High-Pressure Denaturation of Staphylococcal Nuclease Proline-to-Glycine Substitution Mutants†

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ABSTRACT: Our recently reported pressure-jump relaxation kinetics experiments on staphylococcal nuclease folding and unfolding [Vidugiris *et al.* (1995) *Biochemistry 34*, 4909] demonstrated that both transitions exhibit positive activation volumes, with that of folding being much larger than that of unfolding. Thus high pressure denatures proteins by slowing the rate of folding more than that of unfolding. In the present work, we take advantage of the very slow folding and unfolding rates under pressure to examine the kinetics and volume changes along the reaction coordinate for protein folding−unfolding for an interesting set of mutants of staphylococcal nuclease: P42G, P47G, P117G, and the double mutant, P47G+P117G. Previous studies have shown that replacement of an individual proline residue at position 42, 47, or 117 by glycine leads to paradoxical protein stabilization against denaturation by guanidine chloride, high temperature, or high pressure. In order to observe unfolding over an attainable pressure range, guanidine hydrochloride was employed. Within experimental error, the activation volumes and equilibrium volume changes were independent of the concentration of this denaturant and our analysis of the rate constants is consistent with the generally accepted hypothesis that this denaturant acts both by increasing the rate of unfolding and decreasing the rate of folding. We show that the stabilization resulting from each of the proline-to-glycine substitutions arises primarily from a decrease in the unfolding rate, and to a small degree, from an increase in the folding rate. The changes in rate constants upon proline-to-glycine substitution can be modeled in terms of small stabilization of the unfolded state, a greater stabilization of the transition state, and a still greater stabilization of the folded state. Although the rates were found to change for all of the mutants in the set, no changes greater than experimental error were found in the corresponding equilibrium volume changes and activation volumes for folding and unfolding. At low pressures (well below the onset of unfolding) the pressure-jump relaxation profiles for wild type proteins (both Foggi and V8) showed kinetic complexity. Although the effect was attenuated somewhat in pressure-jump profiles of one proline-to-glycine mutant (P42G), its persistence in data from all the mutants studied leads us to conclude that its origin is not in *cis/trans* peptide bond isomerization at proline 117, 47, or 42.

It has long been known that the application of high hydrostatic pressure results in the unfolding of globular proteins as determined by changes in UV−visible absorption (Brandts *et al.*, 1970; Zipp & Kauzmann, 1973), fluorescence (Li *et al.*, 1976), infrared (Heremans & Bormans, 1986; Wong & Heremans, 1988; Takeda *et al.*, 1995), and NMR (Samarisinghe *et al.*, 1992; Royer *et al.*, 1993) spectra. This effect of pressure on the stability of proteins arises because the volume of the protein−solvent system is smaller in the unfolded state of the polypeptide than in its folded state. The microscopic contributions to this volume change of unfolding, although they involve differential solvation and excluded volume in the two states, are not well-understood (Heremans, 1982; Weber & Drickamer, 1983; Royer, 1995; Mozhaev *et al.*, 1994; Prehoda & Markley, 1996). We have recently demonstrated (Vidugiris *et al.*, 1995) by pressure-jump relaxation kinetics on staphylococcal nuclease that this destabilization by pressure results from the fact that, although the activation volumes for both folding and unfolding are positive, leading to a decrease in both the folding and unfolding rate as a function of pressure, the activation volume for folding is significantly larger in magnitude than that for unfolding. Thus, the application of pressure slows the folding rate much more than the unfolding rate.

The folding and unfolding transitions of WT[1] are reasonably well represented by a simple two-state model (Schechter *et al.*, 1970; Shortle, 1986; Shortle *et al.*, 1988, 1990; Eftink *et al.*, 1991; Ramsay & Eftink, 1995; Royer *et al.*, 1993; Vidugiris *et al.*, 1995), although the kinetic (Chen *et al.*, 1992a,b; Nakano *et al.*, 1993), NMR (Fox *et al.*, 1986; Evans *et al.*, 1987, 1989; Alexandrescu *et al.*, 1989, 1990; Loh *et al.*, 1991; Jacobs & Fox, 1994), and calorimetric (Carra *et al.*, 1994) evidence has revealed small populations of intermediates. Nonetheless, our previous high-pressure unfolding studies on nuclease WT were well-described by a two-state model, except as noted in the low-pressure region where little unfolding occurs (Royer *et al.*, 1993; Vidugiris *et al.*, 1995). We suspected, on the basis of the above-cited kinetic and NMR data, that this non-two-state behavior observed in the low-pressure range could arise in part from prolyl peptide bond isomerization reactions. In order to test this hypothesis, as well as to characterize the energetic and kinetic effects of proline-to-glycine substitutions and to

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[1] WT denotes recombinant protein produced from *E. coli* whose sequence is identical to the nuclease A produced by the Foggi strain of *S. aureus.*

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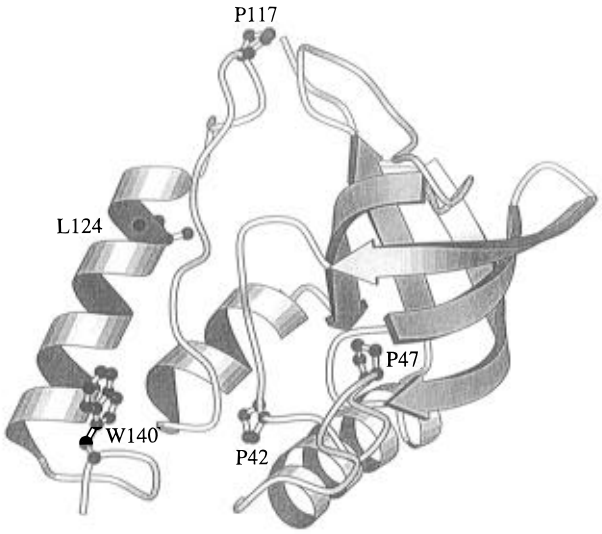

FIGURE 1: Molscript diagram (Kraulis, 1991) of nuclease H124L taken from the crystal structure coordinates of Truckses *et al.* (submitted). Indicated are the positions of residue 124 as well as the positions of the three proline-to-glycine substitutions studied here, P42G, P47G, and P117G. Also shown is the single tryptophan residue (W140), the intensity of which is the observable in the present experiments.

determine which proline, if any, is involved in populating the low-pressure intermediate(s), a study was undertaken to characterize the equilibrium and kinetics of the pressure-induced unfolding of the nuclease H124L[2] and a series of mutants in which proline residues have been substituted by glycine.

Indicated in the ribbon diagram of nuclease H124L (Truckses *et al.*, submitted) in Figure 1 are the positions of the proline-to-glycine substitutions tested in the present studies (P42G, P47G, and P117G, as well as the P47G+ P117G double mutant) and the position of the substitution that differentiates nuclease H124L from WT. It can be seen from Figure 1 that proline 117 is located in a turn connected to the C-terminal α-helix. The tryptophan residue (also shown) which gives rise to the fluorescence signal used to monitor unfolding in the present studies is found at position 140. The loop in which tryptophan 140 is located follows the helix which connects to the loop containing proline 117. The other two prolines whose replacements by glycine have been studied here are found in a loop region that connects the α-helical and β-barrel subdomains of the protein. Studies of protein unfolding by chemical denaturants, heat, and pH have demonstrated that each of the proline-to-glycine substitution mutants is more stable than the parent protein, nuclease H124L (Truckses *et al.*, submitted; Royer *et al.*, 1993). Increased stability brought about by the P117G substitution was also observed in the background of nuclease WT (Chen *et al.*, 1992a,b; Nakano *et al.*, 1993; Hynes *et al.*, 1994).

MATERIALS AND METHODS

Protein Purification. Recombinant staphylococcal nuclease with the sequence of nuclease A from the V8 strain of *Staphylococcus aureus* (H124L) was produced in *Escherichia coli* and purified as described by Alexandrescu *et al.* (1989) and Royer *et al.* (1993). H124L differs from the nuclease from the Foggi strain of *S. aureus* (WT) by replacement of histidine at position 124 by leucine. The purified protein was lyophilized and stored in that form. Solutions of nuclease were prepared from the lyophilate for the folding−unfolding experiments at a concentration of 2−5 μM (as determined by UV absorption at 280 nm and an extinction coefficient of 15 631 cm^{-1} M^{-1}); aggregation can occur in these experiments at higher protein concentration. The buffer solution used was 10 mM bis-Tris at pH 5.5. Bis-Tris provides a pressure-insensitive buffer owing to the fact that the net charge in the protonated and unprotonated forms is equivalent; in the protonated form the bis-Tris bears one positive charge, and in the unprotonated form the dissociated proton bears a positive charge. Thus the buffer exhibits no volume change and has a pK with a negligible pressure dependence. All experiments were carried out at 21 °C.

Pressure-Jump Experiments. The high-pressure generating system used was similar to that described by Paladini and Weber (1981). Pressure-jump relaxation experiments were carried out in a Vascomax high-pressure cell. A UV multifiber bundle (Oriel Corp., Stratford, CT) with focusing optics coupled the exciting light at 295 nm from the monochromator to the pressure cell. Tryptophan emission was monitored at 90° through a Corion 340 nm high-pass filter. Pressure jumps of 75−250 bar were made by closing the valve to the sample compartment, pumping to the desired new pressure, and then rapidly reopening the valve. Fluorescence intensity data were acquired with 2−5 s integration times, which are fast relative to the relaxation time scales. The pressure jumps were small enough that the adiabatic heating of the sample amounted to no more than 0.3 °C, which at the sample temperature of 21 °C would not perturb the folding equilibrium of nuclease significantly. Moreover, the extent of the perturbation of the equilibrium due to the pressure jump itself was less than 10%, such that the linearized rate expression holds (Eigen & de Maeyer, 1963).

Data Analysis. The pressure-jump fluorescence intensity relaxation profiles were fitted to a single-exponential decay by using the time-domain fluorescence global analysis program described by Beechem and co-workers (1992). The intensity of fluorescence at time t, after the pressure jump $I(t)$ was taken to be an exponentially decaying function

$$I(t) = I_o e^{-t/\tau} \quad (1)$$

where I_o is the intensity prior to the jump and τ is the relaxation time. In some cases, data at lower pressures required a long component with a negative amplitude for a good fit to account for the complexity in the profiles. In these cases only the short relaxation time was used in the subsequent estimation of rates and activation volumes.

The effect of pressure on the Gibbs free energy of unfolding necessarily arises from pressure effects on the rates of unfolding, refolding, or both. A reaction rate k_p at a given pressure p can be expressed in terms of the rate at atmospheric pressure $k°$ and the activation volume $\Delta V^{\ddagger}$ for the formation of the transition state (Gladstone *et al.*, 1941):

$$k_p = k° e^{[-p(\Delta V^{\ddagger})/RT]} \quad (2)$$

[2] H124L denotes recombinant protein produced from *E. coli* whose sequence is identical to the nuclease A produced by the V8 strain of *S. aureus*.

Table 1: Equilibrium Parameters for Nuclease H124L and the Proline-to-Glycine Substitution Mutants[a]

protein	[GuHCl] (M)	*m*-value (GuHCl)	ΔG_u (kcal/mol)			ΔV_u (mL/mol)	
			(GuHCl)[b]	eq	kinetics	eq	kinetics
H124L	0.5	5.8 ± 0.1	3.4 ± 0.1	2.4 ± 0.2	3.5 ± 1.4	79 ± 4	135 ± 115
H124L	0.75	5.8 ± 0.1	1.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.9	75 ± 8	134 ± 105
H124L+P42G	0.75	nd[c]	nd	2.4 ± 0.4	2.1 ± 1.5	79 ± 9	67 ± 19
H124L+P47G	0.75	5.7 ± 0.3	2.8 ± 0.4	2.8 ± 0.5	3.0 ± 2.0	75 ± 15	84 ± 15
H124L+P117G	0.75	5.9 ± 0.1	3.2 ± 0.1	3.1 ± 0.4	2.5 ± 1.5	79 ± 11	72 ± 64
H124L+P47G+P117G	0.75	5.28 ± 0.05	3.47 ± 0.05	3.7 ± 0.7	5.2 ± 2.0	76 ± 14	119 ± 44

[a] Standard deviations on the equilibrium free energy change values represent the 67% confidence limits on the value obtained from rigorous confidence limit testing (Beechem, 1992). Deviations on the equilibrium free energy derived from the kinetic rate constants were estimated from the standard errors on the kinetic rate constants. [b] Calculated from reported free energies and *m*-values given in column 3 (Truckses *et al.*, submitted) for the GuHCl concentration used and indicated in column 2. [c] nd, not determined.

For a simple, two-state system undergoing small perturbations near equilibrium, the observed relaxation time τ at a given pressure is represented by the inverse of the sum of the two individual rate constants for the forward and backward reactions, unfolding and folding,

$$\tau = 1/(k_u + k_f) \quad (3)$$

and as such should be independent of the sign of the perturbation (Eigen & de Maeyer, 1963). We have fitted the values of τ across the entire pressure range for the transition in terms of the values of both the folding and unfolding rate constants at atmospheric pressure, $(k^\circ)_f$ and $(k^\circ)_u$, and their individual activation volumes $\Delta V_f^\ddagger$ and $\Delta V_u^\ddagger$ using nonlinear regression (Dugelby, 1984), a computer program for which was kindly supplied by Dexter Northrup. All of the points were equally weighted in the fits.

The values of the intensities 25 min after the jump were taken as the equilibrium intensities, and these equilibrium profiles were fitted using the BIOEQS analysis program for the free energy ΔG and volume change ΔV of the unfolding (Royer *et al.*, 1991; Royer & Beechem, 1992; Royer, 1993) according to the relation

$$d(\Delta G)/dp = \Delta V \quad (4)$$

The BIOEQS program calculates data points at each pressure from the current parameters, ΔV, ΔG, and the asymptotic values of the folded and unfolded state intensities, I_f and I_u, using the equilibrium constant K_{eq}, which is calculated from the intensities measured at each pressure, I_p:

$$\Delta G = -RT \ln K_{eq} = -RT \ln(I_f - I_p)/(I_p - I_u) \quad (5)$$

and minimizes on the basis of the χ^2 difference between observed and calculated data.

Estimates of the experimental error on the equilibrium volume changes and free energies were obtained from rigorous confidence limit testing of these two recovered parameters as described by Beechem (1992). Error estimates on the rate constants and activation volumes were obtained from the nonlinear least-squares error analysis.

RESULTS

Guanidine Hydrochloride Dependence of the Equilibrium Pressure Unfolding of H124L. Since the variant of nuclease produced by the V8 strain of *S. aureus* (H124L) is significantly more stable than that produced by the Foggi strain (WT) and used in our previous studies, the addition of a small amount of guanidine hydrochloride (GuHCl) was required in order to observe unfolding of H124L and the even more stable proline-to-glycine substitution mutants in the pressure range 0−3000 bar available with our present equipment. Since our previous studies were carried out in the absence of GuHCl, the dependence of the pressure unfolding profiles on the concentration of GuHCl was investigated in order to ascertain whether this chemical reagent perturbs the volume change upon unfolding. In Figure 2 are shown the equilibrium pressure unfolding profiles of H124L at 0.5 and 0.75 M GuHCl. Whereas GuHCl has a large effect on the stability of the protein, as evidenced by the shift of the curves to lower pressures as a function of increasing GuHCl, the slopes of the unfolding profiles do not change. Indeed nonlinear least-squares analysis of these unfolding profiles, as described in the Materials and Methods section, yielded values for ΔG_u (Table 1) that were consistent with the destabilizing effect of GuHCl, although indicative of somewhat less stability than obtained from the GuHCl titrations of H124L under similar conditions (Truckses *et al.*, submitted). Analysis of the data in Figure 2 also yielded values of ΔV_u for the unfolding of H124L at the two GuHCl concentrations that were identical within experimental error to each other and to that observed previously for WT in absence of GuHCl (Table 1; Vidugiris *et al.*, 1995). This is an interesting result. It indicates that the effect of exposing protein surfaces and cavities to aqueous solutions of GuHCl is equivalent, in terms of system volume, to exposing them to pure water.

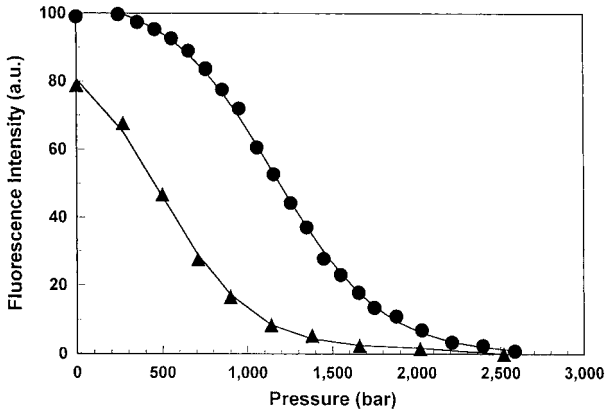

FIGURE 2: Effect of GuHCl on the pressure unfolding profiles of nuclease H124L. Equilibrium fluorescence intensity vs pressure at 0.5 M GuHCl (●) and 0.75 M GuHCl (▲). Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C. Data were obtained as described in the Materials and Methods section. Lines through the points represent the results of nonlinear least-squares analysis of the data as described in Materials and Methods.

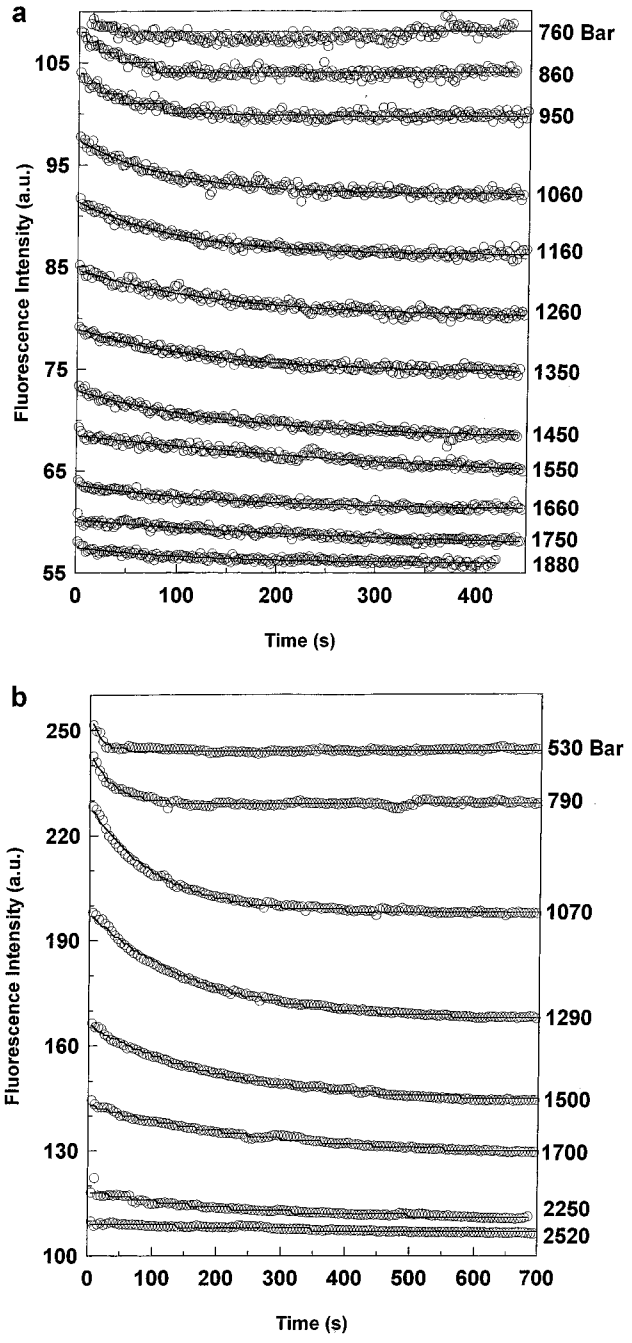

FIGURE 3: Fluorescence intensity vs time profiles for (a) nuclease H124L and (b) nuclease H124L+P42G obtained after small rapid jumps in hydrostatic pressure as described in Materials and Methods. Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C. GuHCl concentration was 0.75 M. Lines through the points represent the results of nonlinear least-squares analysis of the data as described in Materials and Methods.

Guanidine Hydrochloride Dependence of the Kinetics of Pressure Unfolding of H124L. The relaxation kinetic profiles for H124L at 0.5 and 0.75 M GuHCl obtained from small, rapid pressure jumps (in either the positive or negative direction) (Figure 3a) were analyzed for the relaxation time, as described in Materials and Methods and as was done previously with WT (Vidugiris *et al.*, 1995). The relaxation times for H124L increased with increasing pressure (Figure 4a,b), just as for the previously studied WT (Vidugiris *et al.*, 1995). Nonlinear analysis of τ vs pressure was carried out as described in Materials and Methods. A comparison

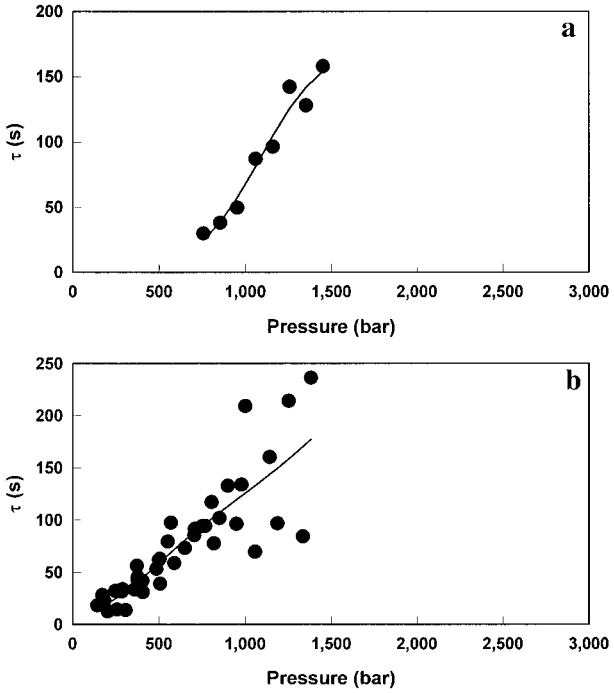

FIGURE 4: Plots of ln τ vs pressure for nuclease H124L solutions containing (a) 0.5 M GuHCl and (b) 0.75 M GuHCl. Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C. Lines represent linear least-squares fits to the data.

of the rate constants recovered from the nonlinear analysis can be found in Table 2. The error on the values is quite large. Nonetheless, the results indicate that the effect of GuHCl on pressure stability arises from an increase in the rate of unfolding and a decrease in the rate of folding. This effect of denaturant on the rates of folding and unfolding has been interpreted as arising from stabilization of both the unfolded and transition states through direct interaction of the denaturant with these states (Tanford, 1970). The unfolding free energy calculated from the ratio of the rate constants at atmospheric pressure is within error of that obtained from analysis of the equilibrium pressure unfolding profile and is in reasonable agreement with those obtained from GuHCl denaturation experiments under similar conditions (Truckses *et al.*, submitted).

The analysis of the plots of τ vs pressure (Figure 4a,b) also yielded large positive activation volumes for folding $\Delta V_f^{\ddagger}$ (Table 2), within error of those previously reported for WT (Vidugiris *et al.*, 1995). The activation volume for unfolding $\Delta V_u^{\ddagger}$ (Table 2) was small and positive, also consistent with our previous results. The difference between the activation volumes for folding and unfolding ($\Delta V_f^{\ddagger} - \Delta V_u^{\ddagger}$) was within error of that obtained from the analysis of the equilibrium unfolding profiles, as would be expected for a two-state unfolding (Table 1). Despite the large uncertainties in the values of the activation volumes, increasing GuHCl does not appear to alter the general phenomenon observed previously (Vidugiris *et al.*, 1995), which is that the folding rate is much more affected by pressure than the unfolding rate because of the large positive activation volume associated with this process. That the activation volumes exhibit behavior in GuHCl solutions similar to that in water is consistent with the observation reported above that the equilibrium volume change for unfolding, which is more

Table 2: Results from Analysis of the Relaxation Times as a Function of Pressure[a]

protein	$\Delta V_f^{\ddagger}$	$\Delta V_u^{\ddagger}$	$(k^\circ)_f$	$(k^\circ)_u$
H124L 0.5 M GuHCl	135 ± 115	0 ± 73	2.3 + 8/− 2.3	0.006 + 0.03/−0.006
H124L 0.75 M GuHCl	154 ± 105	20 ± 13	0.12 ± 0.12	0.02 ± 0.01
H124L+P42G 0.75 M GuHCl	67 ± 19	0 ± 22	0.1 ± 0.003	0.003 + 0.006/− 0.003
H124L+P47G 0.75 M GuHCl	88 ± 15	4 ± 8	0.24 ± 0.14	0.0015 ± 0.0013
H124L+P117G 0.75 M GuHCl	80 ± 64	8 ± 40	0.2 + 0.5/− 0.2	0.003 + 0.01/− 0.003
H124L+P47G+P117G 0.75 M GuHCl	119 ± 44	0 ± 16	4.6 + 12/− 4.5	0.0007 + 0.0013/− 0.0007

[a] Nonlinear analyses. ΔV values in mL/mol; k° values in s^{-1}.

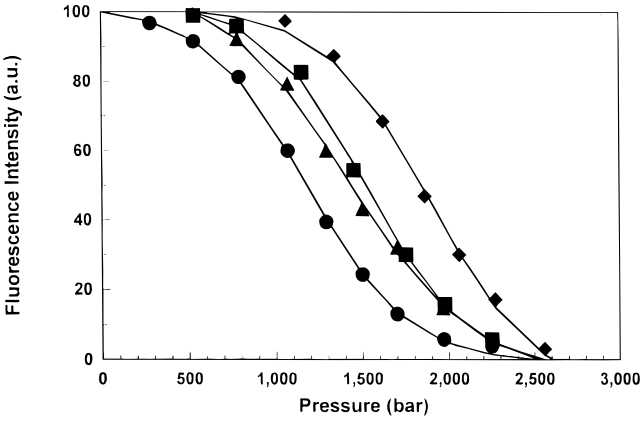

FIGURE 5: Equilibrium pressure unfolding profiles for nuclease H124L+P42G (●), H124L+P47G (▲), H124L+P117G (■), and H124L+P47G+P117G (◆). Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C. The free energies and volume changes of unfolding obtained from analysis of the data can be found in Tables 1 and 2. Lines through the points represent the results of nonlinear least-squares analysis of the data as described in Materials and Methods.

precisely determined than the activation volumes, is unaffected by GuHCl.

Equilibrium Pressure Unfolding of Proline-to-Glycine Mutants of H124L. In Figure 5 are shown the equilibrium unfolding profiles for the proline-to-glycine substitution mutants of H124L at 0.75 M GuHCl. All of the profiles are shifted to higher pressures compared to that of the unsubstituted H124L (Figure 2), consistent with the known increase in stability caused by these mutations. A small amount of hysteresis in refolding was apparent at high pressures for the mutants containing the P47G substitution, presumably due to incomplete equilibration in the return measurements, but the atmospheric pressure intensity value was 100% reversible. This incomplete equilibration in the return profiles at high pressures (data not shown) likely arises from very slow unfolding rates for the mutants containing the P47G substitutions. The equilibrium pressure unfolding profiles of the proline-to-glycine mutants were analyzed to yield the free energy changes ΔG_u (Table 1) and volume changes ΔV_u (Table 1) for the unfolding reaction. In rank order, the stabilities were found to be H124L < H124L+P42G < H124L+P47G < H124L+P117G < H124L+P47G+P117G (see Figure 5). For all of the mutants, the free energies obtained from the pressure profile were in good agreement with those obtained previously in the GuHCl unfolding experiments (Table 1) (Truckses *et al.*, submitted). The values of ΔV_u for all mutants were within experimental error (Table 1). They were also within error of that obtained for WT (Vidugiris *et al.*, 1995), thus demonstrating that the substitution of proline by glycine at position 42, 47, and 117 or leucine for histidine at position 124 does not appreciably alter the volume change observed upon unfolding within the limits of the error on the recovered values.

Kinetics of the Pressure Unfolding of the H124L Proline-to-Glycine Mutants. Values of the relaxation times were obtained from analysis of the relaxation profiles in terms of a single-exponential decay time as shown for H124L+P42G in Figure 3b. Nonlinear analysis of the relaxation time as a function of pressure was carried out as for the H124L nuclease. Examination of the rates obtained from the nonlinear analysis (Table 2) indicates that the major effect on stability arises from the decrease in the rate of unfolding. Only the double-substitution mutant exhibited a large increase in the folding rate constant.

The activation volumes for folding and unfolding $\Delta V_f^{\ddagger}$ and $\Delta V_u^{\ddagger}$ obtained from the nonlinear analysis are given in Table 2. The difference between each pair of values for each mutant should equal the value of the equilibrium volume change of unfolding, ΔV_u. It can be seen from the values in Table 2 that the uncertainty in the activation volumes is quite large, and the differences are well within the error of the ΔV_u values obtained from the equilibrium data (Table 1). The average of the values obtained from the linear analysis for the six proteins/conditions presented in Table 2 is 107 mL/mol for $\Delta V_f^{\ddagger}$ and 5 mL/mol for $\Delta V_u^{\ddagger}$. These values compare reasonably well with our previous values for WT of 92 mL/mol and 20 mL/mol, respectively, for $\Delta V_f^{\ddagger}$ and $\Delta V_u^{\ddagger}$ (Vidugiris *et al.*, 1995). Thus, these mutations do not appear to have any significant effect upon the relative system volumes of the folded, unfolded, and transitions states. There is also reasonable agreement between the free energies of unfolding obtained from the equilibrium and kinetic data (Table 1).

Non-Two-State Behavior in the Low-Pressure Regions of the Kinetics Profiles. The above analyses of the equilibrium and kinetic data from nuclease H124L and its proline-to-glycine substitution mutants assumed a simple, two-state equilibrium between the folded and unfolded proteins, as was previously done with WT (Vidugiris *et al.*, 1995). Within experimental error, this model is adequate to describe the data presented here. The population of folding intermediates, such as that observed in the β-barrel region by hydrogen exchange measurements (Jacobs & Fox, 1994), would only

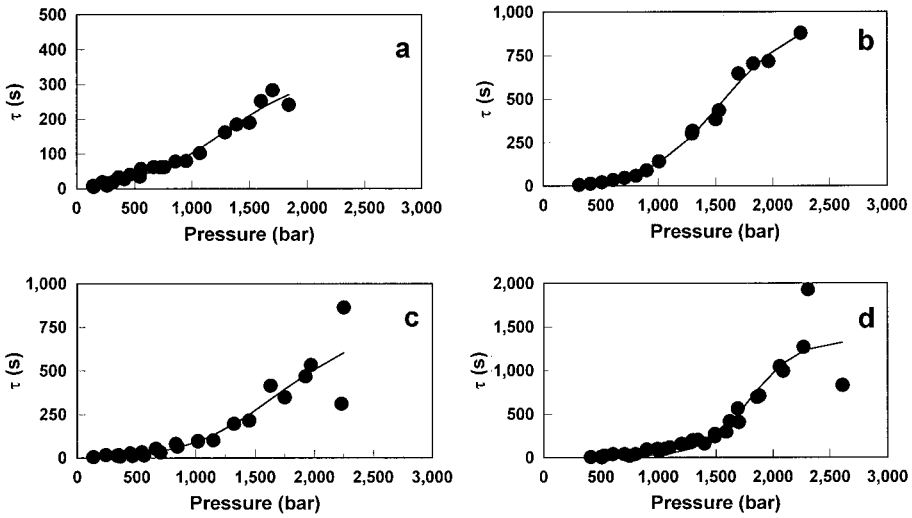

FIGURE 6: Ln τ vs pressure plots for (a) nuclease H124L+P42G, (b) nuclease H124L+P47G, (c) nuclease H124L+P117G, and (d) nuclease H124L+P47G+P117G. Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C. The rates were recovered from analysis of these data according to eqs 2 and 3 and are given in Table 2.

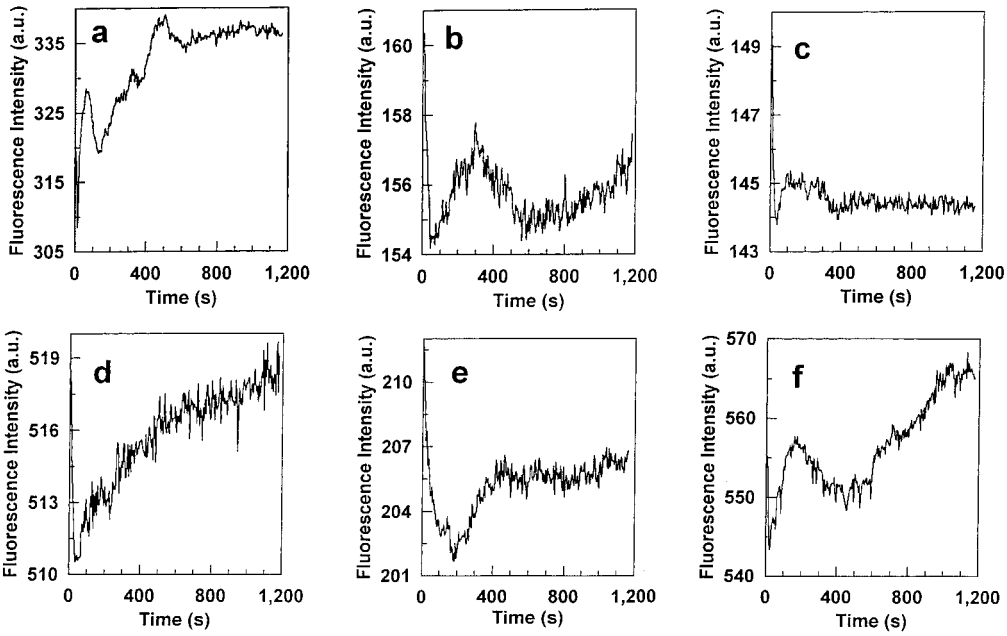

FIGURE 7: Complex, low-pressure, fluorescence intensity profiles for (a) nuclease H124 at 118 bar, (b) nuclease H124L at 86 bar, 0.75 M GuHCl, (c) nuclease H124L+P42G at 145 bar, 0.75 M GuHCl, (d) nuclease H124L+P47G at 55 bar, 0.75 M GuHCl, (e) nuclease H124L+P117G at 80 bar, 0.75 M GuHCl, and (f) nuclease H124L+P47G+P117G at 40 bar, 0.75 M GuHCl. Protein concentration was 2−5 μM in 10 mM bis-Tris buffer at pH 5.5 and at 21 °C.

be observed in the event that these steps became rate limiting to the final folding event, which is apparently not the case. This apparent two-state behavior observed at relatively high pressures breaks down, as previously noted (Vidugiris *et al.*, 1995), at low pressure, between 0 and 500 bar, depending upon the protein and conditions. In the low-pressure range, complex pressure-jump relaxation profiles are obtained. In some cases these could be fitted with two components, a short one and a very long one with a negative pre-exponential factor. In such cases the shorter component was used in the plots of relaxation time vs pressure. Otherwise, if the complexity of the profiles was too great for reasonable analysis, these points were not used in the above two-state analysis.

Since this complex kinetic behavior is not observed at higher pressures, we conclude that increased pressure disfavors the population of the intermediate(s), either through positive activation or equilibrium volume changes. Nonetheless, we were interested to know whether the low-pressure intermediate(s) were related to prolyl peptide bond isomerization in the folded state. In Figure 7a−f are shown representative examples of dozens of collected profiles of these complex pressure-jump relaxation profiles for WT and H124L as well as for the four proline-to-glycine substitution mutants. It can be seen that the complexity is present for all of the nuclease variants studied here and that, given the number of maxima and minima in these intensity profiles, more than one intermediate is populated (Eigen & de Maeyer, 1963). Of the four mutants, the P42G substitution has the greatest effect in attenuating this complexity in the profiles; this suggests that isomerization of this prolyl peptide bond may contribute to some of the kinetic complexity. The profiles for the two mutants bearing the P117G substitution

are, if anything, more complex than those of both WT and H124L.

DISCUSSION

The present pressure-jump relaxation and equilibrium studies on proline-to-glycine substitution mutants of the V8 nuclease (H124L) have demonstrated, first, that the equilibrium volume change observed for H124L is identical within error of that previously obtained for WT (Vidugiris *et al.*, 1995). Moreover, the addition of GuHCl has no significant effect on any of the volume changes. This is an interesting observation. It is believed that GuHCl destabilizes proteins by effectively competing with water for binding to the protein surface (Schellman, 1978, 1987, 1990; Timasheff, 1993). Identical volume changes for solutions of varying GuHCl concentration indicate that the volume change for binding an individual water molecule to exposed protein surface sites is similar to that of binding a molecule of GuHCl. The errors are relatively large for the determinations of the equilibrium and activation volume changes for the proline-to-glycine substitution mutants, but within this error, the values obtained for these mutants are the same as for WT and H124L. The observation of similar ΔV_u values for WT, H124L and the proline-to-glycine substitution mutants is consistent with the similarities in their folded structures (Hynes & Fox, 1991; Truckses *et al.*, submitted) and with the fact that the substitutions are in exposed loop regions of the protein.

Second, the present work demonstrates that the stabilizing effect of the proline-to-glycine substitutions in H124L arises primarily from decreases in the unfolding rate constant, although an increase in the folding rate constant is observed for the double proline-to-glycine substitution mutant. Opposite effects on the folding and unfolding rate constants upon single-site mutation have previously been observed for mutants of dihydrofolate reductase (Garvey & Matthews, 1989). However, in the *arc* repressor system, substitution of proline 8 by leucine decreases the unfolding rate constant but has no effect on the folding rate constant (Schildbach *et al.*, 1995).

In the present case, only relative differences in the stabilities of the unfolded, folded, and transition states can be deduced from these kinetic rate constants. However, they indicate that the free energy difference between the folded and the transition state increases with these proline-to-glycine substitutions, resulting in a significantly slower rate constant for unfolding these proteins. At least in the case of the double mutant, the free energy difference between the unfolded and the transition states decreases, hence increasing the folding rate constant as well. A simple explanation would be that each proline-to-glycine substitution stabilizes the folded state and destabilizes the unfolded state (Figure 8a). Such a result seems unlikely because it is thought that proline-to-glycine substitutions stabilize the unfolded state by an increase in entropy. Moreover, it is unlikely that the transition state would be unaffected by these substitutions if both the folded and unfolded state stabilities are changed. If the proline-to-glycine substitutions alter the stability of the transition state, then three alternatives need to be considered. The unfolded and transition states could be destabilized and the folded state remains unaffected (Figure 8b), or the transition state and folded states could be stabilized and the unfolded state remains unchanged (Figure 8c). We consider

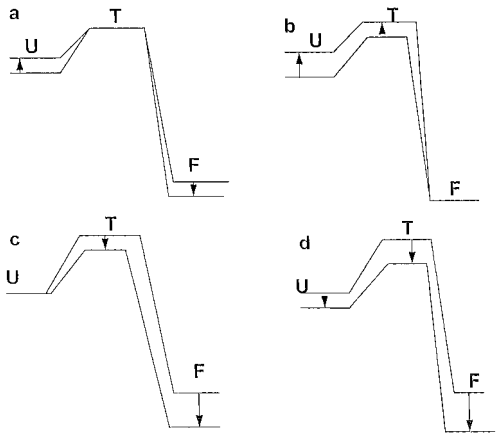

FIGURE 8: Diagram of the possible free energy effects of the proline-to-glycine substitutions. (a) Proline-to-glycine substitutions lead to stabilization of the folded state, destabilization of the unfolded state; (b) proline-to-glycine substitutions lead to destabilization of both the transition and unfolded states, with greater destabilization of the unfolded state; (c) proline-to-glycine substitutions lead to stabilization of both the transition and folded states, with greater stabilization of the folded state; and d) proline-to-glycine substitutions lead to stabilization of all three states in the rank order unfolded state < transition state < folded state, with greater stabilization of the folded state.

these possibilities unlikely because of the expected stabilization of the unfolded state by the proline-to-glycine substitutions. A final possibility (Figure 8d) is that all three states are stabilized by the mutations, with the largest effect on the folded state and the smallest on the unfolded state. An effect of the proline-to-glycine substitution mutants on all three states appears to be the most likely; this pattern is consistent with an entropy increase in the unfolded state. Moreover, by thermodynamic and volume criteria (Serrano *et al.*, 1992; Otzen *et al.*, 1994; Vidugiris *et al.*, 1995), the transition state is thought to lie close to the folded state on the reaction coordinate and should be likewise affected by the mutations, although the structural changes are very small (Truckses *et al.*, submitted).

We have previously reported that the folded states of the H124L proline-to-glycine substitution mutants exhibit very small differences in the fluorescence lifetimes compared to H124L, which, nonetheless track with their relative stabilities (Royer *et al.*, 1993). This suggests that these mutations have an effect on the dynamics of the native state in a manner consistent with our present results indicating that the proline-to-glycine substitutions stabilize the native state. In addition, the percentage intensity change upon unfolding obtained from the equilibrium unfolding profiles was smaller for each of the proline-to-glycine substitution mutants than for parent nuclease and generally follows the trend of increasing stability. The change for H124L was 60% quenching upon unfolding, while that for the double mutant was only 49%. These differences in the quenching efficiency in the unfolded state are indicative of distinct distributions of unfolded conformations for these mutants, consistent with the effects of the mutations on the stability of the unfolded state proposed here.

Finally, these studies have shown that the intermediates in nuclease unfolding that are detected because of their distinct tryptophan quantum yields (this work; Otto *et al.*, 1995; Nakano *et al.*, 1993) arise largely from processes other

than prolyl peptide bond isomerization. Mutation of proline 117 or proline 47 to glycine did not reduce the population of the low-pressure intermediates. A slow phase, not attributed to prolyl peptide bond *cis/trans* isomerization at position 117, has been observed previously in GuHCl jump experiments with nuclease (Nakano *et al.*, 1993). Substitution of proline 42 by glycine did diminish the observed kinetic complexity but did not abolish it. Substitution of proline residues in the *trp* repressor, likewise, does not eliminate or even simplify the kinetic complexity of the folding of that proteins (Mann *et al.*, 1995). Thus, for nuclease H124L at low pressures, multiple intermediates with distinct tryptophan quantum yields are populated, and the majority of these species are not attributable to *cis/trans* isomerizations of prolyl peptide bonds.

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