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Apparently Opposing Effects of Temperature and Guanidinium Chloride in the Denaturation of Ribonuclease A

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The thermal denaturation of ribonuclease A in the presence of guanidinium chloride (GdmCl) was studied by means of circular dichroism (CD). In the presence of GdmCl the transition temperatures decrease with increasing denaturant concentration. However, closer examination of the results obtained shows the following feature: the negative values of molar ellipticity decrease with temperature in the absence of the denaturant; after the addition of the denaturant ellipticity minima appear at temperatures which depend on the denaturant concentration. The higher the GdmCl concentration the lower the temperature of the minimum. In 4 molar (and higher) GdmCl the minimum does not appear and the negative molar ellipticity increases throughout the whole temperature range examined. After reduction of the disulfide bonds, similar behaviour is observed with the minimum at each denaturant concentration being shifted towards a lower temperature. Though there is no obvious explanation for this behaviour, it appears that at the temperatures above the minimum some secondary structure is regained owing to decreased protein denaturant interactions.

INTRODUCTION

Ribonuclease A is one of the most studied enzymes (for a recent review see ref 1). Its denaturation has also been studied extensively[2,3]. It has been found by use of viscometry, difference spectroscopy, and optical rotatory dispersion (ORD) that the product of thermal denaturation, though highly unordered, is not a random coil, but that it retains regions of ordered structure[4-6]. On the other hand, denaturation by guanidinium chloride (GdmCl) yields a randomly coiled product[7,8]. Among the methods which have recently proven particularly useful for the study of protein denaturation is also circular dichroism (CD). Thus there are several studies by CD of various modes of denaturation of ribonuclease A[9-13]. Since no systematic study of the effect of GdmCl on the thermal denaturation of ribonuclease A, has as yet been performed it appeared worthwhile to examine the effect in more detail using CD. In this context the role of the four disulfide bonds present in the ribonuclease molecule is also of interest. Therefore, measurements were also performed with the reduced enzyme, that is, the enzyme in which the disulfide bonds were split. A

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preliminary account of this work has already been published[14]. Somewhat similar studies of ribonuclease A in urea solution have been performed by Foss and Schellman[15]. However, there the situation is immensely more complicated since in urea solutions an equilibrium exists between urea and ammonium cyanate, which shifts with increasing temperature in favour of cyanate.

EXPERIMENTAL

Bovine pancreatic ribonuclease A was obtained from Sigma Chemical Co. (Type III-A, Lot No. 107B-1290). Before using it in measurements, it was dialyzed for 24 h at 4 °C against the solvent. Ultra pure GdmCl was purchased from Schwarz/Mann. All other reagents used were of the best available grades.

Solutions of ribonuclease A (0.3 to 2 mg/ml) were prepared either in 1/15 mol/dm^3 phosphate buffer pH = 8.03 or in 0.01 mol/dm^3 Tris-HCl, pH = 7.7. Reduction of the disulfide bonds was performed either in 0.1 mol/dm^3 2-mercaptoethanol or 0.02 mol/dm^3 dithioerythritol and 8 mol/dm^3 urea or 6 mol/dm^3 GdmCl. The pH of the solutions was adjusted to 8.0 by the addition of 0.1 mol/dm^3 NaOH. The -SH groups were subsequently treated with iodoacetamide. After reduction and carbamidomethylation, solutions were dialyzed against the buffer used in the experiments. Although Tris is less suitable for work at elevated temperatures, no difference in CD spectra was observed between samples prepared in either buffer or by different procedures.

In order to eliminate the possible influence of phosphate, experiments in which the effect of GdmCl on the thermal denaturation of ribonuclease was studied were performed in 0.01 mol/dm^3 Tris-HCl, pH = 7.7.

CD spectra were recorded with a Roussel-Jouan Dichrographe Mark III. In the wavelength range 240 to 320 nm silica cells of 1.0 cm pathlength were used, and in the wavelength range below 240 nm silica cells of 0.01 and 0.05 and 0.1 cm. The instrument was calibrated using epiandrosterone (supplied by the manufacturer) in dioxane. The estimated relative error increases from about 1% above 250 nm to about 10% at the lowest wavelength. The temperature was raised at 5 °C or 10 °C intervals. After allowing about 30 min for thermal equilibration, CD spectra were recorded. During this time the change of CD spectrum at constant wavelength was recorded. Usually, after 15 min constant readings were observed. Over a period of several hours no further changes occurred. Upon cooling, no significant changes of molar ellipticity were observed even at the lowest wavelength of measurement.

The molar ellipticity $[\Theta]_{mrw}$ in deg cm^2/dmol, was obtained by using the equation:

$$[\Theta]_{mrw} = \frac{M_o \Theta}{100\ cl}$$

where M_o is the mean residue molecular weight, 110.5; Θ is the ellipticity, c is the concentration in g/cm^3 and l is the pathlength in dm.

RESULTS AND DISCUSSION

Results of thermal denaturation studies in the presence of GdmCl are given in Figures 1—4. For obvious reasons, only the far-ultraviolet region was investigated. The addition of GdmCl to solutions of the enzyme, as expected, lowers the temperature of the transition to the unfolded state which is reflected in the shift of negative molar ellipticities below 240 nm towards smaller values. However, the negative values of molar ellipticity, which in thermal denaturation decrease invariably with increasing temperatures, reach in the presence of the denaturant a minimum depending on the denaturant concentration. The higher the concentration the lower the temperature of the minimum (Figure 4). Furthermore, in 4 mol/dm^3 GdmCl the minimum does not appear and with increasing temperature negative molar ellipticities increase only. Reduction of

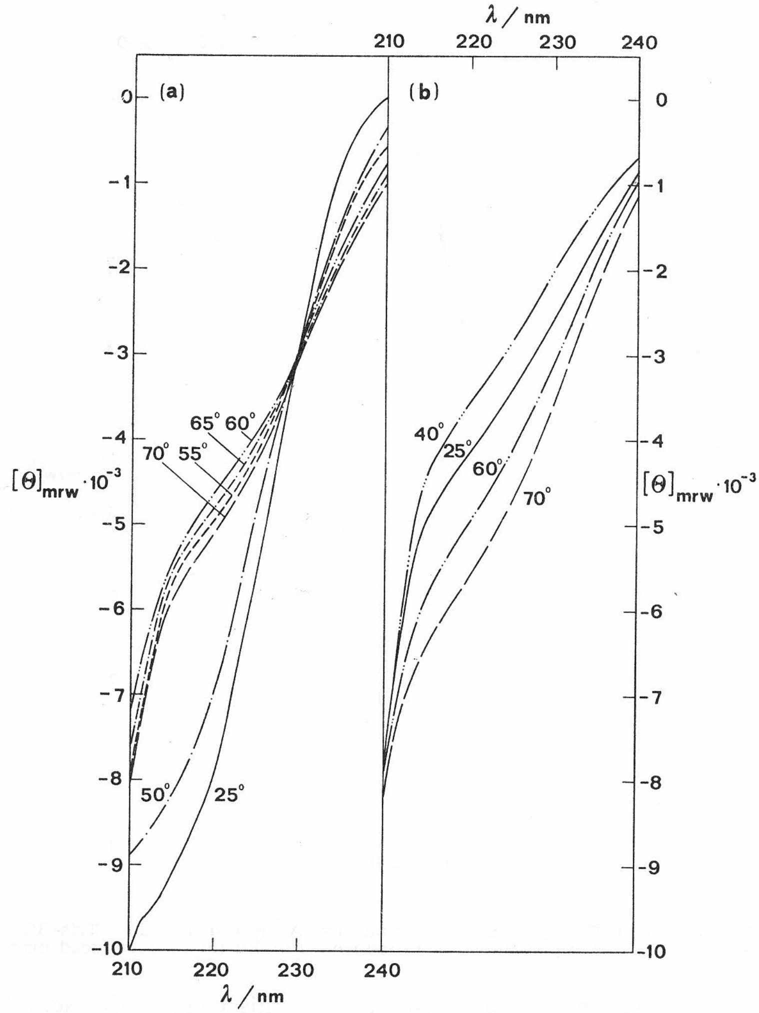

Figure 1. Far-ultraviolet CD spectra of ribonuclease A in 0.01 mol/dm³ Tris-HCl-1 mol/dm³ GdmCl at different temperatures, (*a*) unreduced protein; (*b*) fully reduced protein.

the disulfide bonds does not significantly change this pattern, only the temperature of the minimum becomes lower.

An entirely convincing interpretation of this behaviour is hardly possible. In other words, owing to insufficient knowledge of the processes at molecular level, the attempted interpretation has to be to some extent speculative. If we first consider the trend of the changes in molar ellipticity with increasing temperature, we note that at each GdmCl concentration below 4 mol/dm³ there is a certain temperature of maximal disorder. Additional heating then causes the regaining of some of the secondary structure. This in turn could mean that the interaction (e. g., binding) between the denaturant and the protein decreases with increasing temperature, if we assume that the inter-

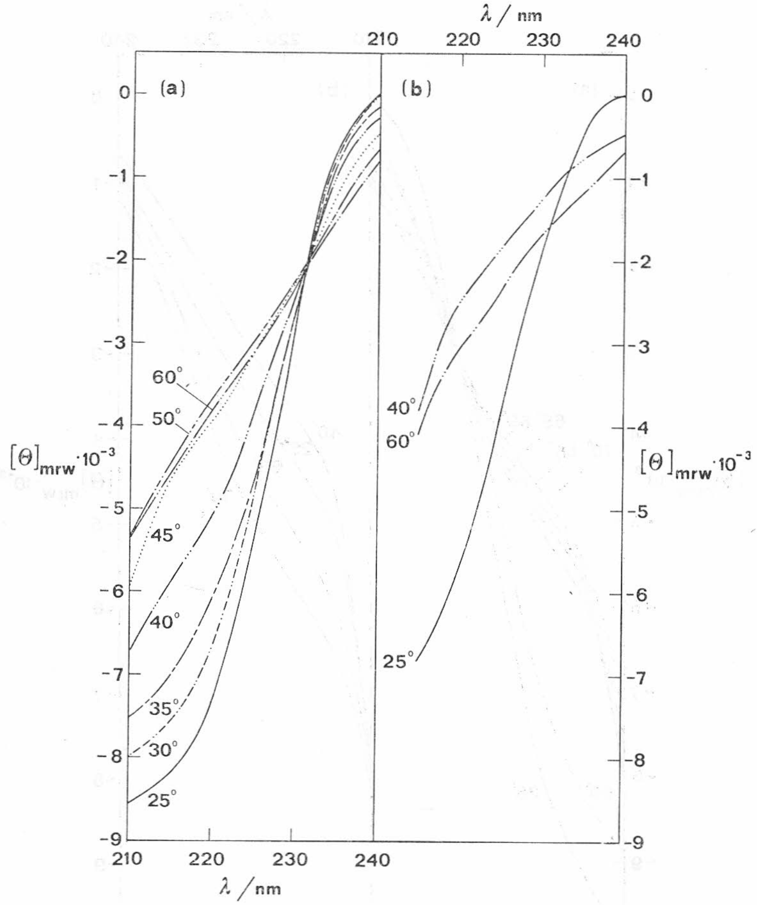

Figure 2. Far-ultraviolet CD spectra of ribonuclease A in 0.01 mol/dm^3 Tris-HCl-2 mol/dm^3 GdmCl at different temperatures, (*a*) unreduced protein; (*b*) fully reduced protein.

action is largely responsible for unfolding[3]. In this context the actual sites of binding are of secondary importance though partial regaining of the ordered structure indicates that among the binding sites which matter are the peptide bonds. We will have more to say about this later on. Furthermore, since the interaction is lessened, the change of spectrum due to the effect of temperature alone should become more and more dominant. In other words, the values of molar ellipticity below 240 nm become comparable to those observed in the absence of denaturant[9–13]. The calculated difference spectrum between 70 °C and 40 °C in 3 mol/dm^3 GdmCl (Figure 3d) seems to support some regaining of ordered structure, considering its course and the position of the minimum. On the other hand, direct proof of the proposed interpretation would also require the knowledge of how the denaturant binding changes with temperature. This, however, cannot be determined in an unambiguous way[16]. Simple calculation also shows that the changes in molar ellipticity observed cannot be attributed to changes in volume with increasing temperature.

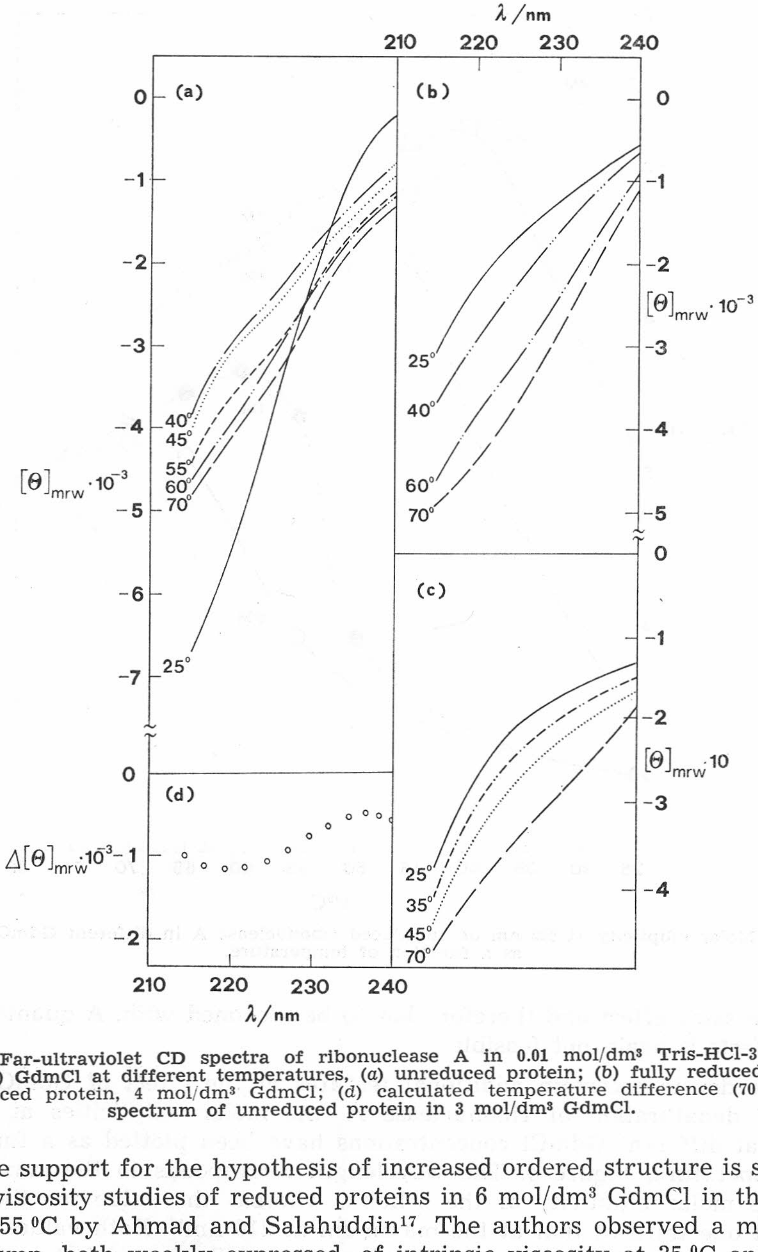

Figure 3. Far-ultraviolet CD spectra of ribonuclease A in 0.01 mol/dm³ Tris-HCl-3 mol/dm³ (4 mol/dm³) GdmCl at different temperatures, (*a*) unreduced protein; (*b*) fully reduced protein; (*c*) unreduced protein, 4 mol/dm³ GdmCl; (*d*) calculated temperature difference (70 °C—40 °C) spectrum of unreduced protein in 3 mol/dm³ GdmCl.

Some support for the hypothesis of increased ordered structure is supplied also by viscosity studies of reduced proteins in 6 mol/dm³ GdmCl in the range 25 °C to 55 °C by Ahmad and Salahuddin[17]. The authors observed a minimum and a hump, both weakly expressed, of intrinsic viscosity at 35 °C and 40 °C, respectively. At higher temperatures the intrinsic viscosity invariably decreased with an increase in temperature. The behaviour was attributed to facilitation of backbone rotations which may cause a decrease in the dimensions and thus the intrinsic viscosity of the randomly coiled protein. While the effect certainly exists, it could hardly explain the large decrease in intrinsic viscosity between 40 °C and 55 °C. On the other hand, regaining of the secondary structure would

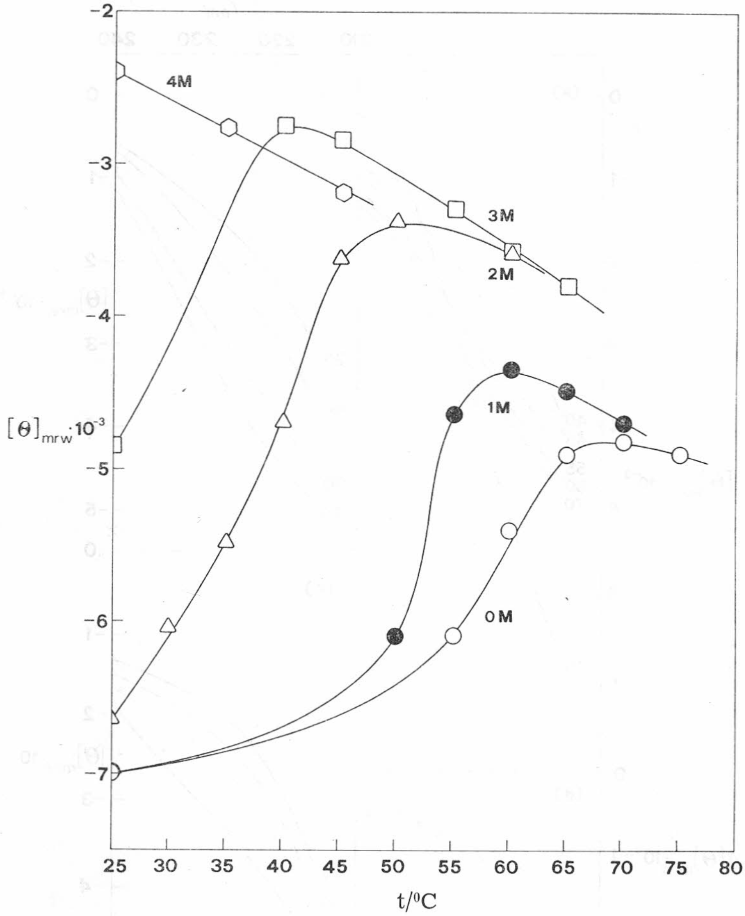

Figure 4. Molar ellipticity at 222 nm of unreduced ribonuclease A in different GdmCl solutions as a function of temperature.

have the same effect and therefore has to be reckoned with. A quantitation of both effects is again not feasible.

In order to ascertain additional features of the effect of GdmCl on the thermal denaturation of ribonuclease A, the molar ellipticities at 222 nm, $[\Theta]_{mrw}$, at different GdmCl concentrations have been plotted as a function of the temperature, Figure 4. The wavelength corresponds to the maximum of negative molar ellipticity of the α-helix, whereas the value for the β-form is about one third of that at the maximum at 216 nm[18]. Both values are considerably larger than that for the unordered form. Changes in $[\Theta]_{mrw}$ at 222 nm should then predominantly reflect changes in secondary structure. Examination of Figure 4 reveals that where unfolding takes place the curve is typical, that is, sigmoidal[2], and - $[\Theta]_{mrw}$ decreases with increasing temperature in the unfolding region. After that, in the post-transition region, - $[\Theta]_{mrw}$ increases with increasing temperature. Accordingly, in 4 mol/dm^3 GdmCl, where unfolding at 25 ^{0}C is complete, only the latter behaviour is observed. Von Hippel and Wong[19] who studied the effect of temperature on the specific rotation at 366 nm, $[\alpha]_{366}$, of ribonuclease A at different GdmCl concentrations observed similar behaviour, only the changes were in the opposite direction, that is, $-[\alpha]_{366}$ increased with increasing temperature (unfolding). Moreover, in 3.75 mol/dm^3 GdmCl a nearly linear decrease in $-[\alpha]_{366}$ was observed with increasing temperature from which it was concluded that at 25 ^{0}C ribonuclease A in 3.75 mol/dm^3 GdmCl was randomly coiled. The decrease was interpreted in terms of a temperature effect, that is, greater flexibility, and possible other factors were not considered. Though the effect of temperature, as in the case of intrinsic viscosity, cannot be neglected, the contribution due to increased order produces the same effect. Aune et al.[6], who followed the optical rotation of ribonuclease A at 400 nm and 56 ^{0}C as a function of GdmCl concentration, also observed its monotonic decrease with increasing temperature above 4 mol/dm^3. The effect was again attributed to greater flexibility which diminishes the optical rotation. Other possible factors have not been considered. There is also a general comment that can be applied to both studies: changes in optical rotation above 300 nm cannot be considered satisfactory indicators of secondary structure and therefore such data have to be supplemented by additional evidence.

In conclusion, it can be stated that the studies by CD of the effect of GdmCl on the thermal denaturation of ribonuclease A have disclosed some unexpected features. The interplay of both factors, that is, the temperature and the denaturant concentration proceeded at lower temperatures as expected, and the transition temperature decreased with increasing denaturant concentration. However, at high temperatures, in the presence of the denaturant, partial regaining of secondary structure was detected. The interpretation offered for the behaviour, though somewhat speculative and highly qualitative, appears plausible. Furthermore, it supports the binding hypothesis of the GdmCl denaturing activity[3]. Namely, in addition to the charged groups on the protein, the backbone peptide groups should be the main binding sites for the guanidinium ion[16]. Lessening of the interaction with temperature would then allow regaining of some ordered structure, that is, partial refolding. Here the role of water molecules, which are also competing for the same binding sites, cannot be ignored. When more data by CD and other methods on such ternary systems at higher temperatures become available, then perhaps a more quantitative and completely unobjectionable interpretation could be proposed.

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POVZETEK

Navidezno nasprotni efekti temperature in gvanidinijevega klorida pri denaturaciji ribonukleaze A

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Termično denaturacijo ribonukleaze A v prisotnosti gvanidinijevega klorida smo raziskovali z metodo cirkularnega dikroizma. V prisotnosti tega denaturanta temperature prehoda padajo z naraščanjem njegove koncentracije. Podrobnejši pregled dobljenih rezultatov pa pokaže, da se pojavijo minima molarne eliptičnosti, ki so odvisni od koncentracije gvanidinijevega klorida. Čim večja je njegova koncentracija, tem nižja je temperatura minimuma. V 4 mol/dm^3 in višjih koncentracijah se minimum ne pojavi več in negativna molarna eliptičnost narašča v celotnem temperaturnem območju. Po redukciji disulfidnih vezi opazimo slično obnašanje, le da se minima pojavijo pri nižjih temperaturah. Preproste razlage za ta pojav ni, vendar se zdi verjetno, da pride nad temperaturo minimov do delne obnovitve sekundarne strukture, kar je posledica zmanjšanja interakcij med proteinom in denaturantom.

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