Hydrogen Exchange in Unligated and Ligated Staphylococcal Nuclease†

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ABSTRACT: The exchange kinetics of over 70% of the 143 backbone amide hydrogens in staphylococcal nuclease H124L (nuclease H124L), both in its unligated state and in its ternary complex with Ca^{2+} and thymidine 3′,5′-bisphosphate, have been quantified by nitrogen-15 resolved proton nuclear magnetic resonance spectroscopy. Protection factors for the slowly exchanging hydrogens in unligated nuclease H124L at 37 °C and pH* 5.5 were found to vary by over one order of magnitude. This range of protection factors has been interpreted in the framework of global and local structural fluctuations. The three most highly protected hydrogens (K24, L25, M26) map to strand 2 of the central five-stranded β-barrel. The free energy change for the opening reaction which exposes these hydrogens to the solvent ($\Delta G°_{op}$) was calculated from the exchange rates in the native and denatured states, the latter values being estimated from model peptide exchange studies [Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11,* 150–158]. Close agreement was found between $\Delta G°_{op}$ and $\Delta G°_{u}$, the free energy change of unfolding as measured by urea denaturation experiments. Exchange of these hydrogens thus appears to occur via global unfolding of the protein. One region exhibited somewhat lower protection factors: it mapped to the C-terminal portions of helix 2 and helix 3 and to part of the intervening segment. This region has been identified as a minor hydrophobic domain of nuclease [Shortle, D., Stites, W. E., & Meeker, A. K. (1990) *Biochemistry 29,* 8033–8041]. The decreased protection factors in this region appear to arise from local structural fluctuations that accompany cis ⇌ trans isomerization about the K116–P117 peptide bond. Inhibitor binding was found to produce global increases in protection factors. The baseline stability increase afforded by inhibitor binding estimated from NH exchange data ($\Delta\Delta G°_{op}$) was found again to be similar to the $\Delta\Delta G°_{u}$ value determined from urea unfolding experiments except for in the region affected by the cis ⇌ trans isomerization of the K116–P117 peptide bond. This region showed additional protection attributed to inhibitor-induced perturbation of the cis ⇌ trans equilibrium to the more exchange stable cis conformation. The results of the present study demonstrate that hydrogen exchange kinetics can be used to estimate the global stability of nuclease H124L in the presence and absence of ligands and to pinpoint local changes in structural free energy. In addition, the exchange rates from the unfolded state appear to be well described by the random-coil values. The results provide no evidence for the existence of any stable hydrogen-bonded structure in the denatured state—as reported by residues L24, K25, or M26—under the conditions of the experiment.

Despite the wealth of structural information available on proteins, the principles underlying their thermodynamic stability are not yet understood. In particular, the effects of chemical, mutational, and ligand binding modifications on the stability of the folded state are not easy to predict, even if the structural consequences of these alterations are known. One approach that offers great promise for solving this problem is NMR[1]-detected hydrogen exchange.

The measurement of hydrogen exchange kinetics provides an effective means for characterizing the structure, dynamics, and stability of proteins in solution. The most successful model for the interpretation of hydrogen exchange data in proteins was introduced by Linderstrøm-Lang (1955). In this scenario, slowly exchanging hydrogens exchange with the solvent only after becoming exposed to it as a result of structural fluctuations (local or global) around the average native conformation. This is illustrated by the following scheme in which exchange takes place from an "open" form of the protein but not from the "closed" form:

$$\text{closed} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{open} \xrightarrow{k_3} \text{exchange} \qquad (1)$$

Implicit in this model is the notion that intramolecular hydrogen bonds are broken when going from the closed to the open states, thus allowing proton transfer to take place between solvent and solute. Under conditions that favor the closed

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[1] Abbreviations: BPTI, basic pancreatic trypsin inhibitor (bovine); GuHCl, guanidine hydrochloride; $\Delta G°_{op}$, standard free energy change of the opening reaction exposing hydrogens to the solvent; $\Delta G°_{u}$, standard free energy change of the unfolding reaction; HSMQC, heteronuclear single- and multiple-quantum correlation; HSMQC–NOE, heteronuclear single- and multiple-quantum correlation with an additional mixing time for nuclear Overhauser effect buildup; *m*, slope of the plot of the standard free energy of unfolding as a function of urea concentration; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; nuclease H124L, staphylococcal nuclease (V8 variant); nuclease wt, staphylococcal nuclease (Foggi variant); pdTp, thymidine 3′,5′-bisphosphate; pH*, glass electrode reading of ^2H_2O solutions without correction for isotope effects.

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form, i.e., when $k_2 \gg k_1$, the observed rate constant (k_{obs}) is expressed by (Hvidt & Nielsen, 1966)

$$k_{obs} = k_1, \quad \text{if } k_3 \gg k_2 \tag{2}$$

$$k_{obs} = k_3\frac{k_1}{k_2}, \quad \text{if } k_2 \gg k_3 \tag{3}$$

For our conditions the slow exchange limit (eq 3) holds, and one can estimate the standard free energy change of the opening reaction from

$$\Delta G^\circ_{op} = -RT \ln\frac{k_{obs}}{k_3} \tag{4}$$

It has been shown in the case of several proteins that the exchange rates of the most highly protected hydrogens are strongly correlated with thermal stability, suggesting that these hydrogens exchange by means of a global unfolding process [Roder, 1989; Delepierre et al., 1983; Wüthrich et al., 1980; Wagner & Wüthrich, 1979; see also Englander et al. (1992)]. Hydrogens with intermediate exchange rates presumably reflect localized structural fluctuations which may or may not be linked to global unfolding (Roder, 1989; Englander & Kallenbach, 1984; Woodward et al., 1982; Delepierre et al., 1983; Wedin et al., 1982; Hvidt & Nielsen, 1966). These studies suggest that the free energy change ΔG°_u of a two-state unfolding transition can be estimated from the exchange kinetics of the most highly protected hydrogens in the native state, provided that the corresponding exchange rates from the denatured state (k_3) can be obtained.

One of the strengths of the hydrogen exchange method over equilibrium denaturation techniques lies in its capacity to pinpoint local changes in structural free energy. We have taken advantage of this property in probing the stability of staphylococcal nuclease H124L [nuclease H124L[2] in its unligated form, and in its ternary complex with Ca^{2+} and the inhibitor thymidine 3′,5′-bisphosphate (pdTp)]. Measurements of the exchange rates for over 70% of the 143 backbone amide hydrogens in unligated and ligated nuclease H124L have delineated regions that are differentially protected from exchange as a result of pdTp complexation. This has provided a basis for interpreting, in a structurally correlated fashion, the changes in stability and dynamics afforded by inhibitor binding. The present results extend previous hydrogen exchange studies of staphylococcal nuclease (Wang et al., 1990b; Torchia et al., 1989; Schecter et al., 1968).

Since it is extremely difficult to measure exchange rates from the native and denatured states under a single set of solution conditions, we have used the k_3 values obtained from the parameters of Molday et al. (1972) to estimate ΔG°_{op} from the hydrogen exchange data. These parameters, which were obtained from model peptides, have been shown to accurately reflect the exchange rates from the thermally denatured states of ribonuclease A (Robertson & Baldwin, 1991), basic pancreatic trypsin inhibitor (BPTI) (Roder et al., 1985), and hen egg-white lysozyme (Radford et al., 1992). In other cases, protection of individual hydrogens has been observed in nonnative states, particularly in acid-denatured ("molten globule") forms (Hughson et al., 1990; Jeng et al., 1990; Baum et al., 1989).

[2] Of the two natural variants of staphylococcal nuclease A, the V8 variant (H124L) was chosen for this study over the Foggi (wt) variant, because of the greater stability of the former.

In the present study, the ΔG°_{op} value for the exchange of the most highly protected hydrogens was found to agree with ΔG°_u, the free energy of unfolding determined from urea denaturation experiments. These results suggest that (1) the hydrogens that exchange the most slowly do so by a global unfolding mechanism, (2) hydrogen exchange can be used in this instance to accurately measure the free energy of unfolding, (3) the Molday parameters adequately predict the exchange kinetics of this subset of NHs from the denatured state, and (4) hydrogen exchange in the open or unfolded state is not hindered by intramolecular hydrogen bonding or other structural factors. These conclusions are strengthened by the observation that ΔG°_u and ΔG°_{op} increase by approximately equal amounts when Ca^{2+} and pdTp are present. In unligated nuclease H124L, cis/trans isomerization of the K116–P117 peptide bond appears to provide an additional exchange mechanism for a local region of the molecule. Recently, it was shown that the trans-P117 form of SNase is both less stable (Hinck, 1993) and less compact (Royer et al., 1993) than the cis-P117 form of the protein. The present results suggest that these effects can be attributed to a disruption of the packing interactions between helix 2 and helix 3 upon cis → trans isomerization.

MATERIALS AND METHODS

Exchange Experiments. Uniformly ^{15}N-labeled nuclease H124L was prepared as described in Wang et al. (1990a). ^{15}N–^1H resonances of the unligated and ligated proteins had been assigned previously at 45 °C, pH* 5.5 (Wang et al., 1990a, 1992). However, the exchange lifetimes of many of the amide hydrogens in the uncomplexed protein were too short for accurate quantitation at this temperature. We therefore reassigned both protein species at 37 °C, pH* 5.5, and subsequently carried out all hydrogen exchange experiments under these conditions.

Samples for exchange studies were prepared by dissolving the lyophilized protein in an aqueous solution (natural isotopic abundance) containing 50 mM succinate-d_4 to a final concentration of 3.0 mM. Samples of the nuclease H124L·Ca^{2+}·pdTp ternary complex contained an additional 9.0 mM pdTp (Pharmacia LKB Biotechnology) and 18 mM $CaCl_2$ (EM Science). The pH was adjusted to 5.1, and the samples were lyophilized to dryness. Immediately prior to NMR data acquisition, the samples were redissolved in 100% ^2H_2O (Cambridge Isotope Laboratories) and placed into the Bruker AM500 NMR spectrometer. Data collection was initiated after a 10-min delay to allow equilibration of the sample to the probe temperature of 37 °C. The HSMQC pulse sequence of Zuiderweg (1990) was used. Each 2D spectrum, consisting of eight repetitions of 256 t_1 increments, required 45 min to complete. This resulted in an experimental dead time of approximately 20 min. A total of 12 and 15 time points, extending out to 22 and 120 h, were collected for the unligated and ligated proteins, respectively.

Prior to Fourier transformation, the t_1 and t_2 dimensions were apodized by Gaussian and squared sine-bell functions, respectively, and zero-filled to digital resolutions of 2 points/Hz in each dimension. Proton occupancies were obtained by using the cross peak volume integration utility in the Felix software package (release 2.1, Hare Research, Inc.). The volumes of the cross peaks within each spectrum were normalized to an external reference of [^{15}N]acetylglycine, which was contained in a capillary inside the 5-mm NMR tube. Rate constants of exchange were calculated from a two-parameter nonlinear least-squares fit of the exponential

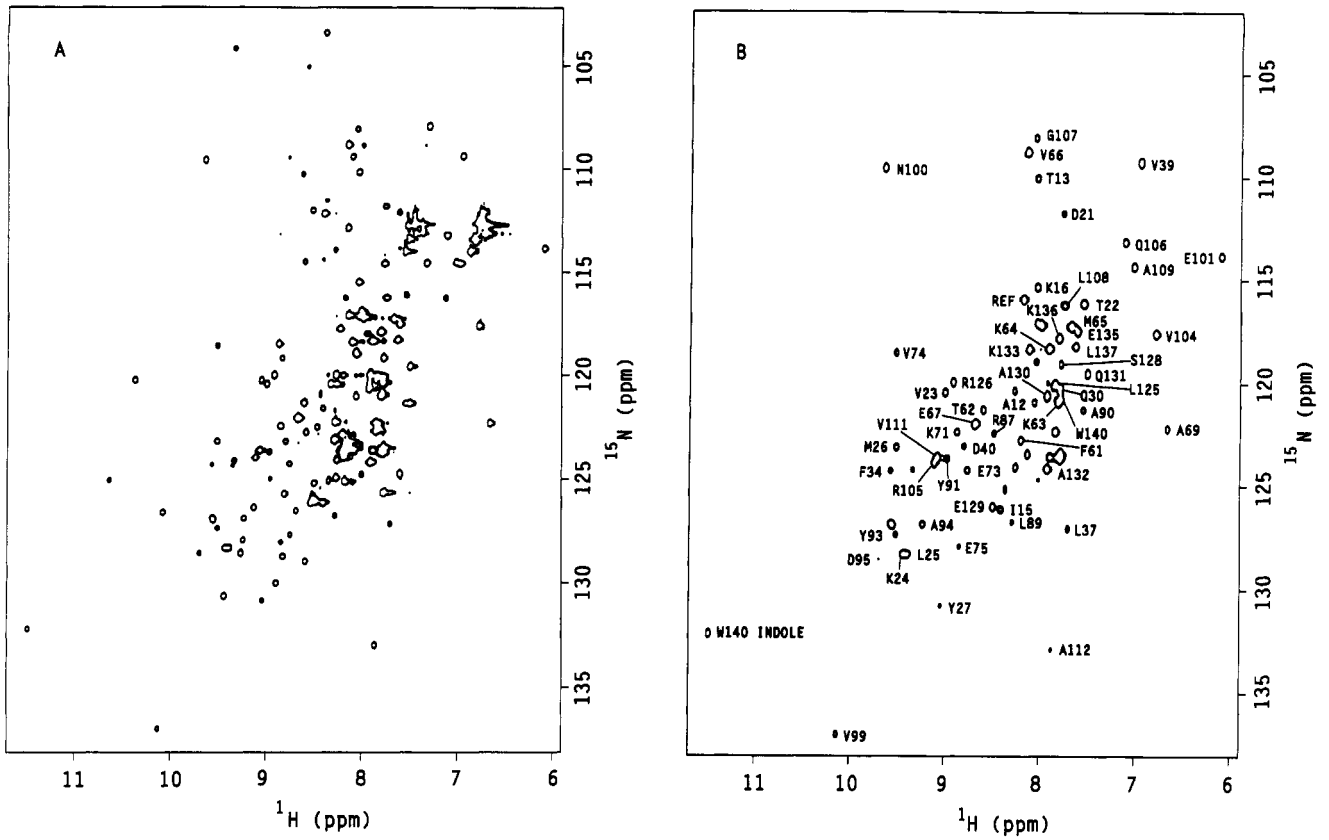

FIGURE 1: 500-MHz ^{1}H–^{15}N HSMQC spectra (Zuiderweg, 1990) of (A) unligated nuclease H124L in 10% $^{2}H_2O$ and (B) unligated nuclease H124L freshly dissolved in 100% $^{2}H_2O$. Sample and spectral conditions are described in the text. Assignments of the slowly exchanging NH cross peaks are shown in panel B.

decay curves. The exchange lifetimes reported are the inverse of these rate constants.

Exchange rates from the denatured state were calculated from

$$k_3 = k_D[^2H^+] + k_{OD}[O^2H^-] \quad (5)$$

The acid- and base-catalyzed rate constants (k_D and k_{OD}, respectively) in eq 5 were obtained from the data of Roder et al. (1985) and were adjusted for sequence effects by applying the parameters of Molday et al. (1972). Slight corrections in k_{OD} and in the intrinsic NH exchange rate for valine were employed, reflecting the more accurate pK_{D_2O} value and the 4-fold slower valine exchange rate determined by Robertson and Baldwin (1991). At 0 °C, the values of k_D and k_{OD} (without correction for side chain effects) were 3.47 min^{-1} M^{-1} and 2.77 × 10^{10} min^{-1} M^{-1}. The activation enthalpies $\Delta H^{\ddagger}_D$ = 15 kcal mol^{-1} and $\Delta H^{\ddagger}_{OD}$ = 2.6 kcal mol^{-1} (Englander & Poulsen, 1969) were used to extrapolate k_D and k_{OD} to 37 °C.

Equilibrium Unfolding Studies. Protein samples for urea unfolding experiments consisted of 3.0 μM protein in $^{2}H_2O$ buffered with 50 mM succinate-d_4 (pH* 5.5). Ternary complex samples contained an additional 1 mM pdTp and 10 mM $CaCl_2$. The stock denaturant solution consisted of 10 M urea in $^{2}H_2O$ containing 50 mM succinate, pH* 5.5. Unfolding was monitored by the decrease in fluorescence intensity of the single tryptophan residue at 325 nm with excitation at 295 nm (ISS Koala spectrofluorometer). The standard free energy change of unfolding at zero denaturant concentration (ΔG°_{op}) was obtained from linear least-squares analysis of

$$-RT \ln K_{app} = \Delta G^{\circ}_u + m[\text{urea}] \quad (6)$$

where K_{app} is the apparent equilibrium constant for unfolding.

RESULTS

As a first step in measuring exchange kinetics of the backbone hydrogens in nuclease H124L, we assigned over 80% of the NH cross peaks in ^{1}H–^{15}N spectra of both unligated and ligated nuclease H124L at 37 °C, pH* 5.5. This was done by first comparing ^{1}H–^{15}N data sets collected at 37 °C with previously assigned spectra obtained at 45 °C. Several peaks were assigned by virtue of their outyling positions in the HSMQC spectra. These served as starting points for further assignments made on the basis of NOE connectivities seen in ^{1}H–^{15}N HSMQC–NOE spectra. A table containing NH peak assignments for complexed and uncomplexed nuclease H124L at 37 °C, pH* 5.5 can be found in the supplementary material (Table S–I).

Approximately 100 NHs were resolved sufficiently well to permit analysis of their exchange rates by two-dimensional ^{1}H{^{15}N} NMR. In unligated nuclease H124L, 49 of these exhibited exchange lifetimes appreciably longer than the 20-min experimental dead time (protection factors between 10^3 and 10^4). Figure 1 contrasts the ^{1}H–^{15}N HSMQC spectrum of nuclease H124L in 90% H_2O/10% $^{2}H_2O$ with that of a sample freshly dissolved in 100% $^{2}H_2O$. The kinetic parameters along with their associated errors are summarized in the supplementary material (Table S–II). Regions of differential protection are mapped to the secondary and tertiary features of the protein in Figures 2 and 3.

Upon binding Ca^{2+} and pdTp, increases were observed both in the number of slowly exchanging amide hydrogens and in their protection factors. The C-terminal half of the sequence exhibited a greater increase in protection than did the N-terminal half (Figure 3). In order to interpret the altered pattern of stability toward exchange in the framework of global

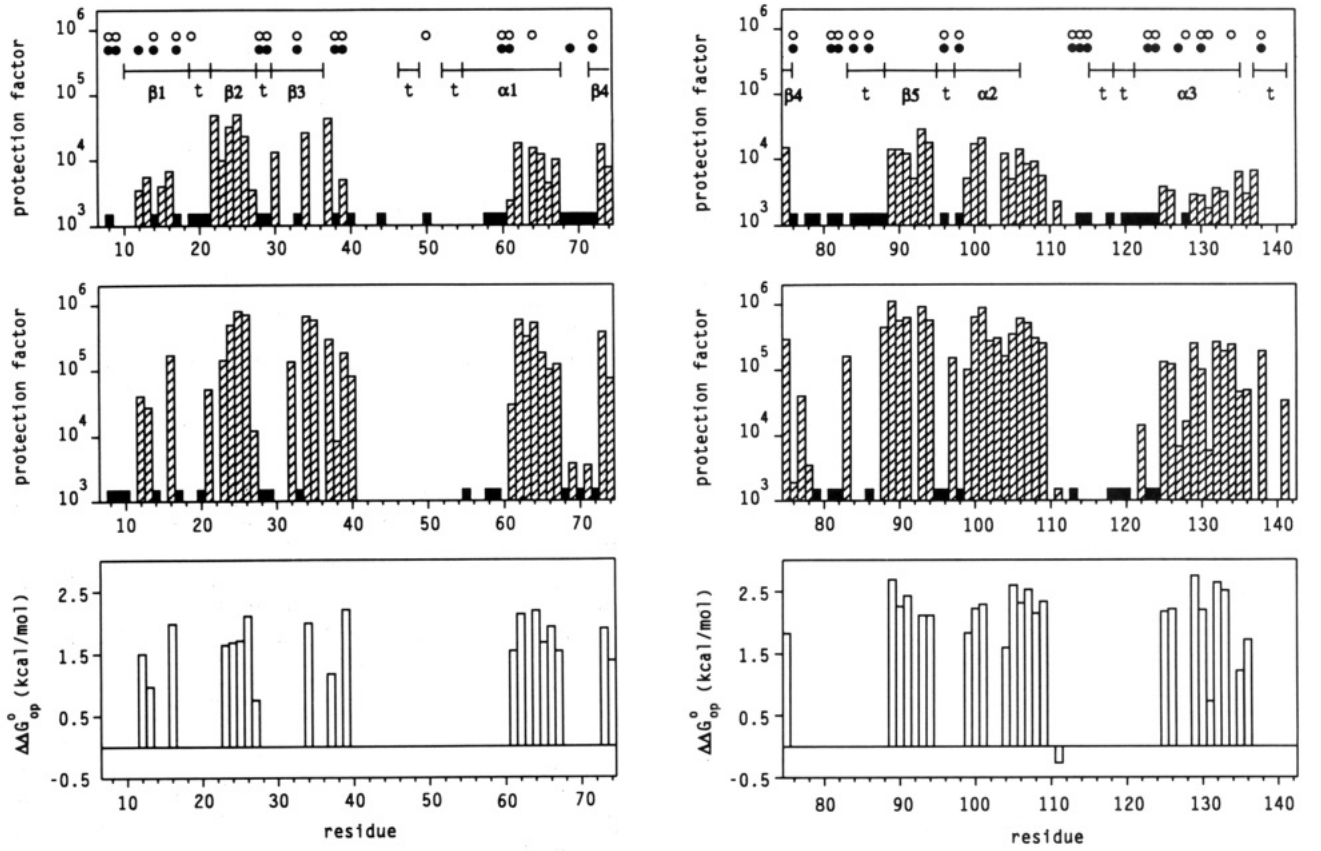

FIGURE 2: Experimental protection factors for the backbone amide NHs of unligated (top) and ligated (middle) nuclease H124L displayed by residue number. Black bars indicated that exchange was nearly complete within the experimental dead time of ~20 min. Open and closed circles indicate residues whose amide group (nitrogen or hydrogen) are exposed to solvent in the X-ray crystal structures of unligated (Hynes & Fox, 1991) and ligated (Loll & Lattman, 1989) nuclease wt, respectively. The secondary structure of nuclease wt (Hynes & Fox, 1991) is shown for reference. The ratios of protection factors for the complexed and uncomplexed enzymes, expressed as $\Delta\Delta G°_{op}$, are shown in the bottom trace.

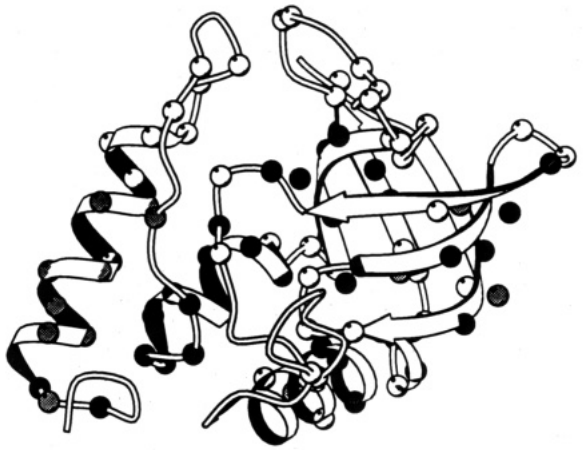

FIGURE 3: Ribbon diagram of unligated nuclease wt (Hynes & Fox, 1991) showing positions of slowly exchanging amide hydrogens coded by level of protection: (white) exchange essentially complete within the dead time of the experiment (20 min); (grey) protection factors between 1×10^3 and 5×10^3; (black) protection factors in excess of 5×10^3. Helix 2 and helix 3 appear as the central and left-most helices, respectively. The MOLSCRIPT program (Kraulis, 1991) was used to draw the structure.

and local structural fluctuations, we first examined the kinetics of the most protected residues in both unligated and ligated nuclease: K24, L25, and M26. If these residues only expose their amide hydrogens to the solvent by a global unfolding mechanism, then one can expect good agreement between $\Delta G°_u$ and $\Delta G°_{op}$. In addition, if their exchange results only from global unfolding, one should find that the increase in stability afforded by inhibitor binding is reflected equally in changes in $\Delta G°_u$ and $\Delta G°_{op}$. These aspects were tested by measuring $\Delta G°_u$ from urea unfolding experiments and by calculating $\Delta G°_{op}$ from eq 4 [with k_3 values derived from the parameters of Molday et al. (1972)]. The results are summarized in Table I. Upon complexation with pdTp and Ca^{2+}, many NHs displayed increases in protection factors beyond the baseline level established by the change in $\Delta G°_{op}$ ($\Delta\Delta G°_{op}$) for K24, L25, and M26. These are mapped to the structure of nuclease in Figure 4.

Table I: Unfolding Free Energies from Hydrogen Exchange and Urea Denaturation Experiments (^2H_2O, 50 mM Succinate, pH* 5.5, 37 °C)

method	$\Delta G°_{unligated}$ (kcal mol^{-1})	$\Delta G°_{ligated}$ (kcal mol^{-1})	$\Delta\Delta G°$ [a] (kcal mol^{-1})	$\Delta\Delta G°$ [b] (kcal mol^{-1})
NH exchange	6.44 ± 0.3	8.25 ± 0.3	1.81 ± 0.4	na[c]
urea unfolding	6.13 ± 0.4	7.36 ± 0.2	1.23 ± 0.4	2.77 ± 0.2

[a] $\Delta G°_{ligated} - \Delta G°_{unligated}$. [b] Calculated from $[urea]_{1/2}$ and m (see text). [c] Not applicable.

A comparison between the free energies calculated from hydrogen exchange and urea unfolding is only meaningful if the unfolding transition is two-state. No folding intermediates are apparent in the urea denaturation profiles (Figure 5), in agreement with previous assertions that folding of wild-type nuclease conforms to a cooperative two-state process (Shortle, 1985; Anfinsen et al., 1971).

DISCUSSION

Correlation of Protection Factors and Structural Features of Nuclease. In order to obtain information on the dynamic

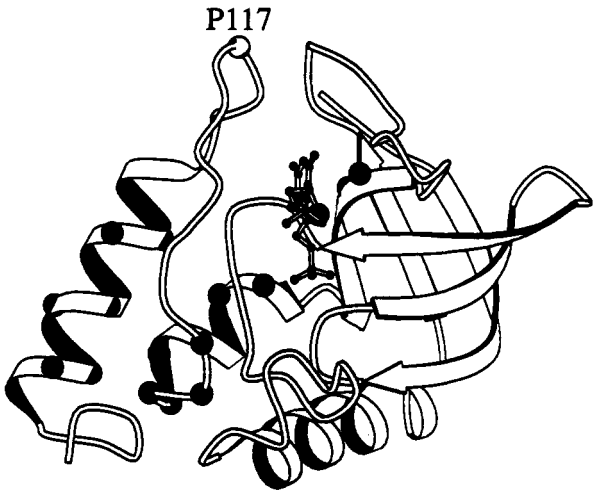

FIGURE 4: MOLSCRIPT diagram (Kraulis, 1991) of the nuclease wt ternary complex (Loll & Lattman, 1989) showing locations (black spheres) of residues with $\Delta\Delta G°_{op}$ values significantly greater than $\Delta\Delta G°_{u}$ values calculated from the protection factors of residues 24–26 in the presence and absence of inhibitor (pdTp and Ca^{2+}). The positions of proline 117 and the bound pdTp molecule are shown.

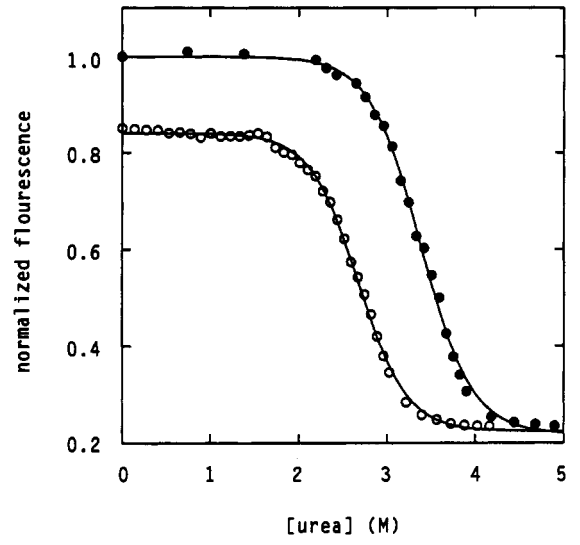

FIGURE 5: Urea denaturation profiles of nuclease H124L (^2H_2O, 50 mM succinate, pH* 5.5, 37 °C) in the absence (○) and presence (●) of inhibitor (pdTp and Ca^{2+}).

processes involved in solvent exposure and exchange, it is of interest to map patterns of differential protection to the secondary and tertiary features of the protein. Almost all of the NH groups that are solvent-exposed in the X-ray crystal structure of uncomplexed nuclease (Hynes & Fox, 1991) exchange within the experimental dead time. This is an expected result, since the mechanism of base-catalyzed exchange in proteins (which far exceeds the acid-catalyzed rate at pH* 5.5; see eq 5) is thought to proceed by direct removal of the amide hydrogen by OH^- (Perrin, 1989). Of the five solvent-exposed NHs that exhibit elevated protection factors in unligated nuclease, four (F61, K64, A130, Q131) can be rationalized in terms of their participation in hydrogen bonds within α-helices. These results suggest that the intramolecular hydrogen-bonding network present in protein α-helices can account for up to a 10^3-fold retardation in NH exchange rates, particularly in the helix center where fraying effects are minimized.

The only other slowly exchanging solvent-exposed amide NH group in unligated nuclease belongs to V39. This result is particularly intriguing since in the crystal structure the V39 amide hydrogen does not participate in either an intramolecular hydrogen bond or in any secondary structural element. Inspection of the crystal structures of nuclease wt reveals that it is not possible, by simple rotation of χ angles, to bring a nearby side chain to within hydrogen-bonding distance of the V39 NH. One possibility is that these solvent-exposed NH groups become protected as a result of protein aggregation. Although dimerization has been noted in unligated nuclease, it is decreased by the addition of Ca^{2+} and pdTp (Alexandrescu et al., 1989). The fact that the addition of inhibitor actually *increases* the protection factors for many of these hydrogens (including V39; Figure 2) argues against protection by aggregation. In a study of the most rapidly exchanging amide hydrogens in BPTI, Tüchsen and Woodward (1985) observed that many non-hydrogen-bonded surface NHs exchanged up to 2 orders of magnitude more slowly than NH groups in model peptides. However, this was attributed to the O-protonation mechanism of acid-catalyzed exchange, which only becomes significant below pH 3 (eq 5). The slowly exchanging, solvent-exposed, non-hydrogen-bonded NHs of K55 observed in turkey ovomucoid third domain at pH* 4.2 (Robertson et al., 1988; Rhyu & Markley, 1988) and C64 in hen egg-white lysozyme at pH 7.5 (Radford et al., 1992) are the closest precedents to V39 of staphylococcal nuclease.

In general, regions of high and low protection correlate well with the presence and absence of α/β secondary structure, respectively (Figure 2). The numerous turns and extended segments rapidly exchange their NHs with the solvent. Two notable exceptions are the stretches of NHs from G107 to V111 and from K136 to S141. These segments, together with the C-terminal portions of helices 2 and 3, constitute an interesting region of the protein. In the X-ray crystal structure, these sequentially discontiguous segments are found to be closely packed. Many intraresidue NOEs (mainly between hydrophobic side chains) link these segments together (J. Wang, unpublished results), confirming that this section remains closely associated in solution and contains extensive hydrophobic contacts. On examining the guanidine hydrochloride denaturation profiles of a large number of alanine, valine, and glycine mutants, Shortle and co-workers (1990) concluded that mutations that decrease $d(\Delta G)/d[GuHCl]$ map to this minor hydrophobic domain. It is possible that the compact nature of this region increases the protection factors of residues 107–111 and 136–141 beyond those normally encountered in extended segments.

Although the majority of NHs in the α-helices of unligated nuclease H124L exhibited protection factors exceeding 10^3, the first few N-terminal residues of all three helices had much faster exchange kinetics. This pattern has been observed in helices of other proteins (Radford et al., 1992; Robertson, 1988; Wand & Englander, 1986; Kuwajima & Baldwin, 1983). Helix 3, the longest of the three, exemplifies the diversity in environments and protection factors of individual α-helical NHs. At the N-terminus, H121, E122, Q123, and L124 exchange within the dead time, although only Q123 and L124 have solvent-exposed NH groups.[3] By contrast, the amide nitrogens of A130 and Q131 are exposed, yet possess protection factors greater than 10^3. The NHs of the first four residues in an α-helix lack amide hydrogen bond acceptors within the helix. The NHs of residues 121–124 are not hydrogen bonded in the X-ray crystal structures of nuclease, and this probably accounts for their lack of protection.

[3] The structure surrounding L124 is uncertain; the closest available X-ray crystal structure is for the Foggi variant of the enzyme which contains His at this position (Loll & Lattman, 1989).

The plot of protection factors as a function of sequence (Figure 2) illustrates an important point: the residues in the C-terminal half of unligated nuclease H124L generally exhibit faster exchange kinetics than do the residues in the N-terminal half. Thus, there appears to be a local mechanism, in addition to global unfolding, contributing to the exchange of the C-terminal residues.

Correlation of Exchange with Stability. Inspection of the ribbon diagram of unligated nuclease (Figure 3) reveals that most of the slowly exchanging hydrogens map to the central β-barrel and to the aforementioned region comprising helices 2 and 3 and part of the extended segment that connects them. Among these hydrogens, the most highly protected (K24, L25, M26) are found buried deeply in strand 2 of the β-barrel. In order to test whether these "core" residues exchange by a process comparable to global unfolding, $\Delta G°_{op}$ was compared with $\Delta G°_{u}$, the free energy change of unfolding determined from urea denaturation experiments carried out in ^2H_2O and at the same temperature and pH as the hydrogen exchange experiments. With k_3 calculated from the parameters of Molday et al. (1972), the average $\Delta G°_{op}$ value was 6.4 ± 0.3 kcal mol^{-1} (Table I). The error incorporates an estimated 50% uncertainty in k_3 [*cf.*, the maximum error of 40% in unfolded BPTI (Roder et al., 1985)] and was determined by standard propagation of errors treatment of the k_{obs} values (and their associated uncertainties) averaged for residues 24–26. Within error, $\Delta G°_{op}$ agrees with the experimental $\Delta G°_{u}$ value of 6.13 ± 0.4 kcal mol^{-1}.

If, as these data suggest, residues 24–26 exchange by a global unfolding mechanism, then one would expect the increase in stability afforded by pdTp binding to be reflected by equal increases in $\Delta G°_{u}$ and $\Delta G°_{op}$. The use of equilibrium unfolding curves to determine differences in stability between proteins has been discussed by Pace et al. (1989). These authors proposed that, instead of estimating $\Delta\Delta G°$ from the difference of individual $\Delta G°$ values, $\Delta\Delta G°$ can be estimated more accurately for certain proteins by taking the difference between the $[\text{urea}]_{1/2}$ values (0.70 ± 0.05 M in the present study) and multiplying this by the average of the m values (3.92 ± 0.1 kcal mol^{-1} M^{-1} in the present case). The rationale for this approach is to circumvent the potentially larger error associated with extrapolating $\Delta G°$ beyond the unfolding transition to zero denaturant concentration. As seen in Table I, the stability increase upon Ca^{2+} and pdTp binding calculated by the two approaches differed by 1.5 kcal mol^{-1}. We note that the values reported in Table I are similar to those obtained in a previous study of nuclease wt (Sugawara et al., 1991). We interpret the fact that $\Delta\Delta G°$ derived from the hydrogen exchange data lies between these values to mean that global unfolding is the likely mechanism for NH exchange at residues 24–26.

Proceedings on this hypothesis, one can now look for regions whose protection factors are raised above and beyond the increment predicted by the baseline level of pdTp-induced stabilization (1.8 ± 0.4 kcal mol^{-1}). Residues that are stabilized toward exchange to a significantly greater extent (≥0.4 kcal mol^{-1}) than this baseline value are indicated in Figure 4. All of these residues are found either near the pdTp binding site or in the aforementioned domain consisting of helix 2, helix 3, and part of the interconnecting segment. The large increase in protection results from the relatively faster exchange rates of these regions in the uncomplexed enzyme rather than from abnormally high protection factors in the ternary complex. The addition of Ca^{2+} and pdTp simply raises the protection factors of these residues to match those of the rest of the protein. Thus, a local opening mechanism, eliminated by Ca^{2+} and pdTp binding, appears to be present in the unligated enzyme which lowers the protection factors of these NHs.

It has been known for some time that unligated staphylococcal nuclease exists in solution as a mixture of four interconverting native states, linked by cis ⇌ trans isomerism about two X–Pro peptide bonds: K116–P117 (Evans et al., 1987) and H46–P47 (Loh et al., 1991). The approximate cis:trans ratios at equilibrium are 10:1 and 1:10, respectively. The known properties of the cis ⇌ trans equilibrium about the K116–P117 peptide bond are consistent with it being the additional mechanism for facilitating exchange in the aforementioned domain of unligated nuclease. First, the protein containing trans-P117 is somewhat more expanded than the cis-P117 isomeric form. This was shown by pressure denaturation studies of mutants with altered K116–P117 cis:trans ratios (Royer et al., 1993). Second, the position of the cis ⇌ trans equilibrium is heavily dependent on interactions between the residues flanking P117 and the residues on the opposing loop (Figure 4). In the most dramatic example, disruption of the hydrogen bonds between D77 and T120 (at the extreme N-terminus of helix 3) by the D77A mutation results in a shift in the cis ⇌ trans equilibrium to ≥98% trans (Hinck, 1993). Finally, addition of Ca^{2+} and pdTp to nuclease H124L has been shown to lead to greater than a 98% population of cis isomers (Alexandrescu et al., 1989).

These observations suggest that the dynamic cis- to trans-P117 isomerization, which occurs under the conditions of the NH exchange experiments at a rate of roughly 0.1 s^{-1}, results in a more "open" form of the protein and that this opening may be coupled to loss of interactions near the N-terminus of helix 3. The extent to which such disruptions might affect the packing of helix 3 with helix 2 and the interconnecting segment (Figure 4) remains the subject of speculation. However, the fact that binding of Ca^{2+} and pdTp simultaneously abolishes the trans-P117 state and eliminates the inordinately low protection factors for this region suggests that the two phenomena are linked.

NH Exchange in Denatured Nuclease H124L. A topic of considerable interest is whether denatured proteins contain residual structure. This issue takes on special significance for staphylococcal nuclease, in light of experimental evidence supporting a non-random-coil denatured state (Shortle et al., 1990; Shortle & Meeker, 1989; Shortle, 1989). However, we find no indication that K24, L25, and M26, the most slowly exchanging residues in native nuclease, are protected to a significant extent in the denatured state. The reasoning behind this conclusion is as follows. The hydrogen exchange and urea unfolding methods yielded similar estimates for the pdTp-induced stability increase, lending credence to the notion that exchange of these residues is controlled by global unfolding. For both unligated and ligated nuclease, general agreement was found between $\Delta G°_{u}$, obtained from urea unfolding curves, and $\Delta G°_{op}$, determined from NH exchange kinetic studies. Since the latter values incorporated the parameters of Molday et al. (1972), it appears that exchange of these residues in denatured nuclease H124L is adequately predicted by model peptide data. The present results, however, hold only for this region and do not rule out the participation of other residues in hydrogen-bonded structures in denatured staphylococcal nuclease.

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SUPPLEMENTARY MATERIAL AVAILABLE

Two tables listing NH peak assignments and exchange lifetimes and protection factors for backbone amide hydrogens of nuclease H124L (12 pages). Ordering information is given on any current masthead page.

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