errors into this estimate. For example, modeling studies indicate that if 10% of gene V protein exists as the folded monomer in the equilibrium in addition to the folded dimer and unfolded monomer, using a two-state model to describe the denaturation would lead to an overestimate of 0.2 kcal/mol for $\Delta G_u°$ in the denaturation transition region, and possibly a much larger error when it is extrapolated to the absence of denaturant (Liang and Terwilliger, unpublished results).

In addition to systematic errors introduced by using simplified models of the denaturation equilibrium, other measurement conditions could also introduce uncertainties into the estimation of $\Delta G_u°$. As the denaturation of gene V protein by GuHCl is strongly affected by pH near neutral pH (Figure 4), any variation in the pH of samples will introduce errors when stabilities of different mutant gene V proteins are measured and compared. On the basis of the dependence of CD_{229nm} in 2.6 M GuHCl on pH (Figure 4), we estimate that a deviation of pH from neutral pH by 0.1 pH unit would lead to an error in the estimation of $\Delta G_u°$ of about 0.14 kcal/mol and an error of 0.03 M in the midpoint GuHCl concentration (C_m).

Kinetics of Folding and Unfolding of the Gene V Protein. Although the gene V protein appears to exist largely as either a folded dimer or an unfolded monomer at equilibrium, it is possible that, during the folding process, there are intermediate states which are transiently highly populated. Such states have been detected in other cases by observation of large deviations in the kinetics of folding or unfolding from those expected from the two-state model (Kim & Baldwin, 1982; Creighton, 1984; Kuwajima, 1989). In the case of the gene V protein, such deviations might be detected by the observation of populations of gene V protein chains which fold or unfold with different time constants. Alternatively, they might be detected by inconsistencies between equilibrium measurements and kinetic measurements, e.g., a difference between the equilibrium

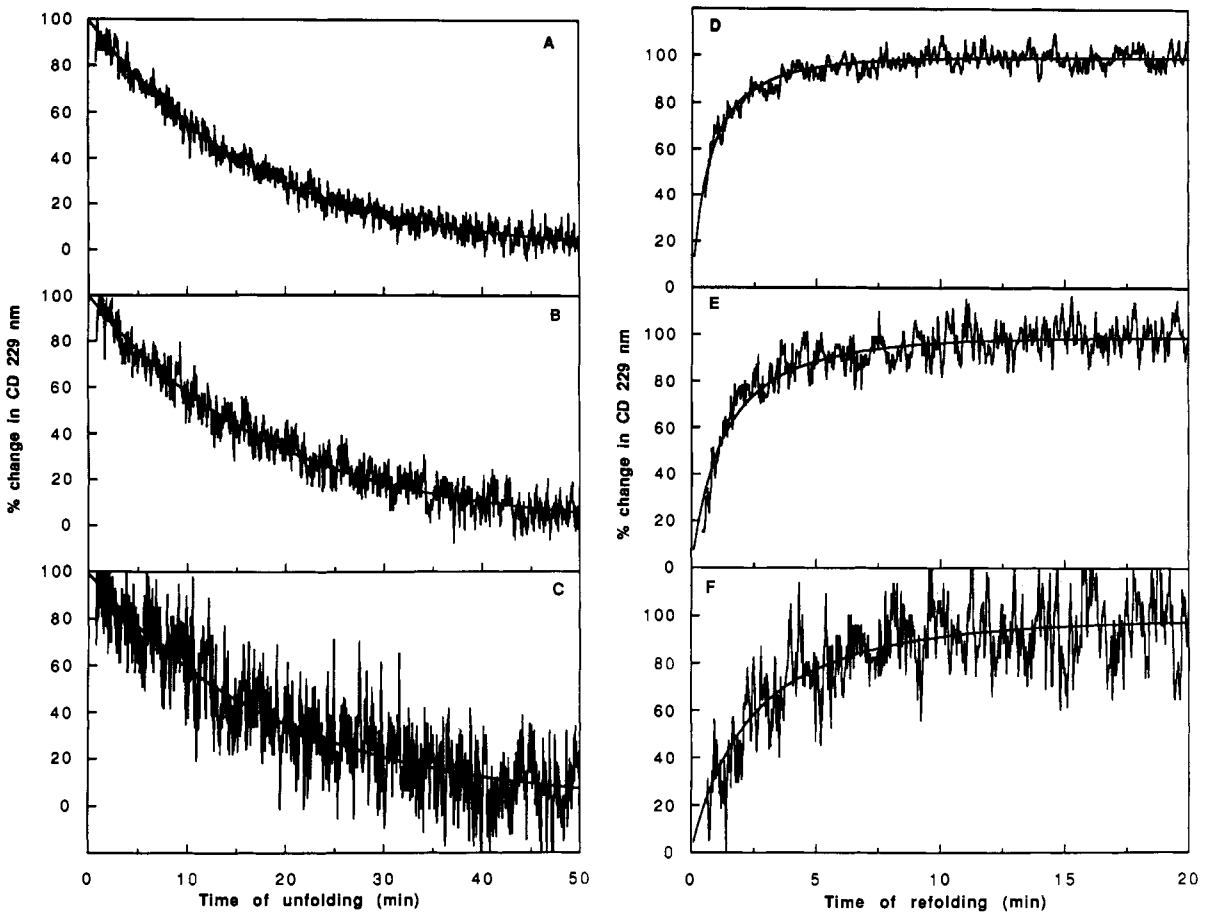

FIGURE 7: Unfolding and refolding kinetics of gene V protein as followed by CD_{229nm}. The relative changes of CD_{229nm} were plotted as a function of time. The unfolding in 3 M GuHCl and refolding in 2 M GuHCl of 2.5, 5, and 10 μM gene V protein were followed at 25.0 °C by CD_{229nm}, as described under Materials and Methods. The reaction time courses for the three gene V protein concentrations observed by CD_{229nm} were scaled according to the relative changes in CD. Panels A, B, and C show the unfolding reactions of 10, 5, and 2.5 μM gene V protein, respectively, in 3 M GuHCl. The smooth lines represent a fit of the kinetic model (eq 13), in which the parameters were $k_1^{app} = 0.046$ min^{-1} and $k_2^{app} = 490$ M^{-1} min^{-1}. The initial and final CD_{229nm} values of the reactions (100% and 0% CD changes) were extrapolated from the observable portions of kinetic time courses. The initial CD_{229nm} value for the unfolding of 10 μM protein (panel A) calculated from the parameters determined in equilibrium denaturation experiments (legend to Figure 5) was identical with the extrapolated value, and the final CD_{229nm} for the same reaction calculated from equilibrium denaturation experiments was 3% lower than the extrapolated value. Panels D, E, and F show the refolding of 10, 5, and 2.5 μM gene V protein, respectively, in 2 M GuHCl. The smooth lines represent a fit of the kinetic model (eq 13), in which the parameters were $k_1^{app} = 0.020$ min^{-1} and $k_2^{app} = 7.5 \times 10^4$ M^{-1} min^{-1}. The final CD_{229nm} values for the refolding reactions (100% CD changes) were extrapolated from the observable portions of the reaction time courses. The final CD value for the refolding of 10 μM protein (panel D) calculated from the results of equilibrium denaturation experiments (legend to Figure 5) was 5% higher than that extrapolated from the reaction time courses. For the initial CD_{229nm} values (0% CD changes), the values calculated from equilibrium denaturation experiments (legend to Figure 5) were used.

constant K_{eq} based on a two-state model (eq 4) and the ratio of the apparent unfolding and folding rate constants k_1^{app} and k_2^{app} (eq 13).

We have used measurements of both the CD_{229nm} and the accessibility of cysteine-33 to chemical modification to measure the rates of folding and unfolding of the gene V protein. Figure 7 shows that it is possible to use CD to monitor the unfolding or refolding processes initiated by a jump in the concentration of GuHCl. Panels A–C of Figure 7 show the CD_{229nm} as a function of time after GuHCl concentrations of gene V protein samples were increased from 2 M, where the gene V protein is largely folded, to 3 M, where the protein is mostly unfolded at equilibrium (see Figures 5 and 6A). As expected for an unfolding reaction, the CD_{229nm} decreases from that of the folded protein to that of the unfolded protein. The half-time of unfolding is essentially unaffected by a 4-fold change in protein concentration from 2.5 to 10 μM, consistent with a unimolecular transition from a folded dimer to two unfolded monomers. The three unfolding reactions at different gene V protein concentrations can be adequately modeled by the simple two-state kinetic model shown in eq 13 with one set of apparent unfolding and refolding rate constant parameters for three experiments.

The rate of unfolding of the gene V protein can also be independently determined by measuring the rate of chemical modification of cysteine-33 in the gene V protein by the sulfhydryl-reactive reagent PDS. At 3 M GuHCl, the first-order rate contant for unfolding of gene V protein estimated by this method was in agreement with result of the CD experiment (Table I).

The refolding of gene V protein was monitored in a similar way, as shown in panels D–F of Figure 7. As predicted from the two-state model for the folding transition, the time course of refolding depends strongly on protein concentration, with gene V protein refolding faster at higher protein concentrations than at lower concentrations. The kinetics of refolding in 2 M GuHCl of the gene V protein at these three concentrations can all be fairly well represented by a single rate constant for refolding, k_2^{app} (eq 13).

In the observed unfolding reactions of the gene V protein (Figure 7, panels A–C), the CD_{229nm} decreased in what appears to be a single kinetic transition from the values expected for

Table I: Apparent Unfolding and Folding Rate Constants in 2 and 3 M GuHCl[a]

	in 2 M GuHCl	in 3 M GuHCl
k_1^{app} (min^{-1})	$(2.0 \pm 0.4) \times 10^{-2}$ (PDS)	$(5 \pm 1) \times 10^{-2}$ (PDS), $(4.6 \pm 0.5) \times 10^{-2}$ (CD)
k_2^{app} (min^{-1} M^{-1})	$(7.5 \pm 0.6) \times 10^4$ (CD)	
k_1^{app}/k_2^{app} (M)	$(2.7 \pm 0.8) \times 10^{-7}$ (PDS, CD)	
K_{eq} (M)	$(2.6 \pm 0.6) \times 10^{-7}$ (CD)	

[a] The apparent first-order unfolding rate constant (k_1^{app}) and the apparent second-order refolding rate constant (k_2^{app}) (eq 13) determined at 25.0 °C in buffer B and 2 or 3 M GuHCl are listed. The methods of determination indicated by the texts in parentheses are as follows: CD, CD at 229 nm; PDS, modification of cysteine-33 by PDS. Details of these measurements are described under Materials and Methods and in the legends of Figures 1 and 7. Also listed in the table are the ratio of the apparent kinetic rate constants and the equilibrium constant (K_{eq}) of unfolding of the gene V protein (eq 4) extrapolated from the equilibrium denaturation experiments monitored by CD_{229nm} (Figure 5).

the folded form to those of the unfolded form. The unfolding reactions do not seem to contain any fast phase. The observable portions of the refolding reactions (Figure 7, panels D–F) are also consistent with a single kinetic transition from the unfolded form of the protein to the folded form. In neither the kinetics of folding nor that of unfolding did we find any evidence of multiple populations of gene V protein with differing kinetics, and only the folding rate was dependent on protein concentration. In particular, the dependence of refolding on gene V protein concentration shows that the refolding of gene V protein monitored by CD_{229nm} is a bimolecular reaction, corresponding to a transition from two monomers to one dimer.

We then compared the ratio of the apparent rate constants for unfolding and folding at 2 M GuHCl (eq 13) with the apparent equilibrium constant (eq 4) for the unfolding transition. If the two-state model were a good description of the unfolding transition, these would be equal. As shown in Table I, in 2 M GuHCl, the ratio of unfolding and folding rate constants is in agreement with the apparent unfolding equilibrium constant extrapolated from the equilibrium denaturation experiments. Therefore, for the unfolding and refolding reactions in 2 M GuHCl, we do not find evidence for the presence of kinetic intermediates.

Dependence of the Folding and Unfolding of the Gene V Protein on GuHCl Concentration. To obtain some clues about the nature of the transition state in the folding and unfolding of the gene V protein, we investigated the effect of GuHCl concentration on the apparent rate constants for folding and unfolding of the protein. The apparent unfolding and refolding rate constants in various concentrations of GuHCl measured by using the chemical reactivity of cysteine-33 are shown in Figure 8. As anticipated (Kuwajima, 1989), the rate of unfolding of the gene V protein increases with increasing concentration of the denaturant, and the rate of refolding decreases with the concentration of the denaturant. The apparent unfolding rate (k_1^{app}) is only weakly dependent on the concentration of denaturant, while the apparent folding rate constant (k_2^{app}) is strongly dependent on denaturant concentration. As GuHCl is thought to affect the equilibrium between the folded and unfolded states by increasing the solubility of apolar side chains which are exposed during unfolding, the weak dependence of the unfolding rate on denaturant concentration suggests that the transition state is not very different from the folded state in its exposure of apolar surface.

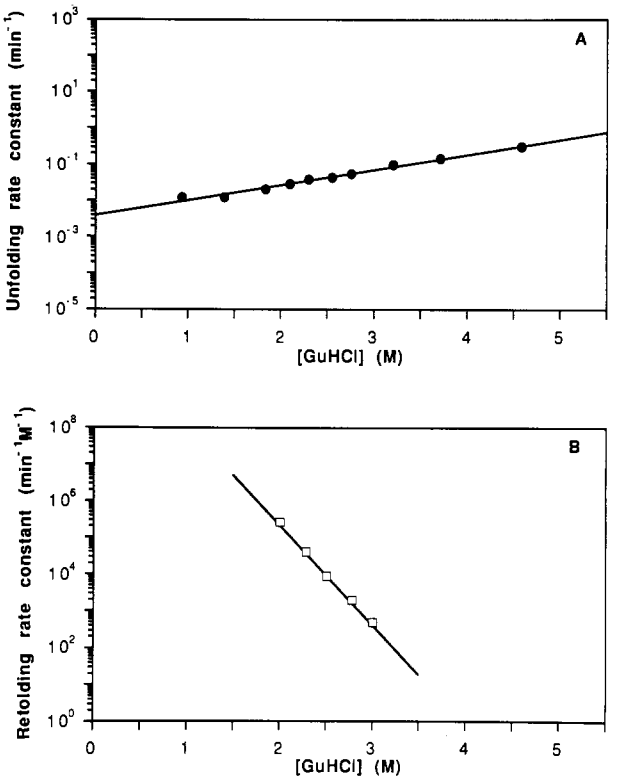

FIGURE 8: Effect of GuHCl concentration on apparent unfolding and folding rate constants. The apparent unfolding rate constant k_1^{app} (panel A, ●) and folding rate constant k_2^{app} (panel B, □) in eq 13 and 14, determined at 25.0 °C by using cysteine reactivity as described under Materials and Methods, are plotted on a logarithmic scale as functions of the concentration of GuHCl. Continuous lines are least-squares fits of a linear relation between the concentration of GuHCl and the logarithms of the rate constants. The apparent folding rate constants (k_2^{app}) were calculated from equilibrium constants (K_{eq}) and unfolding rate constants (k_1^{app}) by using eq 15. As the equilibrium constant (K_{eq}) could not be measured outside the denaturation transition region, only those values of k_2^{app} determined in GuHCl from 2 to 3 M are plotted in panel B. The unfolding rate constant (k_1^{app}) could only be determined for experiments in which the rate of PDS modification is much greater than the rate of refolding of the gene V protein, so that the unfolding of the gene V protein is rate-limiting in eq 13 and 14. On the basis of the refolding rate constants extrapolated from those determined for solutions containing GuHCl between 2 and 3 M in the denaturation transition region, we estimate that the rate of modification is at least 30 times greater than that of refolding under the conditions of our experiments when the concentration of GuHCl is more than 1 M.

Conversely, the strong dependence of the folding rate on denaturant concentration suggests that the transition state is quite unlike the unfolded form in its exposure of apolar groups.

Discussion

Like many other proteins, the gene V protein of bacteriophage f1 undergoes a cooperative structural transition in solutions containing GuHCl. This transition alters the secondary structure of the protein, as indicated by the changes in the peptide backbone CD at 211 nm, the tertiary structure, as indicated by the changes in chemical reactivity of a buried cysteine residue and by the CD at 229 nm due to tyrosine residues, and the quaternary structure, as indicated by the dependence of the unfolding equilibrium on protein concentration. The two CD measurements and the chemical reactivity of cysteine-33 yield very similar estimates of the dependence of the equilibrium on denaturant concentration, indicating that all three probes are monitoring a single transition and therefore suggesting that partially folded forms of the protein are not present at high levels at equilibrium.

Furthermore, the dependence of the denaturation equilibrium on protein concentration is that expected from a two-state model. All of these results are consistent with a simple two-state model for folding and unfolding of the gene V protein involving only a folded dimer and an unfolded monomer. Similar results have been found for many small monomeric proteins (Ghelis & Yon, 1982; Creighton, 1984), as well as small dimeric proteins (Pakula & Sauer, 1989; Bowie & Sauer, 1989). Our results are also consistent with the X-ray structure of the gene V protein (Brayer & McPherson, 1983). In this structure, there are extensive interactions between the monomeric subunits of the protein, and the intersubunit hydrophobic core is contiguous with the individual hydrophobic regions of each monomer. On the basis of this structure, Brayer and McPherson suggested that an isolated monomer would be too extended and nonglobular in its structure to be stable (Brayer & McPherson, 1983).

The kinetics of folding and unfolding of several proteins have been examined in some detail, and in most cases, several steps with very different rates are involved in both folding and unfolding. Very rapid steps in folding include formation of a compact structure and formation of secondary structural elements during folding (Udgaonkar & Baldwin, 1988), and slower steps include proline isomerizations and subtle rearrangements after the tertiary structure of the protein is formed (Kim & Baldwin, 1982). For the gene V protein, both the unfolding reactions in solutions containing various concentrations of GuHCl and the refolding reactions observed in 2 M GuHCl are quite slow. The dependence of the rate of refolding on protein concentration suggests that the rate-limiting step in the refolding is a bimolecular reaction corresponding to a transition from two monomers to one dimer. Although the gene V protein does have six prolines per monomer, the protein concentration dependence of refolding suggests that the proline isomerization is not the rate-limiting step in the folding of the gene V protein in 2 M GuHCl. The slow step in the folding of the gene V protein that we observe seems to involve the association of two monomers. One possibility is that dimerization and the formation of tertiary structure are dependent upon each other and occur essentially simultaneously. Alternatively, the slower dimerization may occur first, followed by a faster tertiary structure formation. It is possible that the simple kinetics we observe for the folding of gene V protein are due to the fact that the refolding experiments were carried out in 2 M GuHCl, a concentration of the denaturant that may be high enough to disrupt any partially folded intermediates. In the absence of GuHCl or in a low concentration of GuHCl, the folding kinetics may be more complex.

The dependence of the unfolding and folding rate constants on denaturant concentration suggests that the conformation of the transition state of the folding reaction of the gene V protein is close to that of the folded state. The transition-state theory in chemical kinetics has been used in the studies of protein folding (Matthews, C. R., 1987). For the folding and unfolding processes of a protein which may include a series of reactions, the transition state for the overall reaction is generally defined as the transition state with highest energy (Matouschek et al., 1989). To assess the relative structural organization of the transition state from the dependence of the kinetic rate constant (k) on the concentration of the denaturant (C), a parameter

$$\Delta b = -RT(\mathrm{d} \ln k/\mathrm{d}C) \qquad (16)$$

has been introduced (Tanford, 1970; Schellman, 1978; Chen et al., 1989; Kuwajima et al., 1989). For the unfolding or folding reactions, the respective parameters $\Delta b_{\text{unfolding}}$ and $\Delta b_{\text{folding}}$ were calculated for the gene V protein from the apparent unfolding and refolding rate constants k_1^{app} and k_2^{app} determined in our measurements. The ratio of $\Delta b_{\text{unfolding}}/(\Delta b_{\text{unfolding}} - \Delta b_{\text{folding}})$ was 0.13, which, according to this model, suggests that during the unfolding reaction, from folded state to transition state, 13% of the apolar surface exposed by totally unfolding the protein is exposed. This suggests that the transition state resembles the native structure of gene V protein in its accessible surface area and is therefore nativelike. Similar conclusions have been reached for a number of proteins (Segawa et al., 1973; Goto & Hamaguchi, 1982; Creighton, 1984). These results are consistent with the idea that in either the folding or the unfolding of a protein, the highest energy barrier is a state in which the protein has a compact and nativelike structure.

Conclusion

The reversible unfolding of the gene V protein in GuHCl appears, to a close approximation, to involve only two states, the native dimer and an unfolded monomer. This observation allows the use of a two-state model to estimate the changes of free energy upon unfolding of the gene V protein and its mutants in the presence of denaturant. The gene V protein differs from most model systems for studying protein folding in that it is dimeric and has no helical structures. As some effects of mutations may depend on the secondary or quaternary structures in which they occur, we suggest that the gene V protein will be a useful model system for studying the effects of amino acid substitutions on protein folding and stability.

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Spatial Arrangement of Coenzyme and Substrates Bound to L-3-Hydroxyacyl-CoA Dehydrogenase As Studied by Spin-Labeled Analogues of NAD^+ and CoA[†,‡]

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ABSTRACT: The synthesis of nitroxide spin-labeled derivatives of *S*-acetoacetyl-CoA, *S*-acetoacetylpantetheine, and *S*-acetoacetylcysteamine is described. These compounds are active substrates of L-3-hydroxyacyl-CoA dehydrogenase [(*S*)-3-hydroxyacyl-CoA:NAD^+ oxidoreductase, EC 1.1.1.35] exhibiting v_{max} values from 20% to 70% of *S*-acetoacetyl-CoA itself. *S*-Acetoacetylpantetheine and *S*-acetoacetylcysteamine form binary complexes with the enzyme and exhibit ESR spectra typical for immobilized nitroxides. In the case of spin-labeled pantetheine, the radical is more mobile. When spin-labeled substrates are bound simultaneously to each active site of this dimeric enzyme, spin–spin interactions differentiate between two alternate orientations of the substrate [Birktoft, J. J., Holden, H. M., Hamlin, R., Xuong, N. H., & Banaszak, L. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8262–8266]. The fatty acid moiety is thought to be located in a cleft between two domains whereas a large part of the CoA moiety probably extends into the solution. NAD^+, spin-labeled at N^6 of the adenine ring, is an active coenzyme of L-3-hydroxyacyl-CoA dehydrogenase (60% v_{max}). Complexes with the enzyme exhibit ESR spectra typical of highly immobilized nitroxides. Binding of coenzyme NAD^+ causes conformational changes of the binary enzyme/substrate complex as revealed by changes in the ESR spectrum of spin-labeled *S*-acetoacetylpantetheine.

L-3-Hydroxyacyl-CoA dehydrogenase [(*S*)-3-hydroxyacyl-CoA:NAD^+ oxidoreductase, EC 1.1.1.35] catalyzes the NAD-dependent interconversion between L-3-hydroxy and 3-oxo fatty acyl CoA thioesters. The fatty acid chain length can vary from 4 to 20 (Wakil et al., 1954). The enzyme has been isolated from various sources (Lynen et al., 1952; Wakil et al., 1954; Stern, 1957; Grassl, 1957), and more recently, the crystal structure of the dehydrogenase from pig heart mitochondria has been determined by X-ray crystallography (Birktoft et al., 1987). 3-Hydroxyacyl-CoA dehydrogenase from this source is a dimer with a molecular weight of M_r = 67000 (Bitar et al., 1980; Birktoft et al., 1987). Some heterogeneity at the amino termini of the chains may be the result of proteolytic processing. Each dimer is composed of two domains with the amino-terminal end of each domain comprising a typical Rossmann fold (Rossmann et al., 1975) for binding of the adenine moiety of NAD. Much less is

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