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Influence of an Extrinsic Cross-Link on the Folding Pathway of Ribonuclease A. Conformational and Thermodynamic Analysis of Cross-Linked (Lysine^{7}–Lysine^{41})-Ribonuclease A†

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ABSTRACT: A cross-linked ribonuclease A derivative, Lys^{7}-dinitrophenylene-Lys^{41}-ribonuclease A, has been prepared and characterized for ultimate use in protein-folding experiments. Immunochemical assays and spectroscopic measurements demonstrated that the introduction of this artificial cross-link does not perturb the native conformation of ribonuclease A. The cross-linked protein exhibited a reversible thermal transition with T_m = 53 °C at pH 2.0, which is 25 °C higher than that of unmodified ribonuclease A under the same conditions. The denatured form of the cross-linked ribonuclease A has a conformational chemical potential that is 4.9 kcal/mol higher than that of the denatured form of unmodified ribonuclease A at 40 °C and pH 2.0, assuming that the cross-linked and the unmodified proteins have the same conformational chemical potential in the native conformation. This is in good agreement with a theoretical value of 5.2 kcal/mol, calculated from the reduction of chain entropy of the denatured form upon introduction of the extrinsic cross-link. Thus, it is concluded that the extrinsic cross-link between Lys^{7} and Lys^{41}, formed by the dinitrophenylene group, does not affect the native conformation of ribonuclease A but destabilizes the denatured conformation, probably by decreasing its chain entropy.

Konishi et al. (1982b) proposed two types of pathways for protein folding. One is designated as a growth-type pathway, in which interactions present in the native conformation play significant roles by forming nucleation sites that subsequently direct the folding of other parts of the chain around the nucleation sites. The other is denoted as a rearrangement-type pathway, in which some nonnative interactions are essential for folding, and the disruption of these intermediates or rearrangement to native ones constitutes the rate-limiting step.

Denton et al. (1982) and Lynn et al. (1984) have studied the influences of the α-helix and β-turn-inducing compact structure on the folding pathways. Both the α-helix and the β-turn are stabilized by short- and medium-range interactions. These authors suggested that not all ordered structures in ribonuclease A (RNase A)[1] are equivalent in terms of their influence on the folding pathway; some can play an essential role, but others may not. The α-helix at the N-terminal region of RNase A and one or more bends that establish the compact structure are among those ordered structures that do not play an essential role in the folding of RNase A. [Since the extrinsic cross-link between Lys^{7} and Lys^{41} discussed in this paper (see below) involves two N-terminal α-helices within the loop, we will report a study of the roles of these α-helices in the folding pathways in a subsequent paper.]

In order to study the role of long-range interactions in the folding pathway, Scheraga et al. (1984) proposed that the introduction of an extrinsic cross-link into RNase A would force some nativelike long-range interactions to exist in the denatured and intermediate conformations and hence would shift the folding pathway from a rearrangement-type to a growth-type one. In this and the following papers, we shall examine the consequences of this proposal.

An intramolecular cross-link is one of the sources of distance information about a protein. Furthermore, cross-links enhance the thermodynamic stability of the protein, e.g., cross-linked (Glu^{35}–Trp^{108})-lysozyme has a thermal transition temperature that is 29 °C higher than that of unmodified lysozyme in 1.94 M Gdn·HCl, pH 2.0 (Johnson et al., 1978). Since RNase A is one of the best characterized proteins, various reagents have been designed to form covalent cross-links between residues, especially lysines, in RNase A. Some of the cross-linking reagents that have been used are 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Marfey et al., 1965a,b), dimethyl adipimidate (Hartman & Wold, 1966, 1967), [2-(*p*-nitrophenyl)allyl]trimethylammonium iodide (ETAC I), and 2-(*p*-nitrophenyl)allyl 4-nitro-3-carboxyphenyl sulfide (ETAC II) (Mitra & Lawton, 1979). Many difficulties, however, have been associated with the use of these cross-linking reagents. Dimethyl adipimidate forms Lys^{7}–Lys^{37} or Lys^{31}–Lys^{37}

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; ^{125}I-RNase A, RNase A labeled with ^{125}I; CL(7–41)-RNase A, cross-linked derivative of RNase A, N^{ϵ},$N^{\epsilon\prime}$-(2,4-dinitrophenylene-1,5)-(lysine^{7}–lysine^{41})-RNase A; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; ETAC I, [2-(*p*-nitrophenyl)allyl]trimethylammonium iodide; ETAC II, 2-(*p*-nitrophenyl)allyl 4-nitro-3-carboxyphenyl sulfide; CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; CMC, carboxymethylcellulose; C>p, sodium cytidine cyclic 2′,3′-phosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Gdn·HCl, guanidine hydrochloride; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; DPE-bis-Ac-Lys-NHMe, N^{ϵ},$N^{\epsilon\prime}$-(2,4-dinitrophenylene-1,5)-bis(N^{α}-acetyl-L-lysine methylamide); PBS, phosphate–saline buffer (300 mM NaCl, 63 mM KH_2PO_4, and 120 mM Na_2HPO_4, pH 7.2); BBS/ovalbumin/azide, borate–saline buffer (75 mM NaCl, 100 mM boric acid, and 50 mM sodium tetraborate, pH 8.4) containing 0.1% ovalbumin and 0.02% sodium azide.

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cross-linked RNase A, but these two derivatives were not completely resolvable on gel or on ion-exchange column chromatography (Hartman & Wold, 1967; Takahashi & Ooi, 1973). In addition, the thermal transition curve of the cross-linked RNase A indicates the presence of some degree of conformational change at low temperature (Takahashi & Ooi, 1973), the existence of a small transition before the main one implying that there is some distortion of the native conformation of RNase A. Both ETAC I and ETAC II produce two pairs of cross-links, Lys^7–Lys^{37} and Lys^{31}–Lys^{41}, in RNase A. However, the reaction requires high pH and high temperature, and we found that a significant amount of the protein is irreversibly denatured during cross-linking under such conditions. On the other hand, DFDNB can cross-link Lys^7 and Lys^{41} of RNase A under mild conditions (pH 8.5 and room temperature) and therefore was adopted for our work.

In this first paper, we investigated the effects of an extrinsic cross-link on the conformational and thermodynamic properties of RNase A. The conformation of the modified protein was examined by measuring enzymatic activity and circular dichroism (CD) spectra and by performing immunochemical assays. Since Lys^{41} is in the active site, the cross-linked RNase A would be expected to have no enzymatic activity. Far-ultraviolet CD (210–240 nm) monitors the backbone structure of the polypeptide chain, and near-ultraviolet CD is sensitive enough to the environments of the tyrosine side-chain chromophores to provide information pertaining to the three-dimensional structure of the protein. Finally, the immunochemical assay, which measures the binding strength between an antibody and an antigen (protein), provides evidence as to whether or not the native surface structure of RNase A is retained (Brown et al., 1967).

A previous study of the thermodynamic properties of cross-linked lysozyme (Johnson et al., 1978) suggested that the extrinsic cross-link destabilizes the denatured conformation of the protein by decreasing its chain entropy. As a result, the relative stability of the native conformation compared to that of the denatured one is increased. In this paper, we analyzed the conformational and thermodynamic properties of $N^{\epsilon},N^{\epsilon'}$-(2,4-dinitrophenylene-1,5)-(Lys^7–Lys^{41})-RNase A [CL(7–41)-RNase A] in order to show that the introduction of an extrinsic cross-link destabilizes the denatured conformation without affecting the native conformation. The effect of the cross-link on the kinetic folding pathway will be reported in the following paper.

Experimental Procedures

Materials. Bovine pancreatic ribonuclease A (Sigma Chemical Co., Type IIA) was purified on a carboxymethylcellulose (CMC) column (Taborsky, 1959). CMC was purchased from Whatman Ltd. (CM-52). Trypsin (Sigma Chemical Co.), which had been affinity purified by Dr. J. A. Nagy using the method of Jany et al. (1976), was purified further on a C-18 high-performance liquid chromatography (HPLC) column following a procedure of Titani et al. (1982). 1,5-Difluoro-2,4-dinitrobenzene, glycine, sodium cytidine cyclic 2′,3′-phosphate (C>p), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), and N^{α}-acetyl-L-lysine methylamide were purchased from Sigma Chemical Co. Ultrapure guanidine hydrochloride (Gdn·HCl) was obtained from Schwarz/Mann and was used without further purification. 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS) and deuterated trifluoroacetic acid were the products of Aldrich. *d*-10-Camphorsulfonic acid was obtained from J. T. Baker Chemical Co. $Na^{125}I$ was purchased from Amersham. Ovalbumin (5× crystallized) was obtained from Calbiochem-Behring. Lactoperoxidase/glucose oxidase beads were purchased from Bio-Rad Laboratories. All other reagents were reagent grade or better.

Preparation of Cross-Linked RNase A and Model Compound. RNase A (150 mg, 11 μmol) was dissolved in 750 mL of 50 mM borate buffer (pH 8.5). While this solution was stirred in the dark at room temperature, 9 mL of a 2% (v/v) methanol/water solution containing 4.5 mg (22 μmol) of DFDNB was added slowly, at a rate of 0.015 mL/min over a period of 10 h. The solution was stirred for another 20 h, and the reaction was then quenched by addition of glacial acetic acid to pH 3.0. The reaction mixture was concentrated to about 50 mL by use of an Amicon ultrafiltration cell and a YM5 Diaflo filter (with a molecular weight cutoff of 5000) and desalted over a gel column (2.5 × 30 cm, Bio-Rad P-6DG) equilibrated with 0.1 M acetic acid. The protein fractions were pooled and lyophilized.

The model compound $N^{\epsilon},N^{\epsilon'}$-(2,4-dinitrophenylene-1,5)-bis(N^{α}-acetyl-L-lysine methylamide) (DPE-bis-Ac-Lys-NHMe) was synthesized from N^{α}-acetyl-L-lysine methylamide and DFDNB according to the procedure of Zahn & Meienhofer (1958). The product was purified further by HPLC using a C-18 column and 33% (v/v) acetonitrile–water isocratic elution. The ^1H NMR spectrum, taken in CF_3COOD on a Brüker 300-MHz Model WM-300 spectrometer, showed chemical shifts (relative to DSS) of 1.65 (4 H, C^{γ}), 1.82 and 2.03 (8 H, C^{β} and C^{δ}), 2.22 (6 H, N^{α}-acetyl), 3.15 (6 H, *N*-methyl), 3.45 (4 H, C^{ϵ}), 4.80 (2 H, C^{α}), and 9.37 ppm (1 H), which is one of the benzene ring (C_3) proton resonances while the other ring (C_6) proton resonance overlapped the contaminating HDO peak and was not resolved.

Purification of CL(7–41)-RNase A. Preliminary purification of CL(7–41)-RNase A was carried out by using a CMC column (1.1 × 30 cm) preequilibrated with 5 mM Tris·HCl (pH 8.0). A linear salt gradient of 0–0.11 M NaCl in 5 mM Tris·HCl (pH 8.0) was applied, and fractions containing the major product were pooled, desalted, and lyophilized. Final purification was achieved with a CMC column (1.1 × 30 cm) preequilibrated with 5 mM Tris·HCl (pH 7.0) with a linear salt gradient of 0–0.3 M NaCl in 5 mM Tris·HCl (pH 7.0). Fractions under the major peak were again pooled, desalted, and lyophilized.

Concentration. The concentration of native RNase A was determined spectrophotometrically; $\epsilon_{275} = 9300\ M^{-1}\ cm^{-1}$ or $\epsilon_{277.5} = 9800\ M^{-1}\ cm^{-1}$ (Sela & Anfinsen, 1957) was used. The extinction coefficients of CL(7–41)-RNase A and DPE-bis-Ac-Lys-NHMe were determined by the micro-Kjeldahl method (Lang, 1958; Noel & Hambleton, 1976), together with absorbance measurements, and used to calculate the concentrations of these two compounds for further measurements.

Spectroscopic Measurements. Absorption spectra were obtained with either a Hewlett-Packard Model 8450A UV/vis spectrophotometer or a modified Cary 14 spectrophotometer (Denton et al., 1982). CD spectra were measured on a Cary 60 spectropolarimeter equipped with a Model 6001 CD attachment. The instrument was calibrated with *d*-10-camphorsulfonic acid (Tuzimura et al., 1977). Protein concentrations and path lengths of the quartz cells were 20 μM, 3 mm and 60 μM, 10 mm for CD measurements at 210–240 and 240–500 nm, respectively.

The enzymatic activities of unmodified RNase A and CL(7–41)-RNase A were measured spectrophotometrically with C>p as a substrate (Crook et al., 1960).

Molecular Weight Determinations. The weight-average molecular weight $\bar{M}_w$ was determined by sedimentation

equilibrium using a Spinco Model E ultracentrifuge equipped with interference optics. The measurements were carried out at 22 °C with a rotor speed of 19 530 rpm over a period of 24 h. The partial specific volume of 0.703 mL/g for native RNase A (Richards & Wyckoff, 1971) was applied to CL-(7–41)-RNase A.

Amino Acid Analysis. The amino acid content of unmodified RNase A and CL(7–41)-RNase A was determined by using a Technicon TSM amino acid analyzer. About 1 mg of protein was dissolved in 0.5 mL of constant boiling 6 N HCl. The solution was repeatedly degassed by the freeze-and-thaw method (5 times) and then hydrolyzed at 110 °C for 24 h in vacuo. Assuming that the alanine content is 12.00 mol per mole of protein (Smyth et al., 1963), the numbers of residues of the other amino acids were calculated from the ratios of their ninhydrin color values.

Peptide Mapping. Procedures for sulfonation of RNase A and for peptide mapping of the reduced, S-sulfonated derivative, developed in our laboratory (T. W. Thannhauser, C. A. McWherter, and H. A. Scheraga, unpublished results; McWherter et al., 1984), were followed for CL(7–41)–RNase A. The trypsin-digested peptide fragments were fractionated on a C-18 column by use of a Spectra Physics Model SP 8000 HPLC instrument (McWherter et al., 1984), and the elution profile was monitored at both 345 and 210 nm. Fractions containing dinitrophenylene, which exhibit absorbance at 345 nm, were subjected to amino acid analysis.

Temperature Dependence of ΔA_{287}. Unmodified RNase and CL(7–41)-RNase A were dissolved in 50 mM glycine hydrochloride (pH 2.0) or 0.1 M acetic acid–Tris (pH 4.0) at a concentration of 7.0×10^{-5} M. The same amount of protein was pipetted into both sample and reference stoppered cells of 1-cm path length. The reference cell was maintained at 22 °C while the sample cell was regulated between 10 and 80 °C by another water bath. The increment of temperature was 2–4 °C, and at each temperature, 10–14 min was allowed for thermal equilibrium before recording the difference in absorbance at 287 nm, ΔA_{287}.

Iodination of RNase A. Unmodified RNase A was radioiodinated by using the solid-phase lactoperoxidase method (Marchalonis, 1969; Thorell & Johansson, 1971; David, 1972; David & Reisfeld, 1974; Krohn & Welch, 1974) in the presence of glucose oxidase (Hubbard & Cohn, 1972). The reaction was carried out on 50 μg of RNase A contained in 25 μL of PBS (phosphate–saline buffer: 300 mM NaCl, 63 mM KH_2PO_4, and 120 mM Na_2HPO_4, pH 7.2) according to the instructions of the lactoperoxidase/glucose oxidase beads supplier. $Na^{125}I$ (0.67 mCi) was used as the trace labeler. Quenching of the reaction and separation of excess $Na^{125}I$ from ^{125}I-RNase A were accomplished by applying the entire reaction mixture to a desalting column (1.0 × 18 cm, Bio-Rad P-6DG) equilibrated with BBS/ovalbumin/azide (borate–saline buffer: 75 mM NaCl, 100 mM boric acid, and 50 mM sodium tetraborate, pH 8.4, containing 0.1% ovalbumin and 0.02% sodium azide). The ^{125}I-RNase A fractions were combined and subsequently diluted with BBS/ovalbumin/azide to give a solution containing approximately 1000 cpm/μL. In the trace labeling of RNase A, 14% of the added ^{125}I was incorporated. This corresponds to <0.02 mol of ^{125}I per mole of RNase A and resulted in a specific activity of approximately 6.3×10^6 cpm/μg of protein.

Antibody Preparation and Binding to ^{125}I*-RNase A.* Four young New Zealand white female rabbits were used to obtain antisera to native RNase A. The primary immunization was carried out with a 1:1 (v/v) emulsion of Freund's complete adjuvant (Gibco) plus a 1 mg/mL solution of unmodified RNase A in PBS; 0.6 mL of the emulsion was administered to each rabbit intramuscularly in the hind leg. Booster injections were administered similarly with Freund's incomplete adjuvant (Gibco) replacing the complete adjuvant in the immunizing emulsion. Blood was collected 10–14 days after each booster injection by making an incision in the marginal ear vein. The blood was left at 4 °C overnight and then centrifuged, and the serum was stored at −20 °C. Booster injections and bleedings were repeated at 1-month intervals.

In order to determine which antiserum binds most tightly to ^{125}I-RNase A, i.e., which antiserum should be used in the subsequent competition assays, titer determinations were carried out as described by Nagy et al. (1982). Serial dilutions of the antibody were made with BBS/ovalbumin/azide; this buffer was also used in the assay samples and for washing the pellets. Antibody titers were determined by the method of Hunter (1978), and corrections for nonspecific binding were made by the method of Farr (1958) and Minden & Farr (1978), using binding data from a preimmune serum as a control. Antiserum from the third booster bleed of one of the rabbits, which bound the most tightly to ^{125}I-RNase A, was used in subsequent competition assays.

The specificity of the antibodies to the native conformation of RNase A was demonstrated by the finding that a 1000-fold excess of the denatured protein [i.e., reduced S-sulfonated RNase A (T. W. Thannhauser et al., unpublished results)], compared to ^{125}I-RNase A, showed no detectable inhibition of the binding of ^{125}I-RNase A to the antibodies.

In order to assess the degree of native conformation present in the CL(7–41)-RNase A, competitive radioimmunoassays (Yalow & Berson, 1970) were carried out at 4 °C and pH 8.4, according to the procedure of Nagy et al. (1982), where varying amounts of CL(7–41)-RNase A competed with ^{125}I-RNase A for antibodies raised against native RNase A. The results of this experiment were compared with results from an experiment in which unmodified RNase A competed with ^{125}I-RNase A for antibodies. All dilutions of the competing proteins and antibodies were made with BBS/ovalbumin/azide as the diluent. The final concentrations of the competing antigen were varied from 0 to 2.40×10^{-7} M for CL(7–41)-RNase A and to 3.16×10^{-7} M for unmodified RNase A. The original antiserum was diluted by a factor of 10^4 in all preparations. All competition experiments were carried out under conditions where the antibody binding sites were saturated with antigen to within experimental error. This implies that all antigenic determinants of ^{125}I-RNase A were involved in this assay.

Data from the competition studies, wherein CL(7–41)-RNase A or unmodified RNase A competed with ^{125}I-RNase A for antibodies raised against native RNase A, were treated by a logit analysis (Feldman & Rodbard, 1971; Rodbard & Hutt, 1974). The logit transformation is a method for linearizing binding data. The data are then plotted as $\ln [(B_i/B_0)/(1 - B_i/B_0)]$ vs. log (competitor), where B_0 is the concentration of ^{125}I-RNase A bound to the antibodies in the absence of competitor and B_i is the concentration of ^{125}I-RNase A bound to the antibodies in the presence of competitor. Thus, $(1 - B_i/B_0) \times 100$ represents the percent inhibition by competitor.

Results

Identification of the Cross-Linked RNase A. The cross-linked RNase A was purified by the procedure described under Experimental Procedures (Figure 1A,B). Only the material from peak d of Figure 1B was collected. The homogeneity

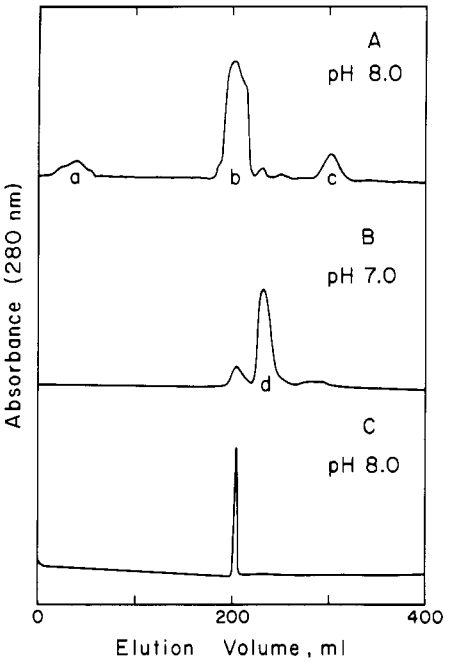

FIGURE 1: Elution profiles for the fractionation of CL(7–41)-RNase A. (A) The lyophilized reacted RNase A (150 mg), dissolved in 50 mM Tris·HCl (pH 8.0), was loaded on a CMC column (1.1 × 30 cm, pH 8.0) and eluted with a 0–0.11 M NaCl linear gradient formed by mixing 250 mL each of 5 mM Tris·HCl and 0.11 M NaCl in 5 mM Tris·HCl (pH 8.0). The flow rate was 0.46 mL/min, and the fractionation was monitored by absorbance at 280 nm. (B) The preliminarily purified protein from peak b (75 mg) was lyophilized, dissolved in 5 mM Tris·HCl (pH 7.0), and loaded on a CMC column (1.1 × 30 cm, pH 7.0). Everything else was the same as in (A) except that a higher gradient, 0–0.3 M NaCl and 5 mM Tris·HCl (pH 7.0), was used. (C) Rechromatogram of 1 mg of final product obtained from peak d. The same procedure as in (A) was followed.

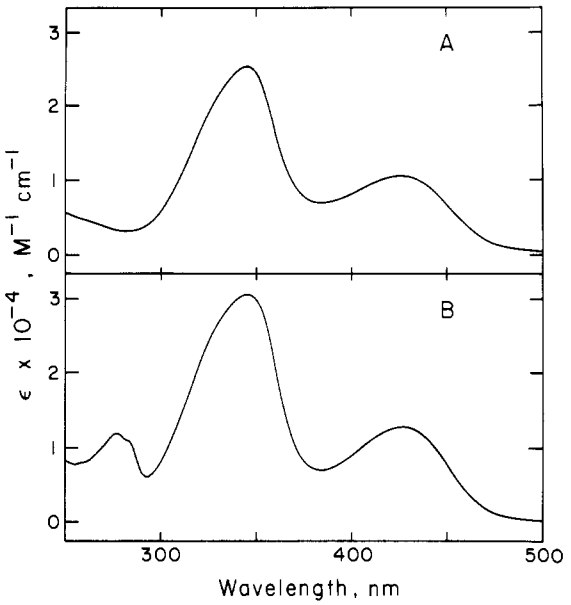

FIGURE 2: (A) Absorption spectrum of the model compound, DPE-bis-Ac-Lys-NHMe (30 μM), in 0.1 M acetic acid. Trifluoroacetic acid (2%) was added to dissolve the model compound. (B) Absorption spectrum of CL(7–41)-RNase A (30 μM) in 0.1 M acetic acid.

of the purified protein was revealed by CMC column chromatography at pH 8.0 (Figure 1C), and its peptide mapping showed >96% homogeneity as described below.

The absorption spectra of the model compound, DPE-bis-Ac-Lys-NHMe, and of the modified RNase A are shown in parts A and B of Figure 2, respectively. The modified RNase A is characterized by ϵ_{345} = 30900 M^{-1} cm^{-1} and ϵ_{430} = 12800 M^{-1} cm^{-1}, which are comparable to ϵ_{345} = 26000 M^{-1} cm^{-1} and ϵ_{430} = 10800 M^{-1} cm^{-1} for the model compound. This shows that one dinitrophenylene group was incorporated per protein molecule.

Table I: Characteristics of CL(7–41)-RNase A

property	this work	Marfey et al. (1965a,b)
enzymatic act. rel to unmodified RNase A (%)	0	15
extinction coeff (M^{-1} cm^{-1})		
ϵ_{278}	11900	15800
ϵ_{345}	30900	31000
ϵ_{430}	12800	11800
mol wt[a]	13600	15300
Lys content after acid hydrolysis	8.0	8.1
residues cross-linked	Lys^7 and Lys^{41}	Lys^7 and Lys^{41}
yield of preparation (%)	35	10

[a] The calculated molecular weight of CL(7–41)-RNase A is 13880.

Sedimentation equilibrium measurements verified that the modified protein is a monomer with an observed $\bar{M}_w$ of 13600, which is very close to a value of 13880 calculated from its amino acid composition and the incorporated dinitrophenylene. The modified RNase A showed a complete loss of enzymatic activity, as determined by its failure to hydrolyze C>p at pH 5.0. Amino acid analysis of the modified RNase A showed that two lysine residues were missing and that the amounts of the other residues remained unchanged to within an experimental error of 5%. These results indicate that two lysine residues are cross-linked by dinitrophenylene.

Peptide mapping of the cross-linked RNase A showed three major yellow peaks containing dinitrophenylene that were identified as fragments from Lys^1 to Arg^{10} and from Cys^{40} to Lys^{61}, fragments from Glu^2 to Arg^{10} and from Cys^{40} to Lys^{61}, and fragments from Lys^1 to Arg^{10} and from Cys^{40} to Phe^{46}. Cleavage of the peptide bond at Phe^{46}-Val^{47} by treatment with trypsin has been reported (Riehm & Scheraga, 1966). Thus, the two lysine residues that were chemically modified, and hence resistant to tryptic cleavage, are Lys^7 and Lys^{41}. The fact that each of these three fragments contains both chemically modified residues (Lys^7 and Lys^{41}) strongly indicates a cross-link between Lys^7 and Lys^{41}. The minor peaks, which could not be identified, contained <4% of incorporated dinitrophenylene. Thus, the purified protein is >96% homogeneous. The presence of the cross-link was further confirmed by the higher thermal stability of the modified RNase A relative to that of unmodified RNase A, as described below. Therefore, we have identified the modified protein as RNase A with a dinitrophenylene cross-link between Lys^7 and Lys^{41}, designated as CL(7–41)-RNase A. These results are summarized in Table I, which also includes the results obtained by Marfey et al. (1965a,b) for comparison.

Conformational Analyses of CL(7–41)-RNase A. The conformation of CL(7–41)-RNase A was examined by an immunochemical assay and by spectroscopic measurements. Since RNase A has several antigenic sites for binding the antibodies produced by rabbits (Chavez & Scheraga, 1979), we examined the retention of these antigenic sites in CL(7–41)-RNase A. Addition of CL(7–41)-RNase A at approximately 200-fold excess relative to ^{125}I-RNase A completely inhibited the binding of ^{125}I-RNase A to the antibodies. This demonstrates that all antigenic sites of ^{125}I-RNase A are found in CL(7–41)-RNase A and implies that neither Lys^7 nor Lys^{41} is involved in an antigenic binding region in RNase A. This is consistent with the work of Brown et al. (1967), who demonstrated that all antigenic determinants present in unmodified

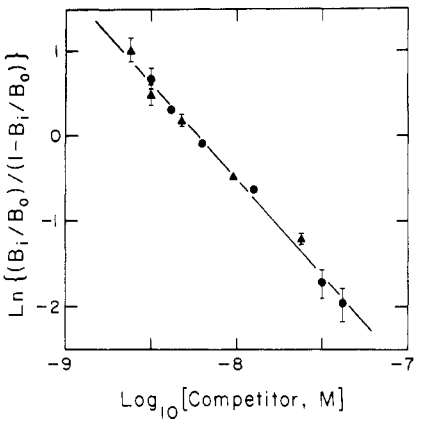

FIGURE 3: Logit transformation (see Experimental Procedures for details) of competitive binding curves for anti-RNase A antibodies and ^{125}I-RNase A in the presence of a competitor: unmodified RNase A (●) or CL(7–41)-RNase A (▲). B_0 is the concentration of ^{125}I-RNase A bound to the antibodies in the absence of competitor and B_i is the concentration of ^{125}I-RNase A bound to the antibodies in the presence of competitor.

RNase A were retained in N^{ϵ}-(dinitrophenyl)-(41-lysine)-RNase A.

We then addressed the question as to whether or not the antigenic determinants are quantitatively retained in CL(7–41)-RNase A. The concentration of ^{125}I-RNase A in the assay solution was determined from binding experiments in which unmodified RNase A and ^{125}I-RNase A competed for the antibodies. The results of the competition experiments are displayed as a logit plot in Figure 3 (see Experimental Procedures for details). When the concentration of unmodified RNase A is equal to that of ^{125}I-RNase A, 50% of ^{125}I-RNase A bound to the antibodies is inhibited, giving ln $[(B_i/B_0)/(1 - B_i/B_0)] = 0.0$. Thus, the concentration of ^{125}I-RNase A was estimated as 6.2×10^{-9} M. Figure 3 also shows the results of competitive binding experiments between CL(7–41)-RNase A and ^{125}I-RNase A. The concentration of CL(7–41)-RNase A needed to inhibit 50% of the binding of ^{125}I-RNase A to the antibodies was determined to be 6.4×10^{-9} M. Furthermore, the value of the slope in Figure 3, as derived from a linear regression analysis of the combined data presented in the logit plot, agrees well with the theoretical value (Rodbard et al., 1969), -2.25 ± 0.07 vs. -2.30 (the latter being the conversion factor between natural and common logarithms). Since the concentration of unmodified RNase A and CL(7–41)-RNase A needed to produce 50% inhibition of the binding of ^{125}I-RNase A is the same to within experimental error, we conclude that the native conformation of the antigenic sites is completely retained in CL(7–41)-RNase A.

CD spectra of unmodified RNase A and CL(7–41)-RNase A at pH 3.0 are shown in Figure 4. Both proteins show similar features in the backbone CD band in the region of 210–240 nm and in the tyrosine side-chain CD bands in the region of 245–285 nm, indicating that the native conformation is retained. The decrease in the magnitude of the molar ellipticity in the far-UV band of CL(7–41)-RNase A is probably due to a band from the dinitrophenylene cross-link. Dinitrophenylene in CL(7–41)-RNase A shows CD bands at 315 and 355 nm, suggesting that the mobility of the cross-link is restricted in the protein. The CD spectra of CL(7–41)-RNase A denatured by 6 M Gdn·HCl at pH 3.0 (data not shown), however, exhibit no peaks in the tyrosine side-chain or dinitrophenylene absorption regions.

Thermodynamics of CL(7–41)-RNase A. Figure 5 shows the changes in the absorbance of CL(7–41)-RNase A induced

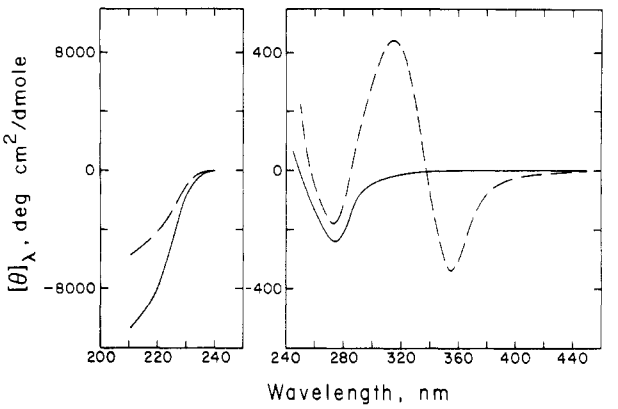

FIGURE 4: CD spectra of CL(7–41)-RNase A (– –) and unmodified RNase A (—) in 50 mM glycine hydrochloride (pH 3.0) at 22 °C. Cells (1-cm path length) containing 60 μM proteins were used for the measurements in the range 240–500 nm, while 3-mm path length cells containing 20 μM proteins were used at 240 nm and lower wavelengths.

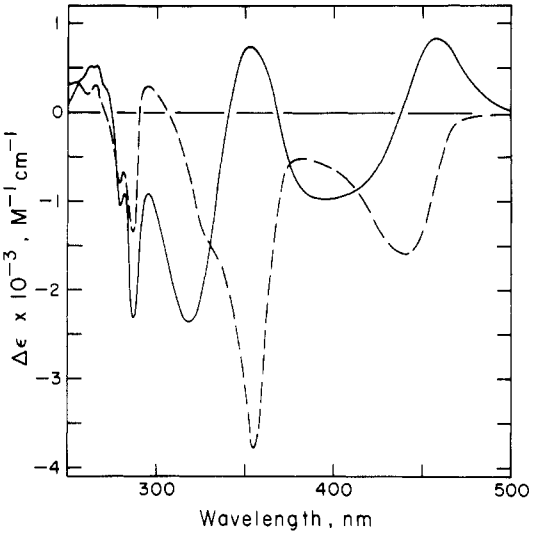

FIGURE 5: Difference absorption spectra of 6 M Gdn·HCl-denatured CL(7–41)-RNase A (—) at 22 °C and thermally denatured CL(7–41)-RNase A (– –) at 70 °C vs. native CL(7–41)-RNase A at 22 °C. All solutions contained 73 μM CL(7–41)-RNase A in 50 mM glycine hydrochloride, pH 2.0.

Table II: Thermodynamic Properties for Unfolding of Unmodified RNase A and CL(7–41)-RNase A at pH 2.0

protein	T_m (°C)	$\Delta H°(T_m)$ (kcal/mol)	$\Delta S°(T_m)$ (eu)
unmodified RNase A	28.0 ± 0.5	64.0 ± 5.5	213 ± 15
CL(7–41)-RNase A	53.0 ± 0.5	90.0 ± 8.6	276 ± 20

by Gdn·HCl denaturation and by thermal denaturation. As in the case of unmodified RNase A (Hermans & Scheraga, 1961), both of these curves exhibit a peak at 287 nm. Hence, this was the wavelength used to monitor conformational changes in both the cross-linked and unmodified proteins.

The thermal transition curves of both CL(7–41)-RNase A and unmodified RNase A at pH 2.0 are shown in Figure 6. The consistency of the denaturation curves (open or closed circles) and renaturation curves (open or closed triangles) demonstrates the reversibility of the thermal transitions. The transition temperature was found to increase dramatically from 28 to 53 °C upon introduction of the extrinsic cross-link. By use of van't Hoff plots, the thermodynamic parameters for the unfolding of these two proteins were estimated and are summarized in Table II.

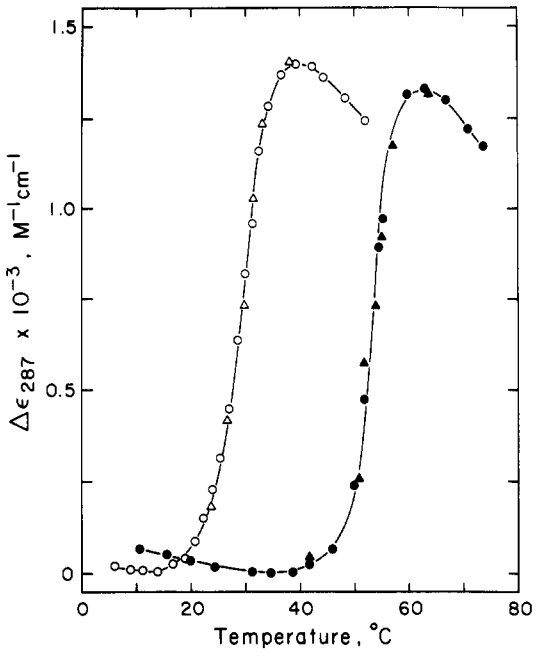

FIGURE 6: Thermal transition curves of unmodified RNase A (○, heating; △, cooling) and CL(7–41)-RNase A (●, heating; ▲, cooling) in 50 mM glycine hydrochloride, pH 2.0.

Discussion

Preparation and Characterization of CL(7–41)-RNase A. CL(7–41)-RNase A was first prepared by Marfey et al. (1965a,b), and their preparation procedures were improved in this paper as follows. Since DFDNB is unstable and is hydrolyzed readily at alkaline pH, it was necessary to find a proper buffer in which to carry out the cross-linking reaction. Among the various buffers tested, we found that 50 mM borate buffer (pH 8.5) leads to significantly less hydrolysis of DFDNB than 1% $NaHCO_3$ (pH 8.4), used by Marfey et al. (1965a,b). Furthermore, the IRC-50 ion-exchange column at pH 6.25 that they used did not purify CL(7–41)-RNase A completely, despite the fact that they rechromatographed the protein twice on the same column. We have used two different CMC columns, at pH 8.0 and 7.0 sequentially; this led to good fractionation (Figure 1). These modifications improved the purity and yield of CL(7–41)-RNase A in our preparation (Table I).

CL(7–41)-RNase A shows slightly higher extinction coefficients than DPE-bis-Ac-Lys-NHMe at 345 and 430 nm (Figure 2). These differences are due to a difference in the environment of the dinitrophenylene group in CL(7–41)-RNase A and in the model compound. A higher extinction coefficient at 278 nm (ϵ_{278} = 15 800 M^{-1} cm^{-1}) and the retention of 15% enzymatic activity by CL(7–41)-RNase A, reported by Marfey et al. (1965a,b), indicate that their preparation was contaminated to some extent. Marfey et al. (1965b) used tryptic digestion of reduced and carboxymethylated CL(7–41)-RNase A in order to identify the location of the cross-link. However, since only 10–20% of their protein was digested by trypsin, they were not able to characterize the impurities. In this paper, we have used a new procedure for carrying out peptide mapping by first sulfonating the disulfide bonds in the protein and then performing tryptic digestion (T. W. Thannhauser et al., unpublished results; McWherter et al., 1984). Because of the increased stability of CL(7–41)-RNase A, the sulfonation of this protein took as long as 1.5 h, compared to only 20 min required for unmodified RNase A. Our results showed *complete* digestion, and the concentration of impurities in the purified CL(7–41)-RNase A was estimated to be less than 4%.

Immunochemical Assay. In the experiments on the competition between CL(7–41)-RNase A and unmodified RNase A for anti-RNase A antibodies, if CL(7–41)-RNase A were denatured to some extent and did not compete as well as unmodified RNase A for anti-RNase A antibodies, then the logit plot data, while still being linear, would be shifted to the right, i.e., in the direction of higher concentration of competitor required for 50% inhibition. Thus, the fact that the 50% inhibition concentrations are the same and the fact that the combined data from the competition experiments are collinear suggest that CL(7–41)-RNase A and native RNase A are conformationally identical, from an immunochemical point of view.

There are several points to consider when applying this immunochemical approach. The first concern is that radioiodination of the protein may disrupt its native conformation. We can eliminate this possibility by considering the following two criteria. The first is that a 200-fold excess of native RNase A (relative to ^{125}I-RNase A) completely inhibited the binding of ^{125}I-RNase A to the antibodies. This demonstrates that all of the antibodies bound to ^{125}I-RNase A have affinity for the native conformation. The second is that a 1000-fold excess of reduced, S-sulfonated RNase A, representative of a denatured form of RNase A, does not inhibit the binding of ^{125}I-RNase A to anti-RNase A antibodies. These two criteria demonstrate that all of the antigenic sites on ^{125}I-RNase A retain their native conformation completely and that all of the antibodies bound to ^{125}I-RNase A bind specifically to the native conformation of RNase A. If these criteria were not satisfied, we could not have used the iodinated protein for this type of assay.

As was mentioned earlier, all immunochemical experiments were carried out under conditions where the antibody binding sites were saturated by excess antigen (Creighton et al., 1978; Creighton, 1980). Thus the total amount of antigen bound to the antibodies in all competition experiments was constant (and equal to B_0).

Complete inhibition of binding occurred in the presence of a 200-fold excess of competitor, CL(7–41)-RNase A. If complete inhibition had not been obtained, it would have implied that an antigenic determinant(s) on the surface of RNase A is modified or blocked. This would have required the substitution of $B_0 - B_\infty$ for B_0, where B_∞ is the concentration of ^{125}I-RNase A bound to the antibodies in the presence of a large molar excess of modified protein. In our experiments, $B_\infty = 0$.

Thermodynamics of CL(7–41)-RNase A. The reversible thermal transition of CL(7–41)-RNase A (Figure 6) shows an absorbance change that is as cooperative as that of unmodified RNase A when monitored at 287 nm. The temperature dependence of the absorbance change at 355 nm (data not shown) exhibited essentially the same transition, but a higher amplitude was observed. This behavior is clearly different from that of Lys^{41}-dinitrophenylated RNase A, which exhibits a change of conformation around the dinitrophenyl groups at temperatures below the main transition (Ettinger & Hirs, 1968).

The thermodynamic properties listed in Table II are of interest. They indicate that the introduced cross-link has enhanced the thermal stability relative to that of RNase A.

Using a model of overlapping and dependent loops (Wang & Uhlenbeck, 1945; Poland & Scheraga, 1965) (see Appendix), we calculated changes in free energy of the denatured forms at 40 °C (due to the reduction of chain entropy by *all*

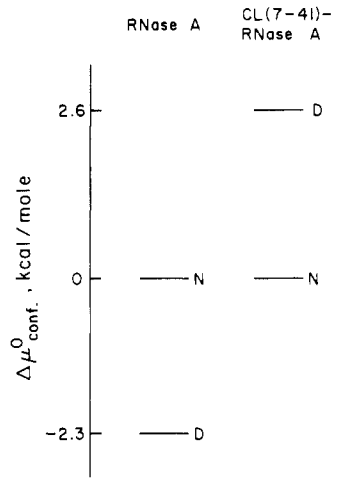

FIGURE 7: Schematic representation of the conformational chemical potential ($\Delta\mu^{\circ}_{conf}$) of the native (N) and denatured (D) conformations of unmodified RNase A and CL(7–41)-RNase A, respectively, at 40 °C, pH 2.0. The $\Delta\mu^{\circ}_{conf}$ was arbitrarily assigned as 0.0 kcal/mol for the native conformations of unmodified RNase A and CL(7–41)-RNase A.

of the cross-links) for the disordered states of CL(7–41)-RNase A and unmodified RNase A as 25.2 and 20.0 kcal/mol, respectively. Thus, the difference in free energy (due to the difference in chain entropy) between the two disordered proteins is 5.2 kcal/mol, theoretically. van't Hoff plots of the data in Figure 6 enabled us to evaluate the difference in the *conformational* chemical potential [defined by Konishi et al. (1982a) and designated as $\Delta\mu^{\circ}_{conf}$][2] between the native and denatured conformations of CL(7–41)-RNase A and of unmodified RNase A as 2.6 and −2.3 kcal/mol, respectively, at 40 °C and pH 2.0. Assuming that the native conformation of CL(7–41)-RNase A has the same value of $\Delta\mu^{\circ}_{conf}$ (arbitrarily assigned as 0.0 kcal/mol) as that of the native conformation of unmodified RNase A, the difference in $\Delta\mu^{\circ}_{conf}$ between the denatured forms of CL(7–41)-RNase A and unmodified RNase A is 4.9 kcal/mol as shown in Figure 7. This is in good agreement with the theoretical value of 5.2 kcal/mol due to the loss of chain entropy in the denatured form due to the extrinsic cross-link. Thus, it is suggested that the artificial cross-link has destabilized the denatured state of RNase A by decreasing its entropy, with little change in the enthalpy. A similar conclusion was arrived at from a study of Glu^{35}–Trp^{108} cross-linked lysozyme (Johnson et al., 1978).

Conclusions

CL(7–41)-RNase A was purified and characterized. Immunochemical assays and spectroscopic methods demonstrated that the native conformation of RNase A was completely retained. The extrinsic cross-link enhanced the thermodynamic stability of RNase A by decreasing the chain entropy of the denatured conformation of the protein.

Acknowledgments

We thank V. G. Davenport for molecular weight measurements, T. W. Thannhauser for nitrogen analyses, amino acid analyses, and peptide mapping, C. A. McWherter for peptide mapping, and E. R. Stimson and J. A. Nagy for assistance in immunizing and bleeding rabbits. We are also indebted to T. Ooi, Y. C. Meinwald, and A. R. Leed for helpful discussions.

Appendix

Entropy Change due to Overlapping Cross-Linking of a Statistical Coil. The introduction of cross-links in *specific* locations of a statistical coil reduces its conformational entropy. We present here a computation of the entropy loss for a statistically coiled macromolecule in which the cross-links overlap, as they do, e.g., in ribonuclease A.

Despite the fact that the ribonuclease chain is short (124 residues) and the segments between cross-links are even shorter, we assume that the chain statistics are Gaussian. Hence, the probability density for the distribution of a chain vector $\mathbf{r}_i$ in a three-dimensional Gaussian manner is

$$P(\mathbf{r}_i) = (3/2\pi n_j a^2)^{3/2} \exp[-3\mathbf{r}_i^2/(2n_j a^2)] \quad \text{(A-1)}$$

where a is the length of a chain element (taken here as the root mean square of all of the virtual bond distances between pairs of successive C^{α} atoms), $\mathbf{r}_i$ is a vector connecting sequential C^{α} atoms of half-cystine residues (C^{α}_i and $C^{\alpha}_{i+n_j}$), and n_j is the number of residues between the C^{α}'s of sequential half-cystines; e.g., $\mathbf{r}_1$ is the vector between the C^{α} atoms of Lys^1 and Cys^{26} and $\mathbf{r}_2$ is the vector between the C^{α} atoms of Cys^{26} and Cys^{40} in ribonuclease A.

Now, suppose that a cross-link is introduced between $C^{\alpha}_{k_i}$ and $C^{\alpha}_{l_i}$, where $l_i > k_i$ (i = 1, 2, 3, 4 for *native* ribonuclease). The vector Φ_i between these two C^{α} atoms is the sum of the vectors representing the intervening vectors, $\mathbf{r}_i$, viz.

$$\Phi_i = \psi_{ik_i}\mathbf{r}_{k_i} + \psi_{ik_{i+1}}\mathbf{r}_{k_{i+1}} + \ldots + \psi_{il_{i-1}}\mathbf{r}_{l_{i-1}} \quad \text{(A-2)}$$

Since eight half-cystines in ribonuclease A provide nine $\mathbf{r}_i$'s (i = 1, ..., 9) between residues 1 and 124, eq A-2 can be written in the following vectorial form:

$$\Phi_i = (\psi_{i1}\ \psi_{i2}\ \ldots\ \psi_{i9})\begin{pmatrix}\mathbf{r}_1\\ \mathbf{r}_2\\ \vdots\\ \mathbf{r}_9\end{pmatrix} \quad \text{(A-3)}$$

where the scalar quantity ψ_{ip} is 1 or 0 depending on whether $\mathbf{r}_p$ is inside or outside the loop, respectively. If there are m loops (m = 4 for native ribonuclease and m = 5 for the extrinsically cross-linked protein), eq A-3 takes account of the overlapping chain segments; then, the probability density for the simultaneous occurrence of m *overlapping* loops (each of which obeys Gaussian statistics) follows from eq 22 of Wang & Uhlenbeck (1945), viz.

$$P(\Phi_1, \ldots, \Phi_m) = (3/2\pi)^{3m/2}|C|^{-3/2} \exp[-(3/2|C|)\sum_{k=1}^{m}\sum_{l=1}^{m} C_{kl}\Phi_k\cdot\Phi_l] \quad \text{(A-4)}$$

where C_{kl} is the cofactor of the element b_{kl}

$$b_{kl} = \sum_{i=1}^{p}\psi_{ki}\psi_{li}n_i a^2 \quad \text{(A-5)}$$

[2] The "conformational chemical potential", μ°_{conf}, was introduced by Konishi et al. (1982a) to compare proteins that differ slightly in their chemical composition, e.g., proteins with and without intact disulfide bonds or unmodified and modified proteins. They expressed the standard state chemical potential of a protein as a sum of two terms. One is an intrinsic chemical potential, which depends on the amino acid composition (including modified residues) and may be estimated essentially as the sum of the chemical potentials of its constituent amino acid residues (Tanford, 1970). The other is the conformational chemical potential, which depends on the specific conformation of the protein molecule. Since CL(7–41)-RNase A has the same native conformation as that of unmodified RNase A, the conformational chemical potential of "native" CL(7–41)-RNase A is assumed to be the same as that of native unmodified RNase A, ignoring the interactions between the cross-link and the protein.

$$\mathbf{b} = \begin{pmatrix} (14 + 18 + 7 + 7 + 12)a^2 & (18 + 7 + 7 + 12)a^2 & (7 + 7 + 12)a^2 & 7a^2 \\ (18 + 7 + 7 + 12)a^2 & (18 + 7 + 7 + 12 + 11)a^2 & (7 + 7 + 12 + 11)a^2 & 7a^2 \\ (7 + 7 + 12)a^2 & (7 + 7 + 12 + 11)a^2 & (7 + 7 + 12 + 11 + 15)a^2 & 7a^2 \\ 7a^2 & 7a^2 & 7a^2 & 7a^2 \end{pmatrix} \quad \text{(A-11)}$$

($p = 9$ for ribonuclease A) and $|C|$ is the determinant of the matrix whose elements are b_{kl}. Hence, the probability for all m cross-links to be present, with their C^α–C^α vectors Φ_i each lying within a corresponding shell Ω_i, is

$$P_m = \int_{\Omega_1} d\Phi_1 \ldots \int_{\Omega_m} d\Phi_m \, P(\Phi_1, \ldots, \Phi_m) \quad \text{(A-6)}$$

Since the distance between *cross-linked* C^α atoms is usually much less than the contour length of the residues in the cross-linked loop, we follow eq 9–11 of Poland & Scheraga (1965) and approximate Φ_i as a zero vector. Hence, the exponential term in eq A-4 goes to unity, and the term $P(\Phi_1, \ldots, \Phi_m)$ becomes a constant $[=(3/2\pi)^{3m/2}|C|^{-3/2}]$.

From Poland & Scheraga (1965, p 382), the shell Ω_i is assumed to have a radius of $a/2$ and a thickness of $0.03a$. Hence, its volume is $0.0943a^3$. Insertion of these values into eq A-6 yields

$$\begin{aligned} P_m &= (0.0943a^3)^m(3/2\pi)^{3m/2}|C|^{-3/2} \\ &= 0.03111^m a^{3m}|C|^{-3/2} \end{aligned} \quad \text{(A-7)}$$

If W is the number of available conformations of a macromolecular chain, then its entropy is

$$S = R \ln W \quad \text{(A-8)}$$

Hence, the entropy change due to the formation of m cross-links is

$$\begin{aligned} \Delta S &= R \ln W_S - R \ln W_R \\ &= R \ln \frac{W_S/W_0}{1 - W_S/W_0} \end{aligned} \quad \text{(A-9)}$$

where W_S and W_R are the number of conformations with and without cross-links, respectively, and $W_0 = W_S + W_R$. Identifying P_m with W_S/W_0, which is $\ll 1$, eq A-9 can be approximated as

$$\begin{aligned} \Delta S &= R \ln [P_m/(1 - P_m)] \\ &\cong R \ln P_m \\ &= R \ln (0.3111^m a^{3m}|C|^{-3/2}) \\ &= R(-3.47m + 3m \ln a - 1.5 \ln |C|) \end{aligned} \quad \text{(A-10)}$$

We are indebted to S. Ihara, W. L. Mattice, and P. W. Mui for this exposition.

As an illustration, we calculate the value of ΔS for native ribonuclease with disulfide bonds at positions 26–84, 40–95, 58–110, and 65–72. For this protein, eq A-5 leads to a 4 × 4 matrix (see eq A-11). For example, according to eq A-5, the b_{12} element of $\mathbf{b}$ is $a^2(\psi_{11}\psi_{21}n_1 + \psi_{12}\psi_{22}n_2 + \ldots + \psi_{19}\psi_{29}n_9)$. Assignment of 0 and 1 to the ψ's, and the appropriate n_i's, leads to $(18 + 7 + 7 + 12)a^2$.

From eq A-11, the matrix $\mathbf{b}$ follows directly, as does its determinant, which is $|C|$. Insertion of this value (together with $m = 4$) into eq A-10 gives

$$\Delta S = 63.85 \text{ eu}$$

or

$$T\Delta S = 20.0 \text{ kcal/mol at } 40 \text{ °C}$$

A similar treatment of CL(7–41)-RNase A, with $m = 5$, the extra cross-link being between residues 7 and 41, yields $T\Delta S = 25.2$ kcal/mol at 40 °C.

Registry No. RNase, 9001-99-4; DPE-bis-Ac-Lys-NHMe, 6367-21-1.

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Independent Folding of the Carboxyl-Terminal Fragment 228–316 of Thermolysin†

Claudio Vita, Daniele Dalzoppo, and Angelo Fontana*

Appendix: Prediction of Stabilities of Thermolysin Fragments‡

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ABSTRACT: The COOH-terminal fragment 206–316 of thermolysin was shown previously to maintain a stable folded structure in aqueous solution comparable to that of the corresponding region in native thermolysin and thus to possess protein domain characteristics [Fontana, A., Vita, C., & Chaiken, I. M. (1983) *Biopolymers 22*, 69–78]. In order to study the effect of polypeptide chain length on folding and stability of an isolated domain, the 111 amino acid residue fragment was shortened on the NH_2-terminal side by removal of a 22-residue segment. Treatment of fragment 206–316 with hydroxylamine under alkaline conditions permitted selective cleavage of the Asn^{227}–Gly^{228} peptide bond, and from the reaction mixture fragment 228–316 was isolated in homogeneous form. This fragment appeared to attain in aqueous solution the folding properties of the corresponding segment in the intact protein, as indicated by quantitative analysis of secondary structure from far-ultraviolet circular dichroism spectra and immunological properties. Thus, double-immunodiffusion analyses showed that fragment 228–316 is able to recognize and precipitate anti-thermolysin antibodies raised in rabbits with native thermolysin as immunogen. The fragment displayed fully reversible and cooperative conformational transitions mediated by pH, heat, and guanidine hydrochloride (Gdn·HCl), as expected for a globular protein species. Thermal denaturation of the fragment in aqueous solution at pH 7.8 showed a T_m of 66 °C and the Gdn·HCl-mediated unfolding a midpoint transition at 2.2 M denaturant concentration. These results correlate well with Rashin's predicted location of stable subdomains in the COOH-terminal portion of thermolysin (see Appendix). An additional interest in the folding and stability properties of fragment 228–316 here described resides in the fact that its nativelike structure is organized as a four-α-helical bundle, a quite recurring protein structural motif [Weber, P. C., & Salemme, F. R. (1980) *Nature (London) 287*, 82–84], herewith shown for the first time to be stable in the absence of disulfide cross-links, bound ions, or cofactors.

Analysis of the three-dimensional structures of monomeric globular proteins has allowed recognition of their specific molecular structural characteristics in terms of a hierarchy of protein substructures, involving domains as one major level of protein structure (Schulz & Schirmer, 1978). Wetlaufer (1973) first surveyed a large set of proteins and by visual inspection identified protein domains as distinct, continuous chain regions that could be enclosed within compact volumes. Later, more quantitative methods to locate structural domains within proteins were developed (Rossmann & Liljas, 1974; Crippen, 1978; Rose, 1979; Sander, 1981; Wodak & Janin, 1981; Rashin, 1981). Some of these approaches (Crippen, 1978; Rose, 1979) allowed a description of protein molecules

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