Spectrophotometric titration of two ribonuclease derivatives

The problem of deciding what role, if any, the tyrosine residues of RNase play in maintaining the tertiary structure has attracted considerable attention in recent years. SHUGAR[1] and TANFORD *et al.*[2] have shown that three of the six tyrosine residues in RNase are anomalous, in that they can not be titrated until very high pH (above 12) is reached, and that when they are titrated, the molecule is irreversibly denatured. The other three residues are titrated normally and reversibly (pK = 10). SCHERAGA[3] has shown that when RNase is acidified, the part of the ultraviolet spectrum due to tyrosine shifts to lower wavelengths.

Two derivatives of RNase have been prepared and studied by spectrophotometric titration to both high and low pH. The derivatives were performic acid—oxidized RNase and the product of a limited pepsin digestion first prepared by ANFINSEN[4], pepsin—inactivated RNase. A Beckman spectrophotometer with a photomultiplier attachment was used. The low-pH experiments were carried out in 0.15 M KCl. The absorbance of the test solution was read using a neutral solution of equal concentration as a reference, and the negative difference in molar extinction at the maximum of the difference spectrum (287 mμ for RNase and pepsin-inactivated RNase; 284 mμ for oxidized RNase) was plotted against pH (Fig. 1).

When this was first done with pepsin-inactivated RNase, it was found that there were two steps in the titration curve, one at pH above 4, and other below pH 3. A plateau separated the two steps. By measuring the difference spectra of solutions at pH 1 and 6 compared to pH 3.5, it was found that only the former had the characteristic two-peaked shape indicative of a tyrosine spectral shift. The second step at higher pH was caused by an impurity extracted from the dialysis membranes in the preparation of the pepsin-inactivated RNase, since this step is absent in untreated RNase, but appears in similarly dialyzed RNase (Fig. 1). Various treatments of the membranes failed to effect any significant improvement. However, as Fig. 1 shows, RNase and pepsin-inactivated RNase are very similar in their spectral behaviour below pH 3, the only difference being that with the latter the shift occurs at a pH 0.5 unit higher than with RNase.

Oxidized RNase clearly behaves quite differently. The largest value of $-\Delta\varepsilon_{284}$ is about 350 and the curve has a slope much more like that expected for a titration curve than do the others. What is being observed here is presumably the neutralization of charged groups near the tyrosine residues, which is the explanation given by EDSALL *et al.*[5] for a similar result with tyrosine. The highest value of $-\Delta\varepsilon_{284}$ observed for oxidized RNase, 350, is about 6 times the value for tyrosine[5], which is 50.

The high-pH experiments were carried out in 0.1 M piperidine buffers adjusted to the desired pH with HCl or KOH. $\Delta\varepsilon_{295}$ (the extinction coefficient at the pH in question minus the extinction at neutral pH) was determined and plotted against the pH[6] (Fig. 2). Since our interest here is in the *shape* of the titration curve, and since the impurity in the RNase and pepsin-inactivated RNase preparations made concentration determinations difficult, we simply assumed that the plots for oxidized-RNase and pepsin-inactivated RNase levelled off at 16,000 (the value found by TANFORD *et al.*[2] for RNase), and that the point at the highest pH for RNase fell on

---

Abbreviations: RNase, ribonuclease; A, absorbance; ε, molar absorptivity or extinction coefficient.

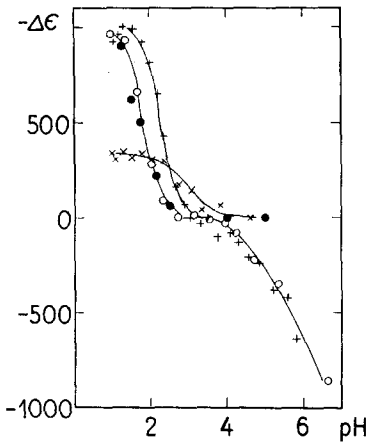

Fig. 1. Titration curves from difference spectra at low pH. ○ Dialyzed RNase. ● RNase (SCHERAGA[3]). + Dialyzed pepsin-inactivated RNase. × Oxidized RNase. All at 287 mμ except oxidized RNase which was done at 284 mμ.

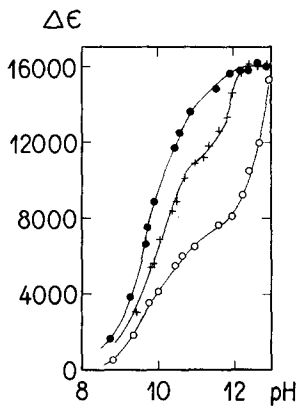

Fig. 2. Spectrophotometric titration curves at high pH. ○ Dialyzed RNase (line through these points is from data of TANFORD *et al.*[2]). + Dialyzed pepsin-inactivated RNase. ● Oxidized RNase.

TANFORD's curve. (The impurity does not significantly change its absorption in this range. The correction necessary was in no case larger than 11 %).

With oxidized RNase it is seen that all six tyrosine residues are titrated together at a pH of 9.8. With pepsin-inactivated RNase there are two steps in the titration curve, but this time they have the relative sizes of about 5:1. We conclude from this that performic acid oxidation, by destroying the secondary and tertiary structure of the molecule[7], has also made all the tyrosine residues normal. In contrast, the limited pepsin digestion appears to have made normal only two of the abnormal residues.

Pepsin-inactivated RNase, therefore, appears to be a derivative in which there is only 1 anomalous tyrosine residue, and yet it still gives the spectral shift at low pH. If we accept SCHERAGA's interpretation of the low-pH effect being due to the breaking of tyrosine-carboxylate bonds, it seems likely that only 1 of the 3 anomalous tyrosine residues of RNase is part of such a bond. If so, the other 2 must be anomalous for some other reason—perhaps they are hydrogen-bonded to non-ionizing acceptors.

We would like to thank Professor K. LINDERSTRØM-LANG for suggesting this problem, and Dr. P. E. WILCOX for many helpful discussions.

C. C. BIGELOW*
M. OTTESEN

Carlsberg Laboratory, Copenhagen (Denmark)

[1] D. SHUGAR, *Biochem. J.*, 52 (1952) 142.
[2] C. TANFORD, J. D. HAUENSTEIN AND D. G. RANDS, *J. Am. Chem. Soc.*, 77 (1955) 6409.
[3] H. A. SCHERAGA, *Biochim. Biophys. Acta*, 23 (1957) 196.
[4] C. B. ANFINSEN, *J. Biol. Chem.*, 221 (1956) 405.
[5] D. B. WETLAUFER, J. T. EDSALL AND B. R. HOLLINGWORTH, *J. Biol. Chem.*, 233 (1958) 1421.
[6] J. L. CRAMMER AND A. NEUBERGER, *Biochem. J.*, 37 (1943) 302.
[7] W. F. HARRINGTON AND J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, 30 (1956) 21.

Received January 9th, 1959

* Postdoctorate Overseas Fellow of the National Research Council of Canada, 1957–59.