One of the primary goals of modern protein chemistry is to understand the structural basis for the thermodynamic stability of proteins. For most proteins, only two states, the native and denatured states, are populated to a significant extent at equilibrium and it is the modest free energy difference between these states that determines the stability of a protein. The origin of the disagreement between predicted and observed pH minima is unknown but may be the nature of the residual structure responsible for this spectrum. The nature of the residual structure responsible for this spectrum is not known. Our results show that it is not stable helix formation by the three α-helices of native ribonuclease A, which would give measurable protection against amide proton exchange.

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; COSY, two-dimensional chemical shift correlation spectroscopy; FID, free induction decay; FMOCS, 5-fluorouracil/methoxy-carbonyl; FPLC, fast protein liquid chromatography; GuHCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; PDLA, poly(D,L-lactic acid); pH*, glass electrode pH reading of D2O solutions without correction for isotope effects; pKd,D, pK of D2O; RELAY, relayed coherence transfer spectroscopy; RNase A, bovine pancreatic ribonuclease A (EC 3.1.27); [3H]TSP, sodium 3-(trimethylsilyl)[2,2,3,3-2H4]propionate.

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detailed structural data is a serious shortcoming in current studies of protein stability, particularly in light of the accumulating evidence suggesting that, contrary to earlier assumptions, denatured states do not always behave like random-coil polypeptides (Tanford, 1968; Grikio et al., 1988; Privalov et al., 1989; Dill & Shortle, 1991).

The present study is an attempt to detect structure in thermally denatured RNase A using hydrogen exchange. There are both theoretical and experimental arguments for the presence of nonrandom structure in thermally denatured proteins. A true random coil will only be obtained in a good solvent, one in which all chemical groups in a protein are well solvated (Tanford, 1968). In this respect, water is not a good solvent and it is likely that interactions, such as those involving hydrophobic groups, will take place in the thermally denatured state. Shakhnovich and Finkelstein (1989; Finkelstein & Shakhnovich, 1989) argue that disruption of tight packing interactions is all that is necessary for denaturation and that this does not necessarily lead to unfolding to a random coil.

Experimental evidence for nonrandom structure in thermally denatured RNase A comes primarily from spectroscopic studies (Aune et al., 1967; Matthews & Westmoreland, 1975; Howarth, 1979; Labhardt, 1982; Privalov et al., 1989). Circular dichroism data, in particular, suggest that there may be a significant amount of structure in thermally denatured RNase A (Labhardt, 1982). In contrast, the calorimetric data of Privalov and co-workers indicate that the hydrophobic groups in thermally denatured RNase A are largely solvated (Privalov et al., 1989). This would argue against close contacts in the clustering of hydrophobic residues in the denatured protein. The reported uncertainty in their $C_p$ determinations ($\pm 0.04$ J K$^{-1}$ g$^{-1}$) results, however, in an uncertainty in the $\Delta C_p$ of unfolding ($\pm 0.06$ J K$^{-1}$ g$^{-1}$), a measure of the solvent-inaccessible hydrophobic surface area in the protein core, that is approximately 15% of the measured $\Delta C_p$ value. The calorimetric data thus cannot be used to rule out the presence of a small, and perhaps energetically significant, amount of nonrandom structure in thermally denatured RNase A.

The possibilities for structure in thermally denatured protein are best exemplified by work on the compact intermediate form, which has been observed in a number of globular proteins at acid pH or at intermediate concentrations of denaturant. The distinguishing features of the compact intermediate form are near-native compactness, high levels of secondary structure, and the absence of specific tertiary interactions (Ptitsyn, 1987; Kuwajima, 1989; Ebwank & Creighton, 1991; Hughson et al., 1991). An important observation, that bears on the present study, is that the thermally denatured forms of some of these proteins appear to be similar to the compact intermediate forms observed at lower temperatures (Ptitsyn, 1987; Kuwajima, 1989).

One of the most promising developments in studies of compact intermediate forms is the use of hydrogen exchange and NMR spectroscopy to obtain information about the involvement of specific amino acid residues in structure (Baum et al., 1989; Hughson et al., 1990; Jeng et al., 1990). The general features of these hydrogen-exchange experiments are derived from studies of exchange in thermally denatured BPTI (Roder et al., 1985). The technique takes advantage of two features of the chemistry of amide groups in the peptide backbone: (1) Amide protons (NHs) are labile to exchange with solvent and (2) NH exchange can be slowed by many orders of magnitude when the NH is involved in hydrogen-bonded structure. For many of the proteins studied to date, a large fraction of the backbone NHs show enough protection to be observed in a $^1$H$-^1$H COSY experiment and it is these NHs that serve as probes in studies of the compact intermediate form. In a typical experiment, protein dissolved in D$_2$O is briefly exposed to conditions favoring the compact intermediate form and then returned to native conditions to quench further NH exchange. Proton occupancies at specific NHs then are assessed by measuring the intensities of assigned cross-peaks in the $^1$H$-^1$H COSY spectrum. This experimental approach has been adopted in the present studies of thermally denatured RNase A.

A further impetus for these studies is the need to check the rules established by Molday et al. (1972) for calculation of the "intrinsic" exchange rates for peptide NHs. Interpretation of the results of hydrogen-exchange studies in proteins depends on knowledge of the exchange behavior for a disordered polypeptide. The contributions of many neighboring side chains toward exchange behavior have been determined using small peptides, but not all side-chain groups have been checked directly. Moreover, there has been only one effort to check the validity of these calculations in a denatured protein; exchange was measured at eight NHs in thermally denatured BPTI (Roder et al., 1985). Results of exchange studies with thermally denatured RNase A provide data for 36 additional NHs.

Materials and Methods

Bovine pancreatic RNase A (type XIIA, Sigma) was further purified by ion-exchange column chromatography (Garel, 1976). Reagent-grade buffers and salts were obtained from J. T. Baker, Inc. D$_2$O was obtained from Cambridge Isotope Laboratories, DCI and NaOD were from MSD Isotopes, and $[^{2}H]TSP$ was from ICN Biochemicals. Peptides were synthesized and purified as described previously (Strehlow et al., 1991). 1D $^1$H NMR spectroscopy was used to check peptide purity, which was >95%, and amino acid composition, which coincided with that of the desired peptides.

$CD$ Spectropolarimetry. CD data were acquired on an Aviv 60 DS spectropolarimeter equipped with a Hewlett-Packard 89100A temperature controller. A 10-mm path length cuvette was used. Ellipticity at 222 nm is reported as mean molar residue ellipticity ($[\theta]_{222}$, deg cm$^2$ dmol$^{-1}$) and was calibrated with (+)-10-camphorsulfonic acid (Chen & Yang, 1977). CD spectra were the average of three scans obtained by collecting data at 0.25-nm intervals from 200 to 190 nm. To follow the thermal denaturation of RNase A, data at 222 nm were collected at 2°C intervals from 0 to 80°C. CD samples were prepared by diluting aqueous stock solutions of protein with buffer containing 1 mM potassium acetate and 10 mM KF (final concentrations). The pH was adjusted to 4.0 using dilute KOH and HCl. Protein concentration was determined by measuring absorbance at 275 nm of the RNase A stock solution diluted 10-fold into 6 M guanidine hydrochloride (final concentration). An extinction coefficient of 8700 M$^{-1}$ cm$^{-1}$ (6 tyrosines $\times$ 1450) was used (Brandts & Kaplan, 1973). Peptide samples were prepared by diluting aqueous stock solutions with buffers containing 1 mM potassium acetate, 1 mM potassium phosphate, and 10 mM KF (final concentrations). pH was adjusted using KOH or HCl. Stock peptide concentration was determined as described for protein; there is a single tyrosine in each of the two peptides studied.

Quenched-Flow Experiments. The quenched-flow apparatus used in these experiments has been described previously (Udgaonkar & Baldwin, 1988). The temperature of the syringe blocks, delay and incubation lines, and stock solutions was maintained at 65°C ($\pm$2°C). The 5 mM solutions of RNase A were prepared in 50 mM sodium citrate buffer.
adjusted to the desired pH at room temperature; the difference in pH values measured at 20 and 65 °C (2-point calibrations at both temperatures) was no greater than 0.03 pH unit in all cases. Protein was unfolded at 65 °C for at least 10 min prior to the mixing experiments. The sample (0.56 mL) was drawn into a syringe in the quenched-flow device where it was equilibrated for at least 3 min. To initiate exchange, the protein solution was rapidly diluted 10.5-fold into buffered D2O at the same pH* and temperature. After a predetermined time, further exchange was quenched by rapidly ejecting the sample into liquid N2. In the continuous-flow mode, which was used for reaction times of less than 1.5 s, exchange times were determined by the speed with which the reacting syringes were emptied, which was followed on an oscilloscope, and the volume of the mixer and delay-line tubing prior to the liquid N2 quench. For longer time intervals, an incubation line was used whose volume was sufficient to hold the entire exchanging sample. There were at least six time points for each experiment, with exchange times ranging from 0.1 to 900 s; consecutive time points typically differed by factors of approximately 3. Following the liquid N2 quench, samples were thawed on ice so that folding would go to completion before any significant further exchange could occur; the time constant for the slow refolding phase of RNase A is about 150 s at 10 °C, pH 3.5 (Schmid & Blaschek, 1984) while the time constant for exchange of an alanine NH is approximately 15,000 s under the same conditions (Englander & Poulsen, 1969). Samples were adjusted to pH* 3.5 with dilute DCI or NaOD and concentrated to 0.5 mL for NMR spectroscopy using a Centricon-10 filter apparatus (Amicon).

1H NMR spectroscopy. 1H NMR spectra of peptides were collected using a 5000-Hz spectral width, 64 scans, a 60° pulse width, and a 5-ms recycle delay. Data were collected in 4096 complex data points. The temperature was 25 °C.

Absolute value mode 1H-H COSY data (Aue et al., 1976) were obtained on a General Electric CN-Omega 500 operating at a proton frequency of 500.13 MHz; 256 blocks of t1 data, each consisting of 32 summed scans, were collected in approximately 160 min. The recycle delay was 1 s, the spectral width was 5814 Hz, and 1024 real data points were acquired in t2. The temperature was maintained at 30 °C. Absolute value mode RELAY data (Wagner, 1983) for peptides were collected for samples containing approximately 2 mM peptide in 90% H2O/10% D2O buffered with 3 mM sodium citrate. Phase cycle was as described by Bax and Drobny (1985). Experiments consisted of 256 blocks of t2 values, each of which was the sum of at least 128 transients. The spectral width was 5000 Hz, 1024 complex data points were collected, the recycle delay was 1 s, and the total mixing time was 40 ms. The solvent resonance was suppressed by preirradiation.

Determination of Exchange Rates. Data were processed using FTNMR (kindly provided by Hare Research, Inc.) running on a VAX 8550 computer. Prior to Fourier transformation, t2 and t1 data were multiplied by squared sine bells that were phase-shifted by 5° and 10°, respectively. The t1 data were zero-filled twice. Volume integrals were measured using the LOCATE routine in FTNMR; peak volumes were normalized to the volume of the nonexchanging aromatic cross-peak of tyrosine 25.

For exchange from peptides, NH resonance intensities were normalized to the sum of the intensities of the nonexchanging aromatic resonances.

Peak volumes, for protein data, and peak intensities, for peptide data, were fit to an exponential function, \( A = A_0 \exp(-kt) \), using a nonlinear least-squares fitting program

![FIGURE 1: Thermal denaturation of RNase A monitored by circular dichroism spectropolarimetry. \( \theta_{222} \) is the mean residue ellipticity at 222 nm. Experimental details are described under Materials and Methods. Note that half of the signal remains after thermal denaturation.](image)

(Fairman et al., 1989). All fits were performed using at least four experimental time points.

Calculation of Predicted NH-Exchange Rates. Predicted exchange rates for completely unfolded RNase A were calculated using eq 1.

\[
k_{\text{pred}} = k_D[^1H^+] + k_{OD}[O^2H^-]
\]

The rate constants for acid- and base-catalyzed exchange, \( k_D \) and \( k_{OD} \), respectively, are based on data for exchange from PDLa (Englander & Poulsen, 1969) with corrections for the effects of side chains on exchange (Molday et al., 1972; Englander et al., 1979). We have used a modified value of the second-order rate constant for base-catalyzed exchange, \( k_{OD} \), following the arguments presented by Roder et al. (1985). Our reasoning for this change is as follows.

The data of Englander and Poulsen (1969) are the most frequently used source of values for \( k_D \), 5.9 \times 10^{-2} M^{-1} s^{-1} \text{ at } 0 °C, and \( k_{OD} \), 5.9 \times 10^{-7} M^{-1} s^{-1} \text{ at } 0 °C. Roder and co-workers (1985) adjusted the latter value, which was determined in H2O, for the isotope effect on the ion product of D2O, but the value used by Roder et al., \( k_{OD} \), 15.875, was calculated using an approximate form of the equation of Covington et al. (1966). If the complete equation is used, then the calculated \( k_{OD} \) value is 15.962 and the resulting \( k_{OD} \) value is 5.4 \times 10^{9} M^{-1} s^{-1}. Activation energies used for calculation of exchange rates at temperatures other than 0 °C are those used by Roder et al. (1985).

RESULTS

To confirm that, under the conditions of the hydrogen-exchange experiments, there is little or no native protein, CD spectropolarimetry was used to monitor the thermal denaturation of RNase A (Figure 1). At pH 4.0 and 65 °C, greater than 95% of the RNase A in solution is denatured. The thermal stability of RNase A decreases with decreasing pH (Hermans & Scheraga, 1961) and no exchange experiments are performed at pH values greater than 3.8, so RNase A in these experiments is essentially completely denatured at 65 °C. There is evidence suggesting that the thermal stability of RNase A might be increased at high concentrations (Tsong et al., 1970) and when dissolved in D2O (Talluri & Scheraga, 1990). When compared to the CD results, there appears to be an increase of about 2 °C in \( T_m \) (57 vs 55 °C) when the thermal denaturation of RNase A in D2O at pH* 4.0 is fol-
lowed by $^1$H NMR spectroscopy (data not shown). But thermal denaturation curves for 0.5 and 5 mM protein, as followed by $^1$H NMR spectroscopy, are superimposable, and most importantly, more than 95% of the protein is denatured at 65 °C under all conditions studied.

The probes for study of NH exchange in thermally denatured RNase A are the subset of peptide backbone NHs that show protection from exchange in the native protein. Of the 119 backbone NHs in RNase A, nearly 60 show significant exchange protection at pH* 3.5 and 30 °C, as measured by NMR spectroscopy (Robertson et al., 1989; Rico et al., 1989; Robertson and Baldwin, unpublished results). Of these, 36 NHs are stable enough and show cross-peak intensities sufficient to allow determination of their exchange rates with the quenched-flow technique described under Materials and Methods.

Amide exchange in thermally denatured RNase A was measured at five different pH* values in order to describe the pH dependence of exchange. The pH dependences for exchange at eight NHs in thermally denatured RNase A are presented in Figure 2. Plots for the other 28 sites are available as supplementary material.

We have fitted the pH dependences using the procedure described by Roder et al. (1985) to derive parameters, pHmin and kmin, describing the pH dependence of exchange.

$$k_{ex} = \frac{k_{min}}{2}(10^{pH-pH_{min}} + 10^{pH_{min}-pH})$$  \hspace{1cm} (2)

The assumptions implicit in this procedure are that (1) the acid- and base-catalyzed exchange are first order in catalyst and (2) there is no water-catalyzed exchange (Roder et al., 1985). During the fitting procedure, the observed rate constants were weighted by the reciprocal of their magnitude, which was normalized to the slowest exchange rate at that site, so as not to favor inordinately the points on the limbs of the pH-dependence curves. The results of least-squares fitting of the data for 36 NHs to eq 2 are presented in Table I. Representative data and the best-fit curves are shown in Figure 2.

There is good agreement between most of the observed and predicted k_{min} values; they usually differ by less than a factor of 2. Among the exceptions are eight valine NHs, where the average protection factor, k_{pred}/k_{obs}, is 3.7 ± 1.2 (vs 1.6 ± 1.4 for all sites). The only other clear pattern in k_{min} is observed at NHs bounded in sequence by at least one positively charged amino acid. Exchange at these sites is always approximately 2-fold faster than predicted; the average protection factor is 0.44 ± 0.08 (n = 6). The NHs of Cys 84 (Figure 2) and Arg 85 (data not shown) show anomalous pH dependences with slopes that are less than 1 and no observable minima down to pH* 1.5.

The predicted exchange behavior for valine NHs has been assumed to be identical to that of alanine NHs (Molday et al., 1972), but this has not been confirmed experimentally. We therefore decided to measure NH exchange in small valine-containing peptides. Exchange was measured in the peptide acetyl-PYVPNH-amide (P114-119), which corresponds to residues 114-119 in RNase A. The valine NHs were identified as such using RELAY data, but they have not been assigned
Hydrogen Exchange in Denatured Ribonuclease A

unambiguously to specific residues. Tentative residue-specific assignments are based on NH exchange behavior (Figure 3a) and chemical shift differences between major and minor peaks which probably result from cis-trans isomerism about one or both of the X-Pro bonds. The experiments were carried out at 25 °C, which is the highest temperature at which exchange can be followed by our methods. Comparison of experimental results with the predicted pH* dependences for the two valline NHs shows that exchange of valline NHs in P114-119 is at least 4-fold slower than predicted (Figure 3a).

It is unlikely that P114-119 will form stable structure at 25 °C, but there are some data to suggest that related peptides may contain nonrandom structure at room temperature (Haas et al., 1987). The Tyr and His NHs in P114-119 have exchange rates that agree well with the predicted rates (data not shown), evidence that if there is nonrandom structure, it is not resulting in slowed exchange at these NHs. CD data for this peptide, however, do not provide unambiguous proof that the peptide is indeed a random coil (data not shown). Therefore, as a further control, NH exchange was measured in acetyl-YKAAVAK-amide, where alanine and valline NH exchange can be monitored simultaneously; only the valline NH shows exchange that is slower than predicted (Figure 3b).

The pH minima for exchange from thermally denatured RNase A tend to fall at lower pH* values than predicted; the average ΔpH min value (see footnote to Table I) is -0.16 ± 0.20. Many NHs of hydrophobic residues show ΔpH min that are more negative than -0.15, but the significance of this is not clear. The pH minimum for exchange of valline and alanine NHs from acetyl-YKAAVAK-amide is shifted to lower pH* by about 0.5 unit (Figure 3b), which probably results from the presence of the positively charged Lys residues (Kim & Baldwin, 1982). A similar phenomenon may occur in thermally denatured RNase A, which carries a net positive charge of +19 at acid pH.

**DISCUSSION**

**Absence of Significant Protection against Exchange.** Most of the 36 NHs at which exchange has been measured in thermally denatured RNase A show kmin values that are within a factor of 2 of their predicted values. NHs that exchange more rapidly than predicted include all NHs bounded in the primary sequence by at least one positive residue, so a systematic correction for exchange at such sites appears to be necessary. The slow NH exchange at valline residues in thermally denatured RNase A has been shown to result from their approximately 4-fold lower intrinsic rates of exchange relative to alanine, which heretofore has been the model for exchange at valline NHs (Molday et al., 1972). Two isoleucine NHs in thermally denatured RNase A also show slowed ex-
change relative to alanine (Table I), suggesting that either they are participating in structure or, as we tentatively conclude, alanine may not be an adequate model for exchange from hydrophobic residues, at least for those with β-branched side chains. There are only two other NHs, Cys 72 and Ala 109, that show protection factors of 2.0 or greater (Table I). Given the uncertainties in the extrapolations of the predicted rates to 65 °C (see below), these values fall on the border between insignificant and significant levels of exchange protection. We thus conclude that, at low pH and at 65 °C, thermally denatured RNase A has no stable hydrogen-bonded structure. In this regard, our results are similar to those from studies of the tryptophan indole NHs in thermally denatured hen lysozyme and eight peptide NHs in thermally denatured BPTI (Wedin et al., 1982; Roder et al., 1985).

A number of slowly exchanging NHs were identified in a recent study of hydrogen exchange in RNase A undergoing thermal denaturation at pH* 2.5 (Talluri & Scheraga, 1990). Most of these NHs, observed after approximately 10 min in the thermal transition zone, belong to the valine and isoleucine residues discussed earlier, so at least some of the observed protection is caused by slow intrinsic exchange; the authors observed this as well in studies of a small valine-containing tripeptide (Talluri & Scheraga, 1990). In fact, if we use an activation energy of 15 kcal·mol−1, then the time constants for exchange of valine NHs near pH* 2.5 and at 65 °C, which range from about 80 to 150 s, extrapolate to approximately 320–600 s at 45 °C, which is near the midpoint of the thermal transition in their study. Moreover, the model of Hvidt (1964) predicts some exchange protection in the thermal transition zone; a 2-fold increase in the time constant for exchange is expected at the midpoint of the transition [see eq 10 in Hvidt (1964)]. It is therefore possible that most of the observed slow NH exchange (Rico et al., 1989; Talluri & Scheraga, 1990) is caused by the intrinsically slow rates of exchange at β-branched hydrophobic residues.

Residual Structure Detected by CD. Our results show that the residual structure detected by CD in heat-denatured RNase A is not correlated with any detectable protection against exchange of amide protons. The present results for thermally denatured RNase A are consistent with the findings from a similar study of thermally denatured S-protein, residues 21–124 of RNase A (Loftus et al., 1986). Interestingly, the thermally denatured forms of both RNase A and S-protein show large far-UV CD signals, possibly corresponding to as much as 10–15% helix (the native protein has a helix content of about 20%), which can be abolished with chemical denaturants such as GuHCl (Aune et al., 1967; Labhardt, 1982).

In addition, a large number of thermally denatured proteins show similar CD signals in the far UV (Dolgikh et al., 1981; Kuwajima et al., 1985; Kuwajima, 1989; Privalov et al., 1989), which some authors have interpreted as representing residual secondary structure (Dolgikh et al., 1981; Kuwajima et al., 1985). The results from the study by Loftus and co-workers (1986), who used tritium exchange to detect structure in S-protein, and the present, more comprehensive, set of experiments with intact RNase A show that the CD signal for thermally denatured RNase A probably is not caused by stable hydrogen-bonded structure. In contrast to the results for thermally denatured RNase A, many NHs in the compact intermediate forms of α-lactalbumin (Baum et al., 1989), apomyoglobin (Hughson et al., 1990), and cytochrome c (Jeng et al., 1990) show significant exchange protection in these experiments. It is thus clear that the correlation between the results of UV–CD studies and NH-exchange experiments is poor: large CD signals in the far-UV region of the spectrum are not diagnostic for stable hydrogen-bonded structure. The question remains as to the origin of the signals in the far-UV CD spectrum of thermally denatured RNase A.

Some possible scenarios in which the residual structure detected by CD could be reconciled with the absence of protection against hydrogen exchange are as follows. CD experiments measure an apparent helix content of about 15% in thermally denatured RNase A. The hydrogen-exchange experiments detect no protection factor greater than 2, which means that the equilibrium fraction of any site involved in hydrogen-bonded structure is less than or equal to 0.5 (Hvidt, 1964). If the 36 NHs studied here are assumed to be representative of all NHs in RNase A, then the two conditions, (1) an overall helix content of 15% and (2) no one residue showing greater than 50% helix, can be satisfied mathematically by a variety of combinations of helix and random-coil residues. At one extreme, these conditions are met by 37 residues with helix contents of 50% each and all other residues in random coil, while the other extreme is 15% helix content at each of the 124 residues in RNase A. In either case, the number of residues involved in helix would have to exceed that observed in the native state (Wlodawer et al., 1986). A possible driving force for the formation of secondary structure in thermally denatured RNase A is some degree of compactness caused by hydrophobic interactions (Chan & Dill, 1990) although additional forces may be necessary to explain the relatively high apparent helix content that is observed (Gregoret & Cohen, 1991). Hydrodynamic data for thermally denatured RNase A are consistent with a somewhat compact conformation while calorimetric data argue against extensive hydrophobic contacts of the type seen in native proteins (Privalov et al., 1989). Any degree of compactness engendered by the hydrophobic effect must therefore be the result of a very limited number of such contacts.

One can question whether the residual CD shown by heat-denatured RNase A arises from known secondary structures. The argument in the preceding paragraph is predicated on the accurate measurement of secondary structure in thermally denatured RNase A by UV–CD. The θ222 of about −5000 deg·cm2·dmol−1 (Figure 1) may not in fact correspond to a significant helix content; A number of small peptides show similar signals at elevated temperatures (Kim et al., 1982; Mitchinson & Baldwin, 1986; Shoemaker et al., 1988; O'Shea et al., 1989; Goodman & Kim, 1989). Studies of such peptides are likely to shed light on the types of structure responsible for the CD signals observed for thermally denatured proteins.

Comparison between pH-Exchange Rate Curves for Model Compounds and Heat-Denatured RNase A. There is a clear trend in the observed pHmax values toward lower pH values than predicted; this also is observed in BPTI [see Table I in Roder et al. (1985)]. As mentioned above, the only pattern in this phenomenon to be identified so far is the large number of hydrophobic residues showing pHmax values that are more than 0.2 pH unit below predicted values. One likely contribution to the low pHmax values is the high net positive charge (+19) on thermally denatured RNase A at acid pH; positively charged environments have been shown to result in depressed pHmax values (Figure 3b; Kim & Baldwin, 1982). Another possible explanation for the low pH minima is an error in one or both of the activation energies, which have been determined only over a limited temperature range (Englander & Poulsen, 1969; Englander et al., 1979; Gregory et al., 1983). There is some disagreement in these determinations, with activation
energies ranging from 13 to 15 kcal-mol$^{-1}$ for acid-catalyzed exchange and 1.2 to 2.6 kcal-mol$^{-1}$ for base-catalyzed exchange. In addition, the contribution of the ionization enthalpy of water or deuterium oxide usually is subtracted from the observed activation energy for base-catalyzed exchange; little has been made of the possible effect of the temperature dependence of this enthalpy, which for D$_2$O ranges from 15.37 kcal-mol$^{-1}$ at 5 °C to 12.88 kcal-mol$^{-1}$ at 50 °C (Covington et al., 1966).

Exchange at Cys 84 and Arg 85 shows behavior unlike that at any other sites (Figure 2; data not shown). No pH$_{min}$ is observed down to pH* 1.5, and the slope of the curve is significantly less than one. The titration of Asp 83 and Glu 86 may be complicating the pH profile of exchange at positions 84 and 85. The puzzling data for Cys 84 and Arg 85 could also be explained by water-catalyzed exchange (Englander et al., 1979; Gregory et al., 1983), but this seems unlikely given that, at the high temperatures used in this study, water-catalyzed exchange is predicted to result in a significant flattening of all of the pH-dependence curves (Gregory et al., 1983). This is not seen at most NHs in RNase A and BPTI (Roder et al., 1985). For many of the NHs in thermally denatured RNase A, a minor contribution from water-catalyzed exchange could explain the slight flattening that is observed near the pH minima (Figure 2 and supplementary material).

The results of hydrogen-exchange studies are a significant addition to the growing body of knowledge about the thermally denatured state of RNase A (Adler & Scheraga, 1990). The results of NH-exchange experiments combined with the calorimetric data of Privalov and co-workers (1989) suggest that the thermally denatured state of RNase A contains little or no stable hydrogen-bonded structure and is well solvated.

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SUPPLEMENTARY MATERIAL AVAILABLE

One figure showing plots of log $k$ versus pH* for 28 additional amide protons in thermally denatured RNase A (3 pages). Ordering information is given on any current masthead page.

REFERENCES

DNA Triplex Formation of Oligonucleotide Analogues Consisting of Linker Groups and Octamer Segments That Have Opposite Sugar–Phosphate Backbone Polarities†

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Abstract: The DNA oligomer analogues 3'd(CTTTCTTT)5'-P4-5'd(TTCTTTCTT)3' (IV), 5'd-(TTCTTTCTT)3'-P2-3'd(CTTTCTTT)5' (V), and 5'd(TTCTTTCTT)3'-P2-3'd(CTTTCTTT)5'-P4-5'd-(TTCTTTCTT)3' (VI) (P2= P=P and P4= P+P+P+P, where P = phosphate and * = 1,3-propanediol) have been synthesized. These oligomers consist of a linker group or groups and homopyrimidine oligonucleotides which have opposite sugar–phosphate backbone polarities. These oligomer analogues are designed to form triplexes with a duplex, 5'd(AAAGAAAGCCCTTTCTTAAGAAGAA)3'-5'd(TTCTCTAAGAAAGGCTTTTCTT)3' (I), which contains small homopurine clusters alternately located in both strands. The length of the linker groups, P2, P4 and P6, was based upon a computer modeling analysis. Triplex formation by the unlinked octamers 5'd(TTCTTTCTT)3' (II) and 5'd(TTCTTTCTT)3' (III) and the linked oligomer analogues IV–VI with the target duplex was studied by thermal denaturation at pH 5.2. The order of stabilities of triplex formation by these oligomers was I–V > I–IV > I–II, III. The mixture of I and VI showed two transitions corresponding to the dissociation of the third strand. The higher transition corresponded to the dissociation of 3'-3' linked octamer segments, and the lower one corresponded to the dissociation of 5'-5' linked octamer segments. The Tm of the latter transition was higher than that of the I–IV triplex; thus the triplex formed by the 5'-5' linked octamer segment was stabilized by the triplex formed by the 3'-3' linked octamer segments in the I–VI triplex. Triplex formation of this system was also studied in the presence of ethidium bromide. Only the I–IV triplex and the part of triplex formed by the 5'-5' linked oligomer segments in the I–VI triplex were stabilized in the presence of ethidium bromide, whereas the I–V triplex or the I–II, III triplex was not. In the presence of ethidium bromide, the three octamer segments in VI dissociated from the duplex I in a synergistic manner. Negative bands at 230–210 nm were detected in the CD spectra of I–II, III, and I–IV, I–V, or I–VI at pH 5.2, indicating triplex formation. Addition of ethidium bromide to the mixtures of oligomers changed their CD spectra only slightly. These results are useful when considering the design of oligonucleotide analogues that can bind as third strands to DNA duplexes of higher complexity.

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