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EFFECTS OF NEUTRAL SALTS ON THE CIRCULAR DICHROISM SPECTRA OF RIBONUCLEASE A AND RIBONUCLEASE S

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The circular dichroism (CD) spectra of ribonuclease A, ribonuclease S, and *N*-acetyltyrosineamide were recorded as a function of pH in the presence of various concentrations of inorganic salts. Above pH 9.0 salting-in of tyrosine residues increases their intramolecular associations. This association enhances the contribution from these residues to the CD spectrum leading to an apparent titration curve that is shifted toward lower pH. The data indicate that unfolding of ribonuclease A and S by inorganic salts does not begin with disrupting existing electrostatic interactions. But, as the unfolding process progresses, disruption of electrostatic interactions may take place. This is consistent with our previous calorimetric studies which suggest that unfolding of ribonuclease A by salts proceeds initially by energetically favorable solvation of the folded protein. An increase in ellipiticity at 275 nm of partially unfolded protein in salt was observed as the pH was changed from 7.0 to 4.0. This observation may suggest that the isothermal unfolding of the protein by salts at low pH proceeds through an intermediate step which involves histidine residues and causes a conformational change in the tyrosine's asymmetric environment.

1. Introduction

In our previous studies [1] we have measured the enthalpies of transfer of ribonuclease A from buffer to solutions of guanidine hydrochloride, urea, $CaCl_2$ and LiCl. These experiments suggested that unfolding of the protein in guanidine hydrochloride proceeds by preferential solvation of the folded protein. However, it was uncertain whether the isothermal unfolding of the protein by inorganic salts proceeds in the same manner [1]; specifically, whether there is a significant contribution from disruption of electrostatic interaction to the isothermal unfolding of the protein.

Thermodynamic quantities depend only on the initial and final conformational states. They give, therefore, little detail on subtle conformational changes or structural alteration. Structural information on conformation changes can be derived from spectroscopic techniques. In this investigation, the CD method has been used to extend our previous studies, and to gain a better understanding of the isothermal unfolding process of ribonuclease by inorganic salts.

Von Hippel and Wong [2] have studied the effect of inorganic salts on the thermal unfolding of ribonuclease A at neutral pH by following changes in the specific optical rotation at 366 nm. Their elegant work has contributed greatly to the understanding of the effect of salts on the thermal unfolding of proteins but little structural information could be obtained from measurements of specific optical rotation at one wavelength.

In recent years the CD technique has become a powerful tool for characterization of protein conformations [3]. It has been used to study the effects of various unfolding agents on the structure of ribonuclease [3–7]. Ahmad and Bigelow [4] have studied the isothermal changes in the far-ultraviolet CD region at 220 nm of ribonuclease A resulting from addition of inorganic salts and urea at pH 3.0. They characterized the final end prod-

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ucts of the transition produced by these agents in terms of changes in the secondary structure.

CD studies of the isothermal unfolding process by inorganic salts as a function of pH, in the near-ultraviolet region, can contribute to a better understanding of the role that electrostatic interactions play during that process; i.e., whether electrostatic interactions are involved in maintaining the native structure of ribonuclease and what structural changes, if any, occur upon disrupting these interactions. However, there are few data available in this respect.

The CD spectra of ribonuclease A and S and of *N*-acetyltyrosineamide were recorded, mainly in the near-ultraviolet region, as a function of pH in the presence of various concentrations of alkali and calcium salts. Ellipticity changes in this region represent contributions mainly from tertiary structure (tyrosine residues and disulfide bands). The contributions from tyrosine residues are probably predominant, since changes in the contributions from disulfide bands to the ellipticity should be relatively very small as a function of pH.

2. Experimental

2.1. Materials

Ribonuclease A (type XII-A), ribonuclease S (type XII-S), and *N*-acetyl-L-tyrosineamide were purchased from Sigma Chemical Co. All inorganic salts were obtained from Fisher, and were reagent grade. The proteins were purified by passing through a mixed-bed ion-exchange column (Rohm and Haas Amberlite ME-1). The pH of the ribonuclease A solution (eluted with distilled water) as it came off the column was between 9.6 and 9.7, in agreement with the value of 9.60 reported previously [8]. For ribonuclease S the pH was 9.4–9.5. The protein solutions were lyophilized at concentrations below 1% to avoid aggregation [9]. Protein concentration was determined by dry weight measurements and compared with determinations using a value of 9800 for the molar extinction coefficient of the native ribonuclease at 277.5 nm [10]. LiCl and $LiClO_4$ were heated under vacuum at 150°C for 24 h before use. Doubly distilled water was used throughout.

2.2. Method

CD spectra were measured with a Jasco spectropolarimeter Model ORD/UV-5 with a CD attachment operating at a sample-compartment temperature of 26.8–27.0°C. Samples were prepared in calibrated 5.0-ml volumetric flasks by adding known volumes of appropriate stock solutions of ribonuclease or *N*-acetyltyrosineamide in 0.16 N KCl. Salt solutions at appropriate concentrations were prepared from concentrated stocks, which were filtered (Millipore 0.22 μm) when necessary.

The temperature was controlled by a Haake constant temperature circulator and was measured by inserting a calibrated thermistor attached to an Atkins Thermo Probe inside the empty cell before each measurement. The pH was measured with a Radiometer pH meter.

Measurements were made in fused silica cells of calibrated path length. The light-path length was 1.0 cm for all measurements between 260 and 300 nm and 0.1 cm for measurements below 260 nm. In the near-ultraviolet region 0.6–0.8 mg/ml solutions of ribonuclease A or ribonuclease S and 0.3–0.45 mg/ml solutions of *N*-acetyltyrosineamide were used; in the far-ultraviolet region 0.2–0.25 mg/ml solutions of ribonuclease A were used. Spectra were recorded with the two most sensitive scales (1 or 2 mdegree/cm of chart paper). Molar ellipticity $[\theta]$ in degree cm^2 $cmol^{-1}$ was calculated as

$$[\theta] = \frac{\theta_{obs} \mathrm{MW\ (or\ mrw)}}{10dc}$$

where θ_{obs} is the observed ellipticity (in mdegree), C the concentration (in mg/ml), d the path length (in cm), MW the molecular weight, and mrw the mean residue weight, which for RNase A and RNase S was taken to be 110.5. Baselines were obtained with the same cell and conditions under which the spectrum of the sample was taken.

The instrument was calibrated with D-(−)-phenylglycine (1.03 mg/ml) in 0.1 HCl using a 0.102-cm cell. The maximum $[\theta]$ at 218 nm was assigned as +34 000 degree cm^2 $cmol^{-1}$, the value obtained by Verbit and Heffron [11].

3. Results

3.1. Ellipticity changes in the CD spectrum of ribonuclease upon isothermal unfolding

Inorganic salts such as LiCl or $CaCl_2$ unfold ribonuclease A and S and diminish the ellipticity throughout the CD spectra of the proteins. The changes in the CD spectrum of ribonuclease A are shown (fig. 1 and table 1). Fig. 1 also shows the changes in the near-ultraviolet CD of ribonuclease on addition of 30% dioxane. Addition of 70% aqueous dioxane produced a spectrum that looked similar to that in 7.0 M LiCl. The unfolding curves (shown only for ribonuclease S) in fig. 2 represent a two-state process at 239 and 275 nm. The transition midpoint concentrations for these changes are 2.4 M $CaCl_2$, and 5.7 M LiCl for ribonuclease A and 4.3 M LiCl for ribonuclease S. The values for ribonuclease A are consistent with values obtained from ultraviolet absorption studies [12]. There are few published data on the isothermal unfolding of ribonuclease S by LiCl.

The free energy of unfolding in water ($\Delta G_{und}^{H_2O}$) may be obtained from figures such as fig. 2 by determining the equilibrium constant for the unfolding process using a method of Puett [6]. A severe limitation of the method is the requirement of linear extrapolation to zero concentration of denaturant. Unfortunately, the relation between free energy of denaturation and the concentration of salts such as LiCl or $CaCl_2$ is not linear at low salt concentrations as was shown by Schrier and co-workers [12]. Therefore, any $\Delta G_{und}^{H_2O}$ values obtained by this method should take this into consideration. Assuming that the deviation from linearity would be the same for ribonuclease A and S, a comparison of $\Delta G_{und}^{H_2O}$ for these proteins might be meaningful. The values for $\Delta G_{und}^{H_2O}$ of 10.1 and 8.3

Table 1

The effect of pH and $CaCl_2$ concentration on the ellipticity at 220 nm

The pH was adjusted with HCl or NaOH. At 0 M $CaCl_2$, 0.16 N KCl was present.

pH	$CaCl_2$ (M)	$[\theta]_{220}$ (degree cm^2 $dmol^{-1}$)
3.0	0	8400
6.5	0	8500
13.3	0	2800
7.0	2.0	8700
7.0	4.0	5000

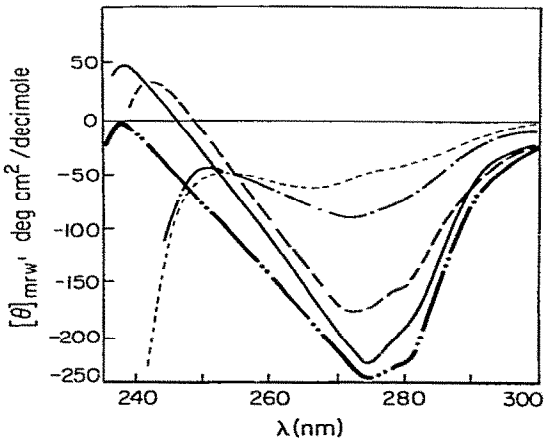

Fig. 1. The near-ultraviolet CD spectra of ribonuclease A in solutions of various molarities of salt at 26.8°C. Salt solution of appropriate concentrations were prepared from concentrated stock solutions; (———) 2.0 M LiCl, pH 6.85; (– –) 5.6 M LiCl, pH 7.15; (–·–·–) 6.0 M LiCl, pH 7.20; (– – – –) 7.0 M LiCl, (–···–) 30% dioxane, pH 5.2.

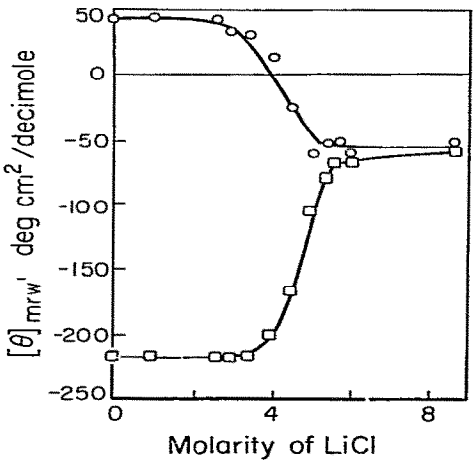

Fig. 2. Changes in the maximum ellipticity of the 239 nm (○) and the 275 nm (□) bands in ribonuclease S as a function of LiCl molarity at apparent pH 6.5–6.9 and 26.8°C.

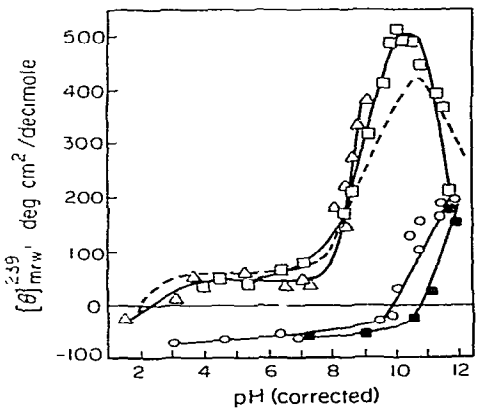

Fig. 3. The effects of corrected pH on the ellipticity of ribonuclease A at 239 nm. Changes in the maximum ellipticity of the 239 nm band in ribonuclease A as a function of corrected pH (see text) in solutions of several salts at 26.8°C: (△) 4.0 M CsCl, (□) 4.0 M LiCl, (○) 6.0 M LiCl, (■) 8.6 M LiCl, (— — —) 0.16 M KCl.

kcal/mol for ribonuclease A and S, respectively, were obtained.

As expected [1], inorganic salts like CsCl, NaCl and RbCl did not diminish the ellipticity of the CD spectra of ribonuclease A and S. Therefore, in the interest of clarity, the CD spectra of these protein in these salts are not shown.

3.2. Ellipticity changes in the CD spectrum of ribonuclease as a function of pH in the presence of inorganic salts

The CD spectra (figs. 3–5) of ribonuclease A and S in various solutions of inorganic salts as a function of pH were measured to assess the effects of neutral salts on the electrostatic interactions in these proteins. In the presence of preunfolding concentrations of up to 4.0 M LiCl, 2.0 M $CaCl_2$, 1.0 M $LiClO_4$, or up to 4.0 M of salts which do not unfold ribonuclease A, the apparent alkaline titration curve (ellipticity changes as a function of pH) was shifted to lower pH, in comparison to the curve in 0.16 N KCl (not shown). The titration curve in unfolding-salt concentrations, however, was shifted toward higher pH. The shift toward lower pH was largest at salt concentrations just below those at which unfolding occurred. The effectiveness of the various salts followed the order 4.0 M LiCl = 2.0 M $CaCl_2$ > 1.0 M $LiClO_4$ ≈ 4.0 M NaCl > 4.0 M CsCl = 4.0 RbCl.

A large part of this shift may result from the

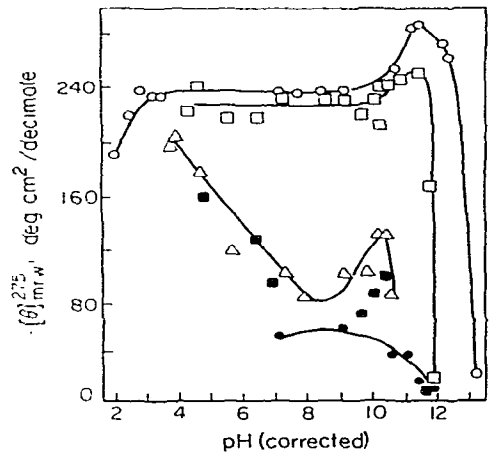

Fig 4. The effects of corrected pH on the ellipticity of ribonuclease A at 275 nm. Changes in the maximum ellipticity of the 275 nm band as a function of corrected pH (see text) in solutions of several salts at 26.8°C: (○) 0.16 M KCl, (□) 4.0 M LiCl, (■) 6.0 M LiCl, (△) 2.6 M $CaCl_2$, (●) 8.6 M LiCl.

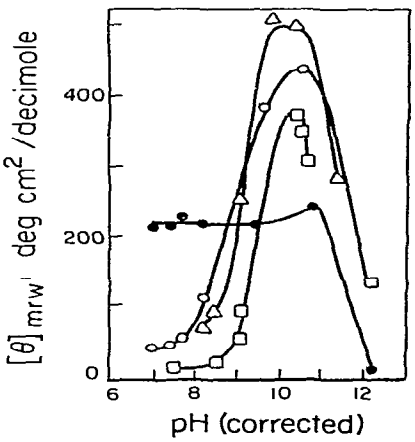

Fig. 5. CD spectra of ribonuclease S as a function of corrected pH at 26.8°C. Changes in the maximum ellipticity of the 239 nm band in (○) 0.16 M KCl, (△) 2.6 M LiCl, and (□) 4.0 M LiCl, and (●) of the 275 nm band in 0.16 N KCl.

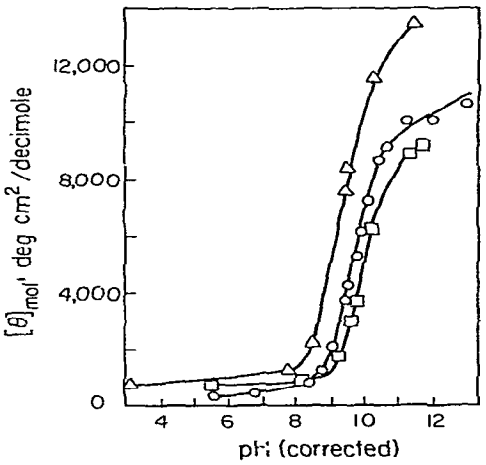

Fig. 6. The effects of corrected pH on the ellipticity of *N*-acetyltyrosineamide at 239 nm. Changes in ellipticity at the 239 nm region as a function of corrected pH (see text) in solutions of several salts at 26.8°C; (△) 4.3 M CsCl, (○) 0.16 M KCl, (□) 4.0 M LiCl.

effects of these salts on the activity coefficient of H^+ and OH^-. These effects can be estimated by a method similar to that used to measure H^+ curves in concentrated quanidine hydrochloride [13]. The pH measured can be corrected for the effect of salt concentration on the activity coefficients by the equations;

$$pH_{(corrected)} = pH_{(measured)} + \log \gamma'H$$

$$pOH_{(corrected)} = pOH_{(measured)} + \log \gamma'OH$$

Table 2

Effect of temperature and pH on the CD spectrum of ribonuclease A

$\Delta[\theta]_{239}$ is the change in ellipticity at 239 nm as temperature is lowered from 26.8 to 11.0°C at specified pH, given in degree cm^2 dmol^{-1} on a mean residue weight basis. $\Delta[\theta]_{275}$ is the change in ellipticity at 275 nm as temperature is lowered from 26.8 to 11.0°C given in degree cm^2 dmol^{-1} on a mean residue weight basis.

pH	$\Delta[\theta]_{239}$	$\Delta[\theta]_{275}$
3.63	53	−2.0
6.35	67	−3.0
8.8	107	5.0
11.3	33	17.0

where $\gamma'H$ and $\gamma'OH$ are the apparent molar activity coefficents of H^+ and OH^- in salt solutions. Titration curves for ribonuclease A and S using changes in ellipticity of the 239 and 275 nm bands corrected for the effects of LiCl or CsCl on the activity of H^+ and OH^- are shown in figs. 3–5. The maximum shift of the titration curve of ribonuclease S at 239 nm to lower pH occurs in the presence of 2.6 M LiCl instead of the observed 4.0 M LiCl for ribonuclease A (figs. 3 and 5). Titration curves in the presence of $CaCl_2$ behave similarly to these in the presence of LiCl but 2.0 M $CaCl_2$ has the same effect as 4.0 M LiCl.

The effects of cooling (to 11°C) on the ellipticities in the near-ultraviolet CD spectra of ribonuclease as a function of pH are given in table 2. Cooling affected only the ellipticity of the 239 nm band and caused little change in the ellipticity near 275 nm.

Titration curves were also taken using the CD spectrum of *N*-acetyltyrosineamide (fig. 6) in order to compare the effects observed above on the titration curve of ribonuclease with the effects of inorganic salts on the titration of a tyrosine model compound [3]. As the pH is raised, there is a decrease in ellipticity in the 224 nm band with a concurrent increase in ellipticity upon formation of the phenolate ion near 239 nm. The 275 nm band's intensity is also decreased at high pH with concurrent increase in ellipticity near 292 nm (not shown). Since for ribonuclease the maximum ellipticity near 239 nm is shifted to 242 nm upon alkaline titration, ellipticities of *N*-acetyltyrosineamide, at wavelengths corresponding to the ellipticity maximum of ribonuclease are given.

4. Discussion

The near-ultraviolet CD spectrum of unfolded ribonuclease A in concentrated salt solution (fig. 1) is similar to the CD spectrum obtained from disulfide bridges [14]. The tyrosine residues appear to rotate freely upon unfolding in neutral salts, so their contributions to the CD spectrum of the protein are minimal, but the intact disulfide bonds do contribute to the spectrum. The α-helix and the

β-structure of the protein are disturbed, since the intensity of the far-ultraviolet band is diminished in the presence of 4.0 M $CaCl_2$ (table 1). However, there is no complete unfolding to random coil structure, as in the case of high OH^- (table 1) or high urea concentration [4].

The alkaline titration of the protein in the presence of unfolding salts (fig. 3) shows a continuous increase in ellipticity of the 239 nm band toward the observed maximum in 0.16 N KCl. In the unfolded state there should be only small environmental constraints on the tyrosine residues so the ellipticity changes correspond only to the ionization of freely rotating exposed tyrosine residues. Therefore, the ellipticity in the presence of unfolding salt concentration is much lower than in the presence of preunfolding concentrations.

Very interesting changes occur in the CD spectrum of ribonuclease upon pH titration in the presence of preunfolding concentration of inorganic salts (figs. 3 and 5). Application of correction factors (as calculated in section 3) for the effects of salts on the activity coefficients of H^+ or OH^- are essential. In the absence of these corrections, erroneous conclusions may be reached, since the ellipticity in particular at 239 nm is very sensitive to changes in pH. In 4.0 M LiCl the correction applied to the apparent pH is very significant, about 1 pH unit.

A careful investigation of figs. 3 and 6 indicates that in the protein titration, factors besides the salting-out and salting-in effects observed for *N*-acetyltyrosineamide should be considered. Salting-out of the tyrosine residues in the protein should shift the aromatic group closer to the peptides units or other amino acid residues of the protein, thus increasing the intramolecular associations of the tyrosine residues. This will enhance the ellipticity contributions from these residues leading to an apparent titration curve in the presence of certain inorganic salts that is shifted toward lower pH. The extent of this association follows the salts' order of effectiveness in salting-out tyrosine residues; LiCl > NaCl > KCl > CsCl. For LiCl the resultant shift of the titration curve of the protein to lower pH (fig. 3) indicates that the positive contribution to the ellipticity at 239 nm from the intramolecular association upon salting-out is larger than the effect of the small increase in pK of the isolated tyrosine residue of *N*-acetyltyrosineamide observed in 4.0 M LiCl (fig. 6).

The decrease in ellipticity above pH 11.0, due to unfolding of the protein by OH^-, begins at about the same pH in the presence of high salt concentrations (up to 4.0 M LiCl) or in 0.16 N KCl (figs. 3–5). It is interesting that such high salt concentration did not contribute to significant destabilization of the protein. This suggests that inorganic salts do not disturb any stabilizing electrostatic interactions that may exist in ribonuclease at high pH. On the contrary, the additional increase in ellipticity just before unfolding by OH^- in the presence of preunfolding salt concentration suggests that inorganic salts may cause local stabilization of the tyrosine residues.

Ribonuclease S is thermodynamically less stable than the native protein (ribonuclease A) toward denaturation by inorganic salts (fig. 2). this is consistent with previous studies with heat and urea [15]. But as was observed before with ribonuclease A, preunfolding salt concentrations did not destabilize further ribonuclease S toward unfolding by OH^- (fig. 5). This suggests that despite their difference in structural stability the intramolecular environment in the region of tyrosine residues is nearly identical in both proteins.

The events that proceed just before unfolding of ribonuclease by inorganic salts are still not clear. However, the data presented above indicate that contributions from disruption of electrostatic interactions by the salts are not significant. This fact further supports our previous findings [1] that before unfolding, energetically favorable solvation of the folded protein is taking place. In the case of salts this will maximize contact with peptide backbone units and will minimize contact with nonpolar groups such as tyrosine residues. This may also explain why cleavage of a single covalent bond in ribonuclease A disrupts conformational stability. Apparently, in the cleaved protein, there is exposure of peptide units thus increasing the section of the protein that can be favorably solvated by inorganic salts, leading to a less stable protein.

The increase in the ellipticity at 275 nm of partially unfolded ribonuclease A by 6.0 M LiCl

or 2.6 M $CaCl_2$ on acid titration to pH 4 (fig. 4) is intriguing. This increase is not observed with the 239 nm band. It may suggest that unfolding of the protein by salts at low pH affects the environment of the various tyrosine residues to different extents. The behavior at 275 nm indicates that electrostatic interactions that are disrupted during unfolding at neutral pH are restored when the pH is lowered thus stabilizing the protein against further unfolding by inorganic salts. This is in contrast to the effect of high concentrations of H^+ on thermal and urea denaturation [4].

The increase in ellipticity that is observed from pH 7.0 to 4.0, in 6.0 M LiCl or 2.6 M $CaCl_2$ can result from a conformational change around tyrosine residues caused by titration of histidine residues, since at this pH range protonation of histidine residues occur. Nuclear magnetic resonance studies following the histidine resonance on acid titration of ribonuclease [16–18] indicate that there must be a conformational change in the vicinity of His-48. It was postulated that this conformational change also involves Tyr-25 and Asp-14. In our investigation, a conformational change around tyrosine residues which might involve Tyr-His or Asp-His interactions is suggested by following the changes in ellipticity contributions from tyrosine residues to the CD spectrum of ribonuclease A. This is the first time, to our knowledge, that such evidence is reported for the isothermal unfolding of the protein by inorganic salts.

On unfolding of ribonuclease by salts, the ellipticities at 239 and 275 nm diminished simultaneously (figs 1 and 2). This observation is in contrast to what is observed in the presence of dioxane (fig. 1) or thermal unfolding (table 2 and ref. 19) where initially only the ellipticity at 239 nm is decreased. It is possible, therefore, that unfolding of the protein by these different agents does not proceed in the same path.

Several possibilities are given for the specific decrease in positive ellipticity at 239 nm upon raising the temperature from 4 to 27°C. Simmons and Glazer [19], and Simons and Blout [20] suggested that a specific local conformational change takes place which causes a disturbance of the environment around a buried tyrosine residue (Tyr-25). This was, however, disputed by others [5] on the basis that exposed tyrosine residues may be contributing to the ellipticity at 239 nm. The data given in table 2 support Simmons' conclusions that the increase in ellipticity upon cooling is probably derived from contributions of buried tyrosine residues.

Table 2 shows that there is an increase in ellipticity at 239 nm upon cooling. This increase becomes larger as the pH is raised to 9.0 and decreases above pH 11.0. This behavior is not expected from exposed residues. The ionization of exposed tyrosine residues is very small below pH 9.0 at room temperature. Since the ΔH for the ionization of tyrosine is 6 kcal/mol, its pK at 0°C is 0.4 units higher than at 25°C [21]. The titration curve of an exposed tyrosine at 11°C should therefore be shifted toward higher pH. A decrease in ellipticity around 239 nm should be obtained rather than an increase. Evidently, there is a conformational change around a buried tyrosine residue on cooling which is pH dependent and which causes an apparent increase in the ellipticity at 239 nm. The decrease in ellipticity around 239 nm might also result from the deepening, broadening or red shifting of the overlapping far-ultraviolet negative band but the data do not support this possibility. Pflumm and Beychok [5] reported that lowering the temperature from 25 to 3°C has no effect whatever on the far-ultraviolet band. Schultz et al. [22] reached similar conclusions for changes in ellipticity at 220 nm between 12 and 27°C. In addition no significant change in the peptide band ellipticity was observed from pH 3.0 to 10, and above pH 13.0 the band is blue shifted (table 1). Above pH 11.0, partial unfolding of the protein has reduced the asymmetric environment around the tyrosine residues. Thus, their contributions to the CD spectrum of ribonuclease have been decreased (figs. 3–5) and are less effected by a change in temperature (table 1).

In the unfolding of the protein by temperature or dioxane, one of the first steps is apparently a local conformational change around a buried tyrosine residue (probably Tyr-25). In the case of thermal unfolding, data obtained from other methods also suggests involvement of tyrosine residues in the first stages of unfolding [23]. There is no CD

evidence, however, that this is also the case in the unfolding of ribonuclease by inorganic salts. The unfolding of the protein by salts at low pH does proceed through an intermediate step in which there is a conformational change which alters the environment of tyrosine residues. This step probably involves a buried tyrosine residue, since it occurs only in the partially unfolded protein.

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