THE DESTABILIZATION OF BOVINE PANCREATIC RIBONUCLEASE BY METAL IONS*

C. L. Herzig and C. C. Bigelow

Department of Biochemistry
University of Western Ontario
London, Canada

Received February 3, 1967

The inhibition of RNase by heavy metal ions has been studied by several workers. Ross, et al. (1962), made a study of the inhibition by Zn(II) ions and concluded that the metal was bound at one or more histidyl residues, and formed a ternary complex with the product cytidine-3'-phosphate. Girotti and Breslow (1966) recently studied the interaction of Cu(II) and Zn(II) with RNase in the presence of product and inhibitor cytidine phosphates, and also report the involvement of histidyl residues. We have studied the effects of Cu(II) and Zn(II) on the denaturation of RNase and have found a highly specific destabilization of the protein by both ions at low molar ratios of ion to protein.

Thermal and urea denaturation were followed by ultraviolet difference spectroscopy. Since the denatured complex tended to aggregate at higher pH's and since the binding of metal ion by RNase decreases at lower pH's, selection of a suitable pH for the denaturation experiments was an important consideration. Difference titration curves were produced as the difference in protons released by RNase at any pH in the presence and absence of metal ion, and the pH of half-maximum proton displacement by metal ion was

*This work was supported by the National Research Council and the Medical Research Council of Canada.

chosen as the pH to be used in the denaturation experiments. This was observed to be pH 5.55 for Cu(II) and pH 7.70 for Zn(II). These pH values actually represent half-maximum proton displacement over a range of molar ratios R, of total metal ion to RNase of 1 to 2; pH control to three significant figures was maintained. In the case of Cu(II), protons released in the region of hysteresis observed at alkaline pH (Girotti and Breslow, 1966) are not included in the estimates of maximum proton displacement. The assumption was then made that, for the first four ions bound, maximum proton displacement in the acid pH range is a linear function of the molar ratio. This assumption is supported by the portion of the titration curves outside the region of hysteresis and by Girotti and Breslow (1966), who also find that the first four binding sites for copper appear titrimetrically equivalent.[1] The metal ions were added as nitrates and no buffers or other materials capable of complexing heavy metal ions were added to the denaturing solutions. The RNase concentration was 2.5×10^{-4} M.

Figure 1 shows the effect of Cu(II) on difference spectra measured with a Cary 15 spectrophotometer during thermal denaturation. The lower difference spectrum in the figure is a result of the usual denaturation blue shift observed in the absence of metal ion (Hermans and Scheraga, 1961). The two upper difference spectra were observed when Cu(II) was present in both sample and reference solutions at molar ratios, R, of total metal ion to RNase of 0.97 and 8.36. The difference spectra in Fig. 1 were recorded near the transition temperature of the particular experiment, but other measurements were made at temperatures high enough to complete the denaturation. All the difference spectra show the

---

[1] There is good agreement between our unpublished titration results and those of Girotti and Breslow (1966). The only significant disagreement is that we find some aggregation in the region of hysteresis at all values of R. There is reason to believe that this aggregation is related to the history of the protein sample.

expected minimum at about 287 mμ, though it is seen that other spectral effects interfere with the observation of the minimum normally observed at 280 mμ. Cu(II) lowers the transition temperature and causes an appreciable displacement of the entire difference spectrum to higher absorbance values. This displacement seems to be due partly to light scattering caused by aggregation in the solution containing the denatured protein and partly to new absorption by the copper-protein complex; the latter is inferred because the aggregation alone is unlikely to cause changes in

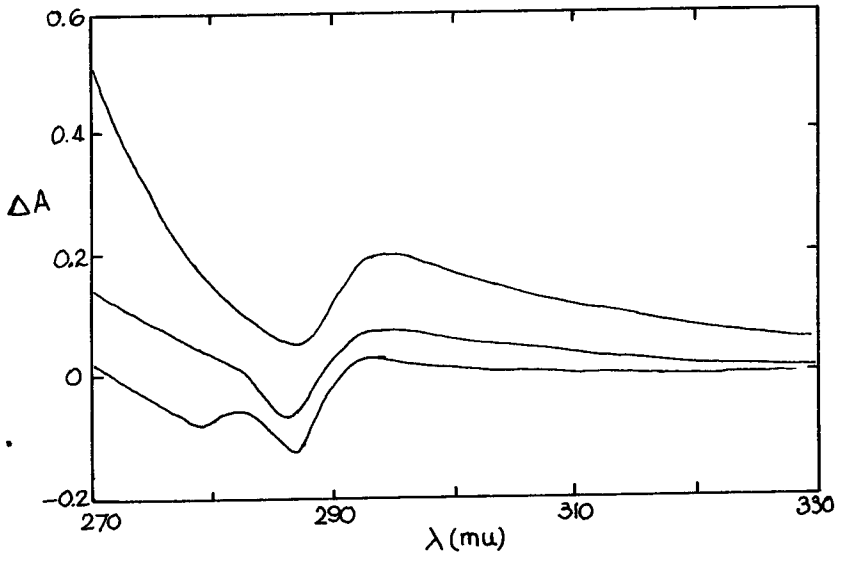

Fig. 1 Effect of Cu(II) on the RNase Denaturation Difference Spectrum R values for the curves, from top to bottom: 8.36, 0.97, 0.00.

---

the essential features of the difference spectra (Bigelow, 1961). It was then necessary to correct the difference spectra for these effects, so that a value for the denaturation blue shift alone could be determined. A plot of the logarithm of ΔA vs. wavelength turned out to be linear for data above 300 mμ. This plot was extrapolated to 287 mμ and the value so determined was subtracted from the uncorrected value. This type of correction has been applied to difference spectra before this (e.g., Bigelow, 1961) to remove light scattering contributions.

The corrected absorbance changes were converted to molar extinction changes and plotted against the temperature in Fig. 2. For clarity, curves for only the same three values of R shown in Fig. 1 are drawn, but curves determined at other values of R are consistent with the

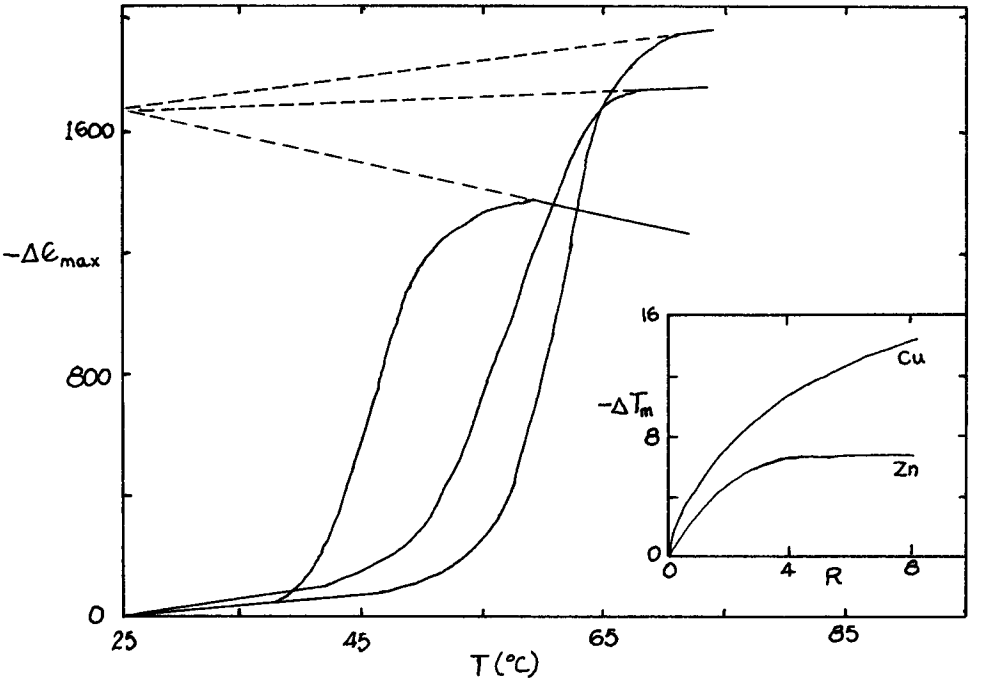

Fig. 2 Thermal Denaturation of RNase in the Presence of Cu(II). R values for the curves, from left to right, 8.36, 0.97, 0.00
Inset: Effect of Cu(II) and Zn(II) on the transition temperature of RNase.

interpretation below. Three effects of the Cu(II) ions can be seen: (i) the transition temperature is lowered as the metal ion concentration rises; (ii) the value of $-\Delta\epsilon$ is lower at higher metal ion concentrations; and (iii) the linear slope of $-\Delta\epsilon$ vs. temperature changes at high temperatures from positive to negative as the ion concentration is raised.

The linear slope of these plots at high temperatures allows us to correct the data for this temperature effect. If all the data are extrapolated back to 25°, they meet at a $-\Delta\epsilon$ value of 1700, the value normally found for thermal denaturation (Hermans and Scheraga, 1961; Bigelow, 1964). Why the value and sign of these slopes should depend

on the ion concentration is not now understood. These extrapolations thus show that the essential features of thermal denaturation are unchanged by the presence of the ions, and the denatured state II, with tyrosyl residues B and A normalized (Bigelow, 1964), is still produced.

Data obtained from thermal experiments using Zn(II), and from urea denaturation in the presence of Cu(II), are similar to the results presented here for melting experiments in the presence of Cu(II). Cu(II) lowers the molarity of urea needed to cause denaturation but the denatured state of the protein, state III (Bigelow, 1964), is again the same as that in the absence of metal ion. A significant finding in the experiments involving Zn(II) is that this ion seems to be about half as effective in the destabilization of RNase as is Cu(II). This is shown in the inset in Fig. 2 where the changes in transition temperatures from melting curves are plotted against R for both metal ions. A few experiments were carried out at different pH values to ensure that the observed differences in the effectiveness of these ions were not due mainly to differences in the pH's at which the experiments were done. Preliminary experiments show that the lowering of the transition temperature observed by difference spectrophotometry is also observed by polarimetry.

Since only some fraction of the first mole of metal ion bound would be expected to be localized at the most conformationally-sensitive site, statistical factors need to be taken into account in the interpretation of the inset of Fig. 2. Such factors are difficult to estimate at present, particularly since Girotti and Breslow (1966) offer evidence for some degree of cooperative binding. We then infer that there is probably no more than one conformationally-sensitive binding site for Zn(II) and probably no more than two for Cu(II); binding at additional sites does not cause destabilization. The fact that RNase is competitively inhibited

by these ions implies that the active center is also a metal-binding site or is influenced by a metal-binding site; thus the inhibition and destabilization of the enzyme may occur at or near the same site. This interpretation demands that both inhibition and destabilization occur at equivalent values of metal ion bound. The work of Girotti and Breslow (1966) provides evidence to support such an interpretation. In addition, Crestfield, Stein, and Moore (1963) have shown that the presence of seven equivalents of Cu(II) inhibits the alkylation of histidyl residues at the active center. An alternative approach should be possible through the use of derivatives of RNase, in which basic amino acid residues at the active center have been modified. Such derivatives are being prepared for further study.

REFERENCES

Bigelow, C.C., J. Biol. Chem., 236, 1706 (1961).
Bigelow, C.C., J. Mol. Biol., 8, 696 (1964).
Crestfield, A.M., Stein, W.H., and Moore, S., J. Biol. Chem., 238, 2421 (1963).
Girotti, A.W. and Breslow, E., J. Biol. Chem., 241, 5651 (1966).
Hermans, J., Jr. and Scheraga, H.A., J. Am. Chem. Soc. 83, 3283 (1961).
Ross, C.A., Mathias, A.P., and Rabin, B.R., Biochem. J. 85, 145 (1962).