Bull. Inst. Chem. Res., Kyoto Univ., Vol. 51, No. 3, 1973

Thermally Induced Denaturation of Intramolecularly Cross-linked Bovine RNase A

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Received May 28, 1973

Thermally induced conformational denaturation of intramolecularly cross-linked bovine RNase A (Lys^{7}-Lys^{37} or Lys^{31}–Lys^{37}) was investigated to compare the non-modified protein. At pH 3.1, a transition temperature of 51° (RNase A, 46.5°) was obtained from difference spectra at 286 nm. Thermodynamic analysis of a transition gave the following functions (the values for non-modified RNase A are in parentheses): $\Delta H°=47$ (51), $\Delta F°=2.32$ (1.91) in Kcal/mole, $\Delta S°=145$ (160) e.u./mole, at 35°. The facts that $\Delta H°$ and $\Delta S°$ per residue in an ordered region seem to be remained constant irrespective of polypeptide species are commented.

INTRODUCTION

Protein is synthesized as a linear polymer of amino acids. Sometimes intramolecular cross-linking is present in a protein molecule, combining distant portions of a polypeptide chain, and plays a role such as to stabilize the secondary or tertiary structure of that molecule or to bring special portions of the chain close together as seen in an active center of an enzyme. A disulfide bridge of cystinyl redidue in proteins serves as an example of naturally occurring intramolecular crosslinkage and it is considered to contribute for moulding the chain into a specific spatial configuration. There are only few knowledges other than a cystinyl bridge as naturally occurring cross-links, even though many organic compounds have been introduced recently to bring artificially a crosslinkage into a protein molecule.[1)]

Introduction of an artificial crosslinkage into a molecule enables us: (1) to estimate the distance between sites which are bridged, (2) to study the effect of a crosslinkage on conformational stability of a protein polypeptide chain. Many studies have been carried out with the first view in these days, applications being even expanded to evaluate the distance between polypeptide chains of protein subunits. With the second aspect we have received almost no reports, whereas many valuable informations on conformational stabilities of protein and also on steric or kinetic effects in enzyme mechanisms are expected in this approach. In this paper we will present the evidence that an intramolecularly cross-linked protein is actually more resistant to heat denaturation. Bovine RNase A treated with dimethyladipimidate was chosen as a model substance.[2)]

EXPERIMENTAL

Materials. Ribonuclease A (RNase A, bovine) was purchased from Miles, England, and purified by chromatography on IRC-50 (3.8×40 cm, prepared according to Hirs,

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Moore and Stein,[3] preliminary equilibrated with 0.14 M phosphate, pH 6.35). IRC-50 was supplied as CG-50 from Mallinckrodt Chemical Works. Elution was carried out at a flow rate of about 120 ml per hour with the aid of a 4-chamber gradienter—four chambers were connected in series from the first, which was the outlet to a column, to the fourth chamber, the first three containing 500 ml of 0.14 M phosphate, pH 6.35 and the last chamber 500 ml of 1.0 M phosphate, pH 6.48. Under this condition, the separation of RNase B from RNase A was as good as a peak of B was well separated from that of A. The fraction containing RNase A were collected, desalted by passing a column of Amberlite MB-1, concentrated by an ultrafiltration, then lyophilized. Trypsin (Type B, lot 900438) was obtained from Calbiochem.

Dimethyladipimidate, prepared from sodium cyanide and 1,4-dichlorobutane according to the method of McElvain and Schroeder,[4] was recrystallized from methanol–dioxane just before use. M.p. 217–219° (m.p. of adipamide into which adipimidate was transformed during melting point measurement). All other reagents used were of the highest grade commercially available and was used without further purification.

Preparation of Cross-linked RNase A. This was performed as described in ref. 2, except for a few slight modifications: pH of the reaction mixture was kept constant by a pH stat (Radiometer, Model TTT1c), and the monomer fractions of Sephadex G-75 chromatography were further purified with chromatography on an IRC-50 column. IRC-50 chromatography was carried out with gradient elution (4-chamber system, each of the first three chambers contains 50 ml of 0.15 M phosphate, pH 6.40, the fourth chamber the same amount of 1.0 M phosphate, pH 6.47) at a flow rate of 50 ml per hour for 100 mg loading on a 0.8×35 cm column). Pooled fraction for modified RNase was dialyzed, lyophilized, and stored frozen.

Spectral Measurements. Difference spectra were obtained with a Shimadzu Multipurpose MPS-50L spectrophotometer equipped with jacketed cell holders. Temperature of the sample-side cell holder was regulated by passing water of desired temperature from an external thermostated circulating bath. In the measurements of thermal denaturation, equilibration of at least 30 minutes was allowed at each temperature before measurements. A reference cell containing the same concentration of protein as a sample cell was kept constant at 20°. Other routine spectral works were carried out with a Hitachi 124 spectro-photometer coupled with a Hitachi QPD-54 recorder.

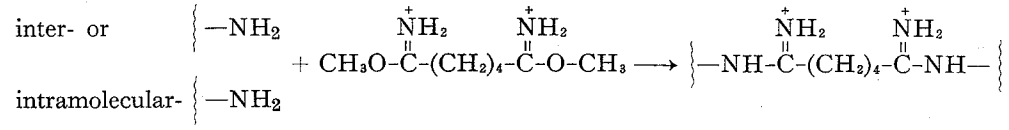
Amino Acid Analysis. Samples were dissolved or suspended in 6 N hydrochloric acid, frozen by dipping into liquid nitrogen, evacuated under 10^{-2} mm/Hg, hydrolyzed for 24 hours at 110°. Hydrolysates were analyzed with a JEOL liquid chromatograph system using the basic procedure of Spackman, Stein, and Moore.[5] Adipamidino-bis-L-lysine was analyzed according to the method of ref. 2.

General. Enzyme activities toward RNA were measured according to the method of Anfinsen *et al.*[6] using the single lot number of RNA which was gifted from Dr. K. Miura. Activities toward 2′,3′-cyclic cytidylic acid were obtained by means of the spectrophotometric method of Crook *et al.*[7] Records of change in difference of absorbance were analyzed by plotting Δ absorbance *vs.* time semilogarithmically to evaluate the apparent first order kinetic rate constants. Rate constants were then plotted against enzyme concentrations (2 to 20 micro g/ml) and the limiting slope toward enzyme concentration= 0 were taken as the activities. SDS-polyacrylamide gel electrophoresis was kindly per-

formed by Mr. H. Ueno in this laboratory in the usual manner. For the purpose of making peptide maps a Toyo Model V high voltage DC generator and Tank were used.

RESULTS

Modification of RNase A. Dimethyladipimidate reacted with ε-amino groups of lysyl residues in RNase A to give a cross-linked polypeptide chain. If the cross-linking reaction occurred only in a molecule, intramolecularly cross-linked RNase A was obtained, but another possibility that dimethyladipimidate reacted with ε-amino groups of lysyl residues in different molecules to afford intermolecularly linked RNase, could not be excluded. Actually the presence of polymeric forms of RNase are shown in Fig. 1, which represents the gel filtration pattern of the reaction products. Good separation of cross-linked products into monomeric, dimeric and higher polymeric fractions was obtained. Electrophoretic patterns of each fraction on polyacrylamide gel in the presence of



sodium dodecyl sulfate accounted for that the fraction denoted as "monomer" in Fig. 1 was almost completely homogeneous in molecular weight of about 14,000, the fraction "dimer", about 27,000. In the following discussions the modified RNase A means exclusively the monomeric form unless otherwise noted.

It was essential to have the modified RNase A being free from the native one, and this was clearly demonstrated to be achieved in our case by chromatography on IRC-50 (Fig. 2). Fig. 2 shows the analytical chromatographic patterns of the modified RNase

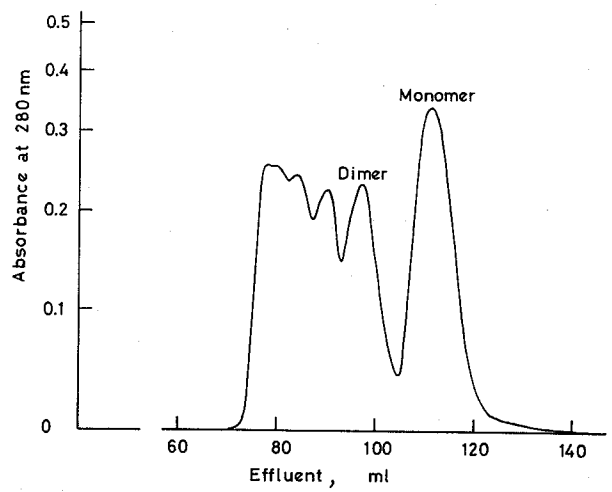


Fig. 1. The gel filtration pattern of reaction products of RNase A and dimethyladipimidate (200 mg protein, Sephadex G-75, 2.0×120 cm). Elution was carried out with 0.1 M ammonium formate and effluent was monitored continuously at 280 nm with a JEOL JLC-BC_2 optical detection system.

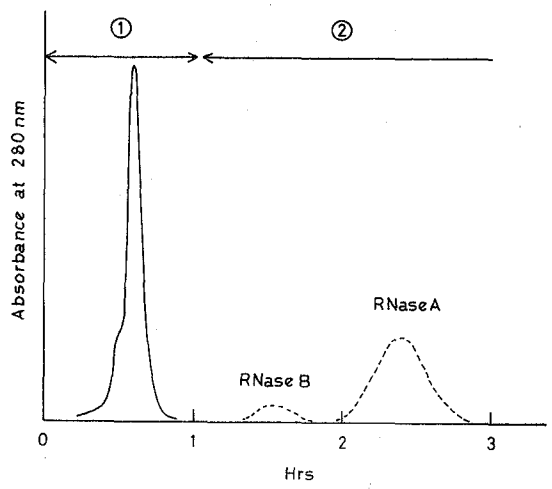


Fig. 2. Chromatography of adipimidate treated RNase A. Eluting condition: 1, 0.1 M phosphate, pH 6.40; 2, gradient elution from 0.15 M (pH 6.40) to 1.0 M (pH 6.47) phosphate. 10 mg of protein on IRC-50, 1.0×30 cm.

A to show the complete absence of the starting native protein.

Identity of our modified RNase A and that of Hartman and Wold[2] was evidenced by comparison of a peptide map of tryptic digests of performic acid oxidized modified RNase A. As apparent in Fig. 2, the chromatographic peak for modified RNase A was asymmetric, perhaps reflecting the fact that modified RNase A was a mixture of two components (one component having a crosslinkage between Lys^{7}-Lys^{37} and the other between Lys^{31}-Lys^{37}) as mentioned by Hartman and Wold.[2] Further attempts to separate the mixture into constituents were not performed in this study. Molecular extinction coefficient for the modified RNase A, monomer, was estimated as 11,600 at 276 nm, where the absorption was maximum. The λ_{max} is slightly shifted to shorter wave length compared to 277.5 nm of the native protein. Dimeric form gave more hypsochromic shift, absorption max. at 275 nm. Activities of modified RNase's are listed in Table I, where relative activities to native protein are shown. Modified RNase A, monomer, showed the activity of 50% higher than the native form when 2′,3′-cyclic cytidylic acid was used as a substrate. Dimeric and polymeric fractions showed values as little as 50% of the reported ones.

Thermally Induced Denaturation. The typical difference spectra, which were obtained when the samples in 0.01 M citrate at pH 3.1 (0.1 M KCl was included) was heated

Table I. Relative Activities of RNase's.

		Substrate	
		RNA	2′,3′-cyclic CMP
Native RNase A		100	100
Modified RNase A	Monomer	83, 85[a]	152, 160[a]
	Dimer	73	80, 135[a]
	Polymer	51	67, 130[a]

a) Values reported in ref. 2.

while the reference was kept at constant temperature (20°C), are shown in Fig. 3 (monomer), 4 (dimer), and 5 (native RNase A). All three proteins showed a decrease in absorbance between 260 nm and 290 nm, the range that usually tyrosyl or tryptophyl residue gave an intense absorption. In the present case, only tyrosines contributed to the absorption around 276 nm because RNase A had no tryptophans, leading to the conclusion that the observed decrease in extinction upon thermal unfolding of the protein polypeptide chain could be originated from the environmental changes of tyrosyl residues. Difference spectrum was a little different for native RNase A and modified proteins, the latter, however, being almost identical to each other. ΔA_{max} was observed at 286–287 nm for all cases, whereas an inflection at about 280 nm in RNase A became a local ΔA_{max} in modified proteins. These change in molecular extinctions with the raise of temperature could be plotted as a function of temperature as in Fig. 6. Qualitatively it is clear that the transition (conformational change) from the native to the denatured state occurred at higher temperature with the modified proteins. As a check of reversibility, it was confirmed that the protein samples heated up to 70° gave the original extinction coefficients within a difference as little as 5%.

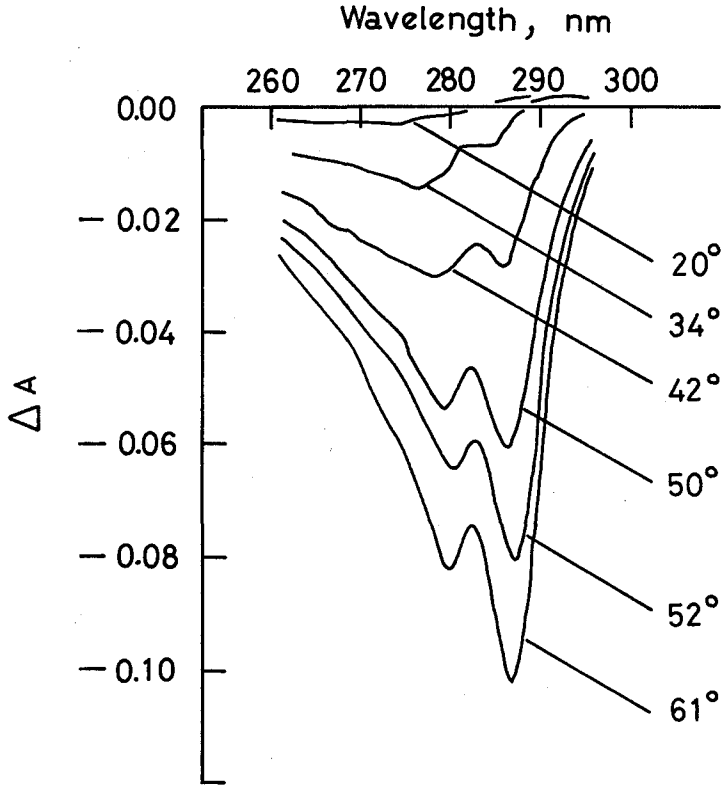


Fig. 3. Examples of difference spectra of modified RNase A, monomer, at several temperatures. Protein in pH 3.1 citrate (0.01 M) and 0.1 M kCl to afford 1 mg/ml solution was divided into two solutions, one of which was placed in a sample cell, the other in a reference cell of a spectrophotometer. Temperature of the sample cell was varied as indicated by inserted figures, while the reference was kept constant at 20°.

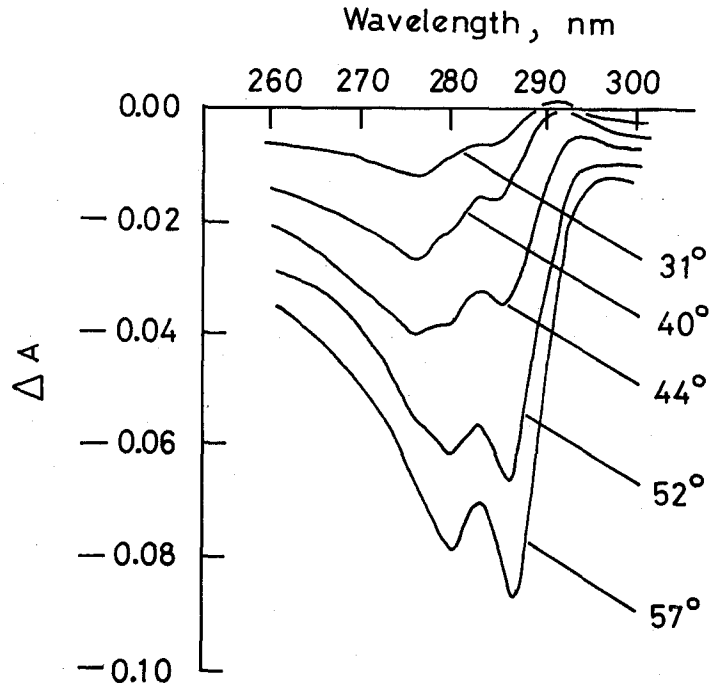


Fig. 4. Difference spectra of modified RNase A, dimeric form.

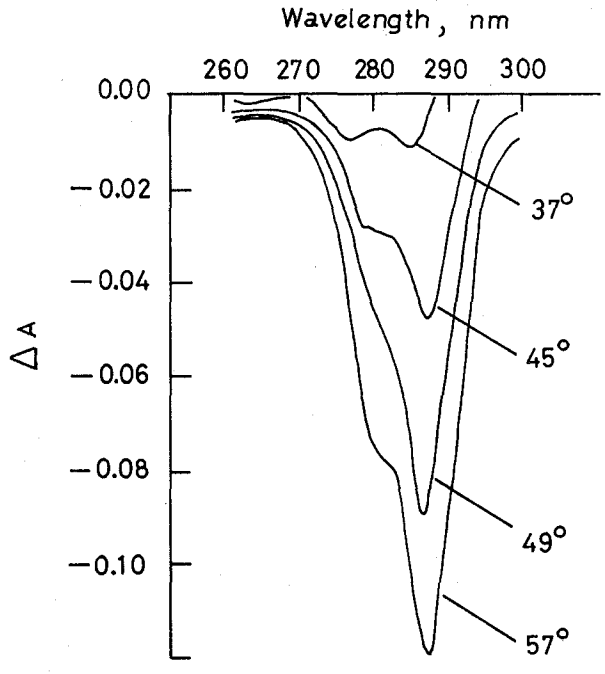


Fig. 5. Difference spectra of RNase A.

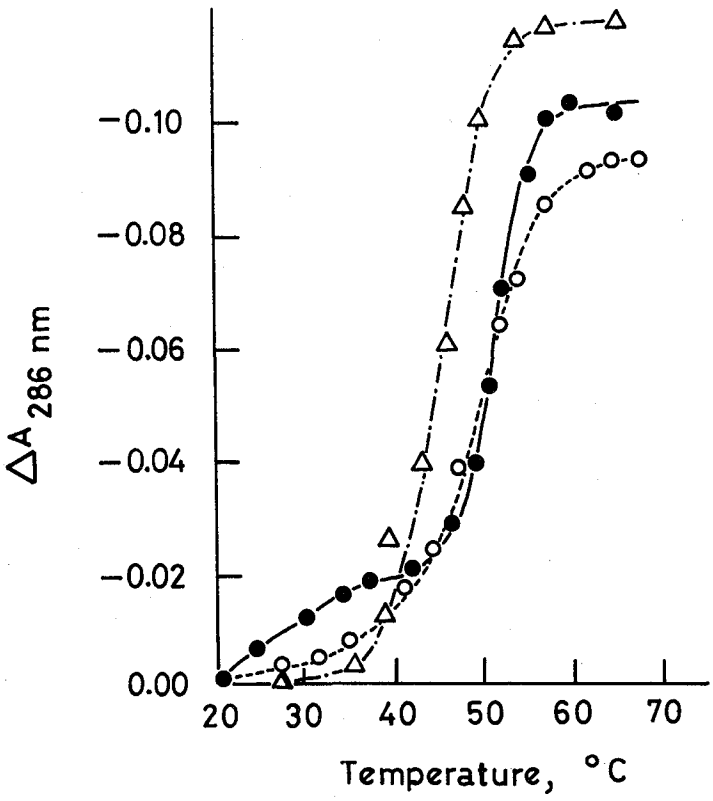


Fig. 6. The relationship between difference in absorbance ($\Delta A_{286\ nm}$) and temperature.
△—·—△ RNase A
●——● Modified RNase A, monomer
○------○ Modified RNase A, dimer

From the spectrophotometric data given in Fig. 6 and assuming that the molecular extinction coefficients are independent of temperature (for adequacy of this assumption, see discussion), we can calculate the equilibrium constant for two state model of thermal transition between the native state and the denatured state at a given temperature according to the equation (1),

$$K(T)=\frac{\varepsilon(T)-\varepsilon_N}{\varepsilon_D-\varepsilon(T)} \tag{1}$$

where ε_N and ε_D are the extinction coefficients of the native and denatured states respectively, which are given as the limiting values as temperature is sufficiently low or high, and ε (T) is the experimental extinction coefficient in the transition region at a temperature T. Transition temperature, T_{tr}, which was defined as the temperature where K was unity, was obtained not by a direct visual inspection of Fig. 6 but by means of a plotting log K against 1/T to avoid uncertainty owing to scattered experimental values on the steepest portion of the curve. Since it was noted that a straight line might be obtained at low temperatures, T_{tr} was calculated from the point where log K=0 on the straight line which was determined initially by a best fit method. In the case of the modified RNase A, monomer, the transition curve of the extinction was not a simple sigmoid but complex

at low temperatures, an extrapolation from the transition region was inevitable. The reason might be attributed partly to steric strain caused by a crosslinkage but mostly remains unexplained. The values of T_{tr} thus obtained were 46.5° for RNase A and 51° for the modified RNase A including the dimeric form.

DISCUSSION

From the evidences such as the peptide maps and activity profiles it is quite probable that our modified RNase A is identical with the sample previously reported by Hartman and Wold.[2] This is an interesting protein derivative since it is one and a half times active as much as the native RNase A if cyclic cytidylic acid was used as a substrate: the reason is still veiled. It is, however, quite apparent that the intramolecular crosslinkage exerted its effects sterically as to loosen a tight steric interaction which might be present in the native protein between a substrate nucleotide molecule and protein reactive sites or by perturbing rigidity of the back-bone polypeptide chain.

Changes in rigidity could be estimated most simply by studying thermally induced denaturation of that protein. When a protein solution is heated above the transition temperature which was characteristic to a species of protein and conditions of the solution, protein molecules begin to lose their original native conformation. Such conformational change would produce a change in environment of a chromophoric group, resulting alterations of a spectrum to which it was concerned. Fairly large amount of such spectral change would be expected in circular dichroic or in optical rotatory dispersion spectra because these spectra reflect directly a change in symmetry of electron distribution of a chromophoric group caused by a back-bone conformational change. Although in less amount, yet a change being accompanied with a conformational change could be observed in an ultraviolet spectrum. We measured ultraviolet spectra under different temperature and established the modified protein was more stable (the higher T_{tr}) to heat treatment than the native one. Although the relationship between rigidity of a polypeptide chain and activity of protein is unknown, this result itself is quite reasonable.

Thermodynamic functions were calculated from the values of K obtained by eq. 1. $\Delta H°$, an apparent heat of denaturation, was obtained by

$$\Delta H° = -R \frac{d \ln K(T)}{d(1/T)}$$

and the free energy change, $\Delta F°$, by

$$\Delta F° = -RT \ln K(T)$$

Our experimental results gave $\Delta H°$=51 Kcal/mole, $\Delta F°$=1.91 Kcal/mole (35°) for RNase A well agreed with the published values.[8~10] For the modified protein, $\Delta H°$ was calculated as 47 Kcal/mole and $\Delta F°$ as 2.32 Kcal/mole.

In the above treatments a two state reaction mechanism and an independency of molecular extinction coefficients on temperatures were assumed. On the application there have been no serious objections at least the transition region was concerned.[11] For the second assumption, though it has been noted that ε_N and ε_D do depend to some extent on temperature, in particular ε_D, upon close inspection of the ultraviolet absorption data, the deviation from a fixed value of ε_N or ε_D is usually little as to allow that the assumption

holds true. Actually the value of $\Delta H°$, 51 Kcal/mole, for RNase A obtained on the basis that both of ε_D and ε_N were independent on temperature is well agreed with the value, 53 Kcal/mole, from an analysis where extinctions were treated as a function of temperature.[9)]

The change of entropy, $\Delta S°$, associated with the transition from the native state to the denatured state is given by

$$\Delta F° = \Delta H° - T\Delta S°$$

as 160 e.u. for RNase A and 145 e.u./mole for modified RNase A. The lesser value of the modified protein will represent the expected property that the intramolecular cross-linking reduces degrees of freedom in movement of segments in the denatured state.

From surveying the accumulated data on the thermodynamic functions for conformational change of a polypeptide chain,[12~18)] some interesting generalizations on the relationships between these functions and the nature of the transition have come out. Details would be published elsewhere in the near future, one of the conclusions is, that if a transition from an ordered structure (α-helix, β-structure, *etc.*) to a denatured (random) one is considered, $\Delta H°$ and $\Delta S°$ are roughly remained a constant value irrespective of the nature of the ordered structure. Table II listed some of the published values of $\Delta H°$ and $\Delta S°$ associated with a helix-coil transition of polypeptides. Even though an electric charge in the coiled state on the side chain was different from poly-L-lysine or -ornithine (positive) to poly-L-glutamic acid (negative), nearly the same value, $\Delta H° \sim$ 1000 cal/residue mole and $\Delta S° \sim 3$ e.u./residue mole was obtained. Application of this finding to the present experiment revealed that conformational change in protein might be also considered as the case and yielded an interesting conclusion. Assuming $\Delta H° = 1$ Kcal/residue mole and $\Delta S° = 3$ e.u./residue mole for the transition from an ordered structure to a random coiled state, the observed value of $\Delta H° = 51$ Kcal/mole for RNase A possibly corresponded to about 51 residues which were responsible for a transition into a random state with the accompanying entropy change of $51 \times 3 = 153$ e.u./mole, being consistent with the observed value, 160 e.u./mole. In the case of modified RNase A, 47 residues were responsible for the transition and were accompanied with the entropy change of $\Delta S =$ 141 e.u./mole which was also consistent to the experimental value, 145 e.u./mole. Comparison of 47 residues of modified RNase A to 51 residues of the native protein showed introduction of the intramolecular crosslinkage presumably constrained additional 4 residues to resist a transition by heat. The conclusion that the 51 residues (out of 124

Table II. Published values of $\Delta H°$ and $\Delta S°$ associated with helix-coil transition of synthetic polypeptides in solution.

	$\Delta H°$ cal/mole	$\Delta S°$ e.u./mole	ref.
Poly-L-ornithine	675	2.4	12
Poly-L-lysine	1210		13
	885	2.7	14
	790	2.38	15
Poly-L-glutamic acid	1120	3.43	14
	975	2.67	16, 17
	1100		18

residues) of RNase A should have been in ordered structures might be supported by the results of the crystallographic analysis of crystalline RNase S (subtilisin-modified RNase A),[19] which showed 24 residues of the enzyme were in α-helices and 34 residues in β-structures.* It is quite conceivable that the number obtained from the experiment of thermally induced denaturation should be smaller than that from the crystallographic analysis owing to the presence of disulfide bridges.

ACKNOWLEDGMENT

We are indebted to Dr. K. Miura for his kind gift of yeast RNA and to Mr. H. Ueno for electrophoretic analysis.

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* One hydrogen bond between an NH and a CO was counted as one residue for both of an α-helix and a β-form.