Thermal Effects on the Circular Dichroism Spectra of Ribonuclease A and of Ribonuclease S-Protein*

E. R. Simons, E. G. Schneider, and E. R. Blout

From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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SUMMARY

The thermal denaturation of ribonuclease A and of S-protein in neutral aqueous solutions has been studied by means of their circular dichroism spectra. For RNase A, the positive circular dichroism extremum near 241 nm shows a biphasic dependence upon temperature, a linear change between 10° and 50° being followed by a sharp one above 52°. The negative circular dichroism extremum at 277 nm is observed in spectra of S-protein below 25°, as well as a negative circular dichroism extremum at 277 nm; both of these extrema disappear upon heating. In contrast to RNase A, the thermal changes observed for S-protein are not reversible.

In a previous communication (1), we have shown the circular dichroism spectra between 215 and 300 nm for RNase A, RNase S, S-protein, and S-peptide. A small positive extremum near 240 nm was present in the spectra of RNase A and RNase S, absent in the spectrum of S-protein, and observed (with lower magnitude) in that of RNase S'. The same extremum decreased when RNase A and RNase S were treated with acid; the CD data could be interpreted as an optical titration curve with an inflection point which corresponded to approximate pK values of 2.0 for RNase A and 3.5 for RNase S. On the basis of model compound studies (2, 3), the CD region from approximately 220 to 240 nm should include contributions from tyrosyl residues. It has been shown previously that the removal of S-peptide from RNase S (4, 5) and the acidification of RNase A (6) were accompanied by the "normalization" of a single tyrosyl residue. Therefore, the changes in the CD extremum at 240 nm were tentatively attributed to some interactions involving this tyrosyl residue, Tyr-25 (1). A small, flat, negative CD extremum at 276 to 278 nm has also been associated with tyrosyl residues (1-3). We now report the use of these CD bands as probes for conformational changes in RNase A and in its derivatives. We describe here some studies of the thermal transition of RNase A, at neutral pH, from which it can be concluded that at least two steps are involved.

The thermal denaturation of RNase in neutral aqueous salt solutions exhibits a midpoint at approximately 62° for RNase A (5), 46° for RNase S (6), and 34° for S-protein (7). The denaturation has been followed by changes in molar absorbance at 287 nm (5-9), in optical rotation at 366 or 436 nm (5, 10, 11), or in viscosity (10). The possibility that this thermal transition exhibits more than one step has been raised (5, 12), but no experimental proof has yet been reported.

RNase A contains 6 tyrosyl residues, 3 each exhibiting normal and abnormal (or "buried") behavior (13, 14). Each of these abnormal tyrosyl residues has been shown to interact with, or be very near to, an aspartyl residue exhibiting an unusually low pK (15, 16). X-ray studies of crystalline RNase A (17) and RNase S (18) agree with these findings. S-protein contains all of the tyrosyl groups of RNase, but only two are buried; a third becomes buried when an equimolar quantity of S-peptide is added to form active RNase S' (7).

Characteristic ORD and CD spectra of native RNase A have been reported (1-3, 19-22). The CD extrema near 240 and 277 nm are at least in part attributable to side chain contributions, probably from the buried tyrosyl residues (19, 21, 22). Urea (2) or dodecyl sulfate (3), as well as substitution of all 6 tyrosyls (19, 21, 22), causes marked changes in the CD spectrum of RNase.

We have utilized CD spectra as a means of following the thermal denaturation of RNase. Specifically, we have attempted to determine whether this denaturation exhibits one or more steps by following the temperature dependence of both the 241 and 277 nm extrema.

EXPERIMENTAL PROCEDURE

Experimental methods have been published previously (1). For these measurements RNase A (Worthington Lot RAF 7A

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† The abbreviations used are: RNase A, the principal chromatographic component of ribonuclease; RNase S, subtilisin-modified RNase A; S-protein, the protein component obtained from RNase S; S-peptide, the peptide component (residues 1 to 20 of RNase S); RNase S', the reconstituted RNase S obtained from equimolar quantities of S-protein and S-peptide; CD, circular dichroism; ORD, optical rotatory dispersion.
and RAF 8CA was dissolved in 0.1 M acetate, phosphate, or sulfate buffer, pH 6.46 ± 0.04. Ultraviolet spectra were obtained with Cary 14 and Cary 15 spectrophotometers. The optical density at 276 nm was used as a measure of concentration ($\varepsilon = 0.709$ per mg per ml). pH was measured on a Radiometer pHM-4 meter. Circular dichroism spectra were obtained with a Jasco ORD/UV-5 spectropolarimeter with a CD attachment which, since our previous communication (1) has been adjusted to twice the sensitivity. Glycol-water solution from an external thermostat was circulated through the jacket of a 10.00-mm quartz cell. Temperatures were measured continuously by means of a YSI thermistor probe inserted directly into the solution in the neck of the cell. Thirty minutes of equilibration were allowed at each temperature before measurement. Base lines were obtained with the same cell at room temperature.

Activities were measured by means of the Anfinsen et al. adaptation (25) of the Kunitz assay, with a single batch of ribonucleic acid (Sigma Lot 10500250) as substrate.

S-protein was prepared by a modification of the Doscher and Hirs technique (26), lyophilized from 50% acetic acid, and dissolved in buffers as for RNase A above.

### RESULTS

#### RNase A

A representative CD spectrum of RNase A at various temperatures is shown in Fig. 1. The results were the same in 0.1 M acetate, phosphate, and sulfate buffers at pH 6.46 ± 0.04. In each buffer, cooling led to a larger positive CD extremum at 241 nm, but no change in the 277 nm extremum. Heating the solutions above 15°C led to a decreased ellipticity at 241 nm, but again an unaltered 277 nm extremum until a temperature above 50°C had been reached. Above this temperature there was a marked change in both CD regions. Upon cooling...
from 65° to approximately 23°, the 277 μm extreum was reversibly recovered but the 241 μm extreum was not. The heated and cooled solutions exhibited full enzymic activity.

We have calculated a thermal denaturation curve (Fig. 2) from the data in Fig. 1. In each solvent the ellipticity difference between temperature, T, and the lowest temperature, T_m, was calculated as Δ[θ]_x and plotted as a function of T. As Fig. 2 shows, the results were essentially identical for the three buffers. There are two distinct melting steps: (a) a broad transition which begins at approximately 15° and proceeds linearly to 50°, and (b) a sharp cooperative transition which begins above 50°.

We have made no attempt to measure the midpoint of the 277 μm thermal transition since we specifically wished to avoid irreversible denaturation of RNase A (5, 10); the temperature was never raised above 66°. Recovery of full enzymic activity between temperature, T, and the lowest temperature, T_m, was estimated as a function of T. As Fig. 2 shows, the results were essentially identical for the three buffers. There are two distinct melting steps: (a) a broad transition which begins at approximately 15° and proceeds linearly to 50°, and (b) a sharp cooperative transition which begins above 50°.

The CD extremum observed for RNase A near 237 μm appears upon cooling and disappears upon heating. The negative extremum at 277 μm does not change upon cooling but does increase on heating. The thermal denaturation curve, shown in Fig. 4, is the same in 0.1 M sulfate or phosphate buffer; a midpoint for the 277 μm ellipticity is observed at about 34°.

**S-Protein—**Representative CD spectra of S-protein at various temperatures are shown in Fig. 3. A positive CD extremum near 237 μm appears upon cooling and disappears upon heating. The negative extremum at 277 μm does not change upon cooling but does decrease on heating. The thermal denaturation curve, shown in Fig. 4, is the same in 0.1 M sulfate or phosphate buffer; a midpoint for the 277 μm ellipticity is observed at about 34°.

**DISCUSSION**

That RNase A undergoes a two-step thermal transition between 10° and 65° is evident from Fig. 2. It remains, however, to explain this observation in terms of the conformational changes that accompany this transition.

The results presented here imply that the thermal denaturation of RNase A fits the two-step thermodynamic model of Hermans and Scheraga (12). According to these investigators, one step would be noncooperative and unaccompanied by a conformational change. They further predicted that this portion of the thermal denaturation would involve the normalization of a single aspartyl-tyrosyl residue pair, as well as noncooperative destruction of some other interactions. The second denaturation step would then occur cooperatively and exhibit a midpoint near 60°. These predictions fit our experimental data (Fig. 2).

The CD extremum at 277 μm has been clearly associated with tyrosyl transitions (2, 3) involving, in large part, the buried residues (20–22). Thus the extremum is deeper for RNase A than for S-protein (Table I). Neither its magnitude nor its breadth changes when RNase A is observed between 10° and 50°. The tyrosyl residues responsible for the contributions in this region are thus probably unaffected by mild heat treatment, just as they were unaffected by mild acidification (1). No definitive assignment of this extremum to specific tyrosyl residues has yet been made, however.

The CD extremum observed for RNase A near 240 μm has been attributed to a tyrosyl contribution (1, 2, 19). The asymmetry of this band can result from the superposition of a positive tyrosyl band centered near 228 μm in model compounds upon a large negative CD extremum centered at much shorter wave lengths (19, 27). Model studies have shown (2, 3) that a small tyrosyl-attributable CD extremum appears at 228 μm at neutral pH and shifts to longer wave lengths, around 242 μm, in alkali. While the buried tyrosyl residues in RNase would not be expected to be ionized at neutral pH, their proximity to charged aspartyl side chains (15, 16) would probably lead to a red-shifted extremum. The normalization of such a tyrosyl by temperature or solvent changes would then cause a parallel blue shift of the extremum and a correspondingly larger overlap (smaller total positive magnitude) in the overall CD extremum near 240 μm.
In S-protein only 2 buried tyrosyl residues are present; one would thus anticipate a different shift of the CD band from that observed in RNase, which has 3 such residues. Our observations that the maxima occur at 241 m\(\mu\) for RNase A and 237 m\(\mu\) for S-protein would fit such a band assignment. Since earlier experiments in our laboratory indicated that carefully prepared RNase monomer and dimer (23, 24) exhibited the same CD spectrum, it is unlikely that either the 241 m\(\mu\) or the 277 m\(\mu\) extremum is related to any intermolecular association of the protein.

The lack of reversibility in the CD extremum near 240 m\(\mu\) has been observed earlier, both for reduced and reoxidized but fully active RNase A (2, 19) and for RNase S (1, 19). It seems likely that this reflects the sensitivity of this extremum, explained above, to very small changes in the tyrosyl residue environment.

It is at this time impossible to give a definitive explanation of our thermal denaturation curve since there is no method for studying the exact environment of specific side chain residues in solution without concomitantly altering these residues. It is, however, tempting to speculate on possible conformational changes which could give rise to the observations shown in Figs. 2 and 4. We would thus consider it possible, albeit unprovable as yet, that these correspond to an initial detachment of non-covalent bonds between the S-peptide and S-protein portions of RNase, followed by a cooperative unfolding of some of the rest of the chain.

CONCLUSIONS

The CD spectra obtained during the thermal denaturation of RNase A reflect two distinct denaturation steps. A noncooperative transition occurs between 15 and 50\(^\circ\), followed by a cooperative one at higher temperatures.\(^4\) These transitions are identical in 0.1 m phosphate, sulfate, and acetate buffers at pH 6.46 ± 0.04. The low temperature transition is accompanied by a linear temperature-dependent change in ellipticity at 241 m\(\mu\). The high temperature transition is accompanied by ellipticity changes in both the 241 and 277 m\(\mu\) regions. Upon cooling full enzymic activity is recovered, as is the ellipticity at 277 m\(\mu\); that at 241 does not quite return to its initial value.

The thermal denaturation of S-protein is accompanied by CD spectral changes at low temperatures at 237 m\(\mu\), and at higher temperatures at both 237 and 277 m\(\mu\).

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REFERENCES

CORRECTIONS

In the papers by A. Martonosi, J. Donley, and R. A. Halpin (Vol. 243, No. 1, Issue of January 10, 1968, page 61) and by Anthony Martonosi (Vol. 243, No. 1, Issue of January 10, 1968, page 71), on page 65, in the legend to Fig. 4, and on page 75, in the legend to Table 1, the medium used for treatment of microsomes with phospholipase C contained, in addition to the components listed, "0.5 mM CaCl₂".

In the paper by E. R. Simons, E. G. Schneider, and E. R. Blout (Vol. 244, No. 15, Issue of August 10, 1969, page 4023), on page 4024, the legends for Figs. 1 and 3 should be replaced with the following:

**FIG. 1.** CD spectra of RNase A in 0.1 M acetate, pH 6.42, 0.65 mg per ml, 10.00-mm light path: a, 16.8°; b, 27.3°; c, 41.8°; d, 56.3°; e, 58.7°; f, 65.2°; g, recooled from 63.2 to 22.0°.

**FIG. 3.** CD spectra of S-protein in 0.1 M phosphate, pH 6.63, 1.087 mg per ml, 10.00-mm light path: a, 14.5°; b, 20.3°; c, 23.2°, initial temperature before cooling started; d, 37.6°; e, recooled from 37.6 to 20.0°; f, 45.0°; g, recooled from 45.0 to 26.0°.

In the paper by J. R. Hunsley and C. H. Suelter (Vol. 244, No. 18, Issue of September 25, 1969, page 4815), on page 4817, Fig. 2 is printed incorrectly as the result of an error by the printer. The photograph of the polyacrylamide gel should be shifted 3 mm to the right. The black dot which appears is a printing artifact. The correct figure and legend is printed below.

![Disc gel electrophoresis of 50 μg of Fraction VII. The enzyme was electrophoresed as outlined in "Materials and Methods" in 6.0% polyacrylamide gel. The gel was split longitudinally, one half was stained, and the other was sliced into 2-mm pieces and each piece dispersed in 0.10 ml of 50% glycerol-10 mM Na phosphate, pH 6.5. Then 10 μl were tested for activity in the standard assay. The enzymatic activity resided in the dark staining band near the left cathodic end. The band to the extreme left was the opaque spacer gel.](image)
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