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A STUDY ON THE POSITIVE ELLIPTICITY IN THE CIRCULAR DICHROISM OF RIBONUCLEASE A

Rami ALMOG

Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A.

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The small positive ellipticity near 239 nm in the CD spectrum of RNase has been investigated as a function of pH. Theoretical calculations using CD parameters representing buried or exposed tyrosine residues have been carried out. A comparison of the theoretical calculations with experimental data suggests that the changes in the band's intensity, as a function of pH, arise mainly from electronic transitions associated with the tyrosine residues. The buried tyrosine residues are the major contributors to the ellipticity in this region at neutral pH. At higher pH contributions from exposed residues are also observed.

1. Introduction

It is generally agreed that the ellipticity around 239 nm, in the CD spectrum of ribonuclease A, is a consequence of overlap between a strong negative band resulting from amide groups and a positive band resulting mostly from tyrosine residues [1–7]. Disagreement exists, however, about which type of tyrosine residue contributes to the band. Simons and co-workers [3–5] argued that the ellipticity near 239 nm is due to buried tyrosine residue and they used this band to follow changes in the degree of exposure of Tyr-25 (buried tyrosine) to aqueous solvents. Pflumm [6] and then Pflumm and Beychok [7] used titration, acetylation and nitration to show that the 239 nm band is due totally or at least in part to transitions from exposed tyrosine residues.

There is growing evidence that changes in the 239 nm band may be used as a sensitive probe for the initial unfolding of this protein by various agents [8]. A better understanding of the various contributions to the optical activity of this band is therefore essential. A rather simple method has been used in this investigation to assess the various contributions from tyrosine residues to this band. It has been shown before [6] that the apparent ellipticity near 239 nm is enhanced and is shifted to a longer wavelength upon increasing the pH from 6 to 10. This change is apparently due to a red shift of the positive band. It is also possible that this enhancement results from minor alteration in the strong amide band but experimental data do not support this possibility [8]. Information on the type of tyrosine residues which contribute to the ellipticity near 239 nm can, therefore, be derived by comparing an experimental pH titration curve to a calculated curve where parameters representing buried and exposed tyrosine residues are used to analyze the red shift of the positive tyrosine band. The calculated curve which best represents the experimental curve should suggest the class of tyrosine residues which contribute to the experimental titration curve.

2. Experimental

Ribonuclease A from bovine pancreas, type XII-A, and *N*-acetyl-L-tyrosineamide were

purchased from Sigma Chemical Co. The protein was purified as described before [8].

CD measurements at 26.8–27.0°C were carried out with a Jasco spectropolarimeter Model ORD/UV-5 with a CD attachment. A 1.0 cm light path cell was used in all measurements between 260 and 300 nm, while a 0.1 cm light path was used at wavelengths below 260 nm. Concentrations of 0.6–0.8 mg/ml ribonuclease A and 0.3–0.45 mg/ml *N*-acetyltyrosineamide were used. The method is described in detail in ref. 8.

3. Results and discussion

The effects of varying alkaline pH on the near-ultraviolet CD spectrum of ribonuclease were measured at 24 pH values ranging from pH 6.17 to 13.15. A representative portion of the total data is given in table 1. The ellipticity of the positive 239 nm band increases as pH increases and reaches a maximum at pH 10.8. This increase in ellipticity is accompanied by a relatively small red shift. The changes in maximum ellipticity of the negative 275 nm band as the pH is raised are smaller than the changes near 239 nm. However, the red shift is much more apparent at 275 nm than near 239 nm.

The changes in ellipticity near 239 nm in the CD spectrum of *N*-acetyltyrosineamide, a model compound for exposed tyrosine residues [1,9] are given in table 2. The wavelengths chosen in table 2 correspond to the position of maximum ellipticity

Table 1

Ellipticities of ribonuclease A as a function of pH at 26.8°C

Ellipticity, $[\theta]$, values are given in degree cm^2 $dmol^{-1}$ on a mean residue weight bases.

pH	239 nm region		275 nm region	
	λ_{max} (nm)	$[\theta]$	λ_{max} (nm)	$[\theta]$
7.2	239	65	275	231
8.9	240	190	276	230
9.7	241	310	279	240
10.8	241.5	433	284	282
11.0	242	425	285	280
12.6	243	247	288	220
13.2	244	112	295	31

Table 2

Ellipticities around 238 nm of *N*-acetyltyrosineamide as a function of pH at 26.8°C

$[\theta]_{mol}$, ellipticity in degree cm^2 $dmol^{-1}$ on a mole basis. $[\theta]_{mrw}$, ellipticity in degree cm^2 $dmol^{-1}$ on a mean-residue-weight basis. $[\theta]_{mrw}$ is calculated by dividing $[\theta]_{mol}$ by the total number of amino acid residues (124) in ribonuclease A.

pH	Wavelength (nm)	$[\theta]_{mol}$	$[\theta]_{mrw}$
6.82	239	446	4
9.10	240.5	2037	16
10.90	242	9647	77
11.47	242	10093	81
13.05	243	10648	86

near 239 nm in the CD spectrum of ribonuclease A at a given pH.

The apparent enhanced intensity and red shift near 239 nm in the CD spectrum of ribonuclease were calculated assuming that the ellipticity at neutral pH is a result of overlap between a strong amide negative band in the far-ultraviolet region and a weaker positive tyrosine band at a longer wavelength. In calculating the resultant apparent parameters resulting from a shift of the band at 239 nm to longer wavelengths it is presumed that these changes simulate the changes occurring in alkaline titration of ribonuclease.

The calculations were performed according to a method used by Wellman et al. [10]. The CD spectrum in this region is considered to be a superposition of two Gaussian curves with positive and negative amplitudes. For any desired values of the parameters A_i, λ_i, λ and Δ_i the resultant spectrum would be represented by the equation,

$$F(\lambda) = A_1 \exp\left[-(\lambda-\lambda_1)^2/\Delta_1^2\right] - A_2 \exp\left[-(\lambda-\lambda_2)^2/\Delta_2^2\right] \quad (1)$$

where Δ_i is the half-bandwidth, A_i the ellipticity, λ_1 and λ_2 the positions of the bands, and λ a variable wavelength for which the corresponding $F(\lambda)$ is calculated. The parameters A_1, λ_1 and Δ_1 represent properties of the intense negative amide band, while the parameters A_2, λ_2 and Δ_2 belong to the smaller positive tyrosine band. Three values have been used for A_2, λ_2 and Δ_2, representing exposed tyrosine residues only, buried

Table 3

Parameters for theoretical calculations of changes in ellipticity around 239 nm in the CD spectrum of ribonuclease A at neutral pH

A_1	λ_1 (nm)	Δ_1 (nm)	A_2	λ_2 (nm)	Δ_2 (nm)
−6500 [a]	216 [a]	12 [a]	400 [b]	225 [b]	13 [b]
			380 [c]		

[a] Ellipticity given in degree cm^2 dmol^{-1} on a mean residue weight basis. Parameters for the amide band are similar to those assigned by Puett [11] to the major transition of the far-ultraviolet band.

[b] Value represents exposed tyrosine residue (see text). A smaller amplitude than in ref. 7 is used in this work in order to achieve a better fit with the given amide band.

[c] Value represents buried tyrosine residues (see text). A_1 [c] and A_1 [a], were calculated from respective rotational strengths given in ref. 11.

tyrosine residues only, and the superimposition of both types of residues. The values for the parameters are given in table 3.

Based on available data from model compounds it is generally accepted that exposed tyrosine residues at neutral pH have a positive band near 225 nm [1,9]. The position of the band may shift to longer wavelength in buried tyrosine residues [5]. In order to achieve a good fit to the observed positive ellipticity near 240 nm, Puett [11] found it necessary to use a positive band, assigned to tyrosine residues, at 235 nm. In table 3 values similar to those used by Puett, therefore, are assigned to possible contribution from buried tyrosine residues. Pflumm and Beychok [6,7], in order to obtain a good fit, used a positive contribution from tyrosine residues at 225 nm. Parameters similar to theirs describe the possible contribution from exposed residues in table 3.

The data in table 4 were obtained assuming that the positive band in ribonuclease near 239 nm at neutral pH results only from contributions of buried tyrosine residues while contributions from exposed tyrosine residues are superimposed on these from the buried residues only upon ionization at higher pH values. The calculations were done as follows: Initially, λ_2 was taken to have a position of a buried tyrosine residue at 234 nm with $A_2 = 380$ degree cm^2 dmol^{-1} and $\Delta_2 = 7$ nm. Then λ_2 was shifted to 239 nm. The resultant

Table 4

Calculated resultant apparent CD titration curve for ribonuclease A using the Gaussian bands [a]

λ_2 (nm)	λ_3 [b] (nm)	A_3 [b]	Resultant apparent λ_{max} (nm)	Resultant apparent A_{max} (nm)
234			239.5	65
235			293.5	111
235.5			239.5	140
236			240.0	155
237			240.0	197
238			240.5	234
239			241.0	257
240	239	100	241.0	389
240	240	130	241.0	415
240	241	180	241.5	468

[a] Using superimposition of exposed tyrosine residues on buried tyrosine residues (explained in text).

[b] λ_3 and A_3 represent the additional contribution from exposed tyrosines on shift from pH 9.0 to 10.8.

apparent changes in the band near 239 nm are given in the first seven rows of table 4. At this point a third Gaussian band with $\lambda_3 = 239$ nm, $A_3 = 100$ degree cm^2 dmol^{-1} and $\Delta_3 = 7$ nm was added. The initial effect on the resultant apparent CD spectrum is given in the eight row of table 4. The effect of rise in intensity with concurrent red shift of the third band on the apparent CD spectrum is shown in the last two rows in table 4. Changes in the apparent parameters resulting from a red shift of the tyrosine band, considering contributions from only exposed or only buried tyrosine residues, were also calculated according to eq. 1 (tabulated data are not shown).

The overall changes in the apparent ellipticity of the CD spectrum of ribonuclease near 239 nm versus changes in the apparent position of the band as a function of pH using calculated data (such as in table 4) and experimental data (such as in table 1) were plotted in fig. 1. The theoretical calculated titration curve (as shown in fig. 1) in best agreement with the experimental titration curve, should suggest that class of tyrosine residues which contributed to the experimental curve.

Clearly, the calculated curve obtained using parameters representing both buried and exposed tyrosine residues most closely approximates the

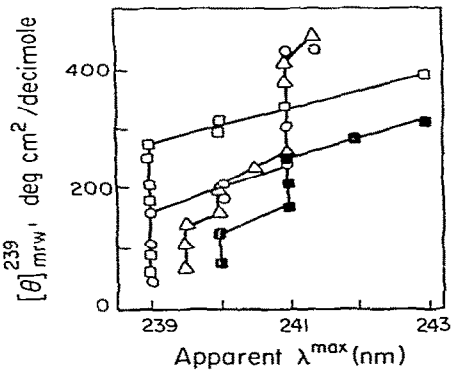

Fig. 1. Experimental and calculated titration plots of Ribonuclease A near 239 nm. Apparent maximum band ellipticity versus apparent band position as a function of pH: (○) using experimental data; (□) calculated using parameters representing exposed tyrosine residues; (■) calculated using parameters representing buried tyrosine residues: (△) calculated using parameters representing buried and exposed tyrosine residues (see text).

experimental titration curve as contrasted with curves in which only buried or exposed tyrosines data have been included. This observation is not affected by small changes in the corresponding parameters (λ, A). The experimental curve shows an initial increase in the apparent ellipticity at 239 nm without a noticeable shift in wavelength (fig. 1). This change in ellipticity takes place when the pH is raised from 6.0 to 9.0. In the calculated curve this change has been modeled by considering a small red shift of the actual position of the buried tyrosine residue from 234 to 239 nm. Such a displacement might be caused by a conformational change around the buried tyrosines which enhances hydrogen bonding. In the second segment of the experimental curve, which extends from pH 9.0 to 10.8, there is an increase in the apparent ellipticity at 241 nm, with a slight shift to 241.5 nm at pH 10.8 (fig. 1). This change was modeled by superimposing contributions from ionization of exposed tyrosines upon the contribution from the buried tyrosine residues. The exact manner in which this is done can be formulated in various ways. Agreement with the experimental titration curve (between pH 9.0 and 10.8) was obtained when the position of a third band, assigned to ionization of tyrosine residues, was shifted from 239 to 241 nm, and ellipticity was increased from 100 to 180 degree cm^2 dmol^{-1}. This increase in ellipticity, 80 degree cm^2 dmol^{-1} based on mean residue weight, compares favorably with the increase in ellipticity, 61 degree cm^2 dmol^{-1} based on mean residue weight, observed upon titration of *N*-acetyltyrosineamide from pH 9.1 to 10.9 (table 2). Sears and Beychok [1] and then Woody [9] have summarized the available data on application of CD spectra of simple model compounds for interpretation of proteins' spectra. Their data suggest that, in the alkali region, simple model compounds such as *N*-acetyltyrosineamide can be used as model compounds for the ellipticity contribution from exposed tyrosine residues to the 230–250 nm region in proteins. Using ellipticity values for *N*-acetyltyrosineamide, at pH 10.9, of about 77 degree cm^2 dmol^{-1}, based on mean residue weight, at 242 nm (table 2), it can be concluded that two or three exposed tyrosine contribute to the CD spectrum of ribonuclease at this pH. The possible contribution from disulfide bridges was not considered in the above treatment, since changes in ellipticity as a function of pH of these groups should not be significant.

Further support for the calculated values can be obtained by comparing the experimental alkaline titration of ribonuclease to that of *N*-acetyltyrosineamide. At pH 10.8 the maximum ellipticity for RNase A at 241.5 nm is about 433 degree cm^2 dmol^{-1} (table 1). If all three exposed tyrosines contribute to the intensity of the phenolate band in the enzyme (at pH 10.8 the buried tyrosine residues are not yet exposed) each exposed group should, by comparison with *N*-acetyltyrosineamide, contribute 77 degree cm^2 dmol^{-1}, or a total of about 231 degree cm^2 dmol^{-1} per mean residue weight. In our calculations a values of 180 degree cm^2 dmol^{-1} per mean residue weight was assigned to contributions from exposed tyrosine residues to the theoretical titration curve. The comparison with *N*-acetyltyrosineamide suggests that the additional increase in ellipticity observed near 239 nm in the titration of ribonuclease (433–231 = 202 degree cm^2 dmol^{-1} per mean residue weight) comes from buried tyrosine residues. In the calculated titration curve, 257–65 = 192

degree cm^2 $dmol^{-1}$ per mean weight was taken as the contribution of the buried tyrosine residues to the changes in ellipticity around 239 nm upon alkaline titration (table 4). There is a good agreement between the experimental and calculated values. It seems, therefore, that at neutral pH the contributions to the 239 nm band are mainly from buried tyrosine residues. However, as the pH is raised above pH 9.0 contributions from exposed residues are also observed.

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