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Disulfide Bond Formation During Renaturation of Bovine Pancreatic Ribonuclease A. Biochemical Assignments of Location of Cystinyl Residues in the Intermediate Protein Species and Theoretical Considerations on the Folding Pathway

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The order of disulfide formation of four cystinyl residues in bovine pancreatic ribonuclease A (RNase A) during renaturation was studied. Protein intermediates were quenched by carboxymethylation at several stages of renaturation, digested with proteolytic enzymes, and analyzed by column chromatography for cystine-containing peptides. Analysis showed that random disulfides formed at the initial stage were reshuffled to specific cysteine pairs in the following sequence. Cys 58, 65, and 72 were most rapid to give specific cystines, then Cys 84 followed. These experimental evidences are understood as one of the two wings observed in the three dimensional structure of the native RNase A is generated at first. Almost all of the available data on renaturation of RNase A support this folding scheme. Theoretical basis in favor of the assigned folding pathway was also given.

INTRODUCTION

Protein biosynthesis has been considered as a sequence of preliminary synthesis on ribosomes of a polypeptide chain encoded a definite primary structure, and the subsequent folding of the nascent chain into a biologically active three-dimensional structure, a protein.[1] The folding of a biosynthetically nascent polypeptide chain into a protein is observed in "renaturation" experiments *in vitro*, the process is thought to mimic the one actually happening *in vivo*.[1~3] In this instance, a protein is denatured in advance by a change of medium such as by perturbing pH, by addition of denaturants, and so on. The denatured protein could be converted into an active form again as a proper surrounding condition was achieved. Although renaturation of a denatured protein might contain a self-assembly process of protein subunits if proteins having subunit structure were concerned, we could restrict the problem in its simplest sense for proteins without subunits.

The idea that both of a biosynthetically nascent polypeptide chain and denatured protein should be conformationally random has been confirmed in many instances. Since a protein has a definite three-dimensional structure, the problem could be expressed as how a structureless polypeptide chain was folded into a conformationally definite one. This problem has been attracted much attention yielding many discussions.[3,4]

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There are fundamentally two aspects to treat the problem. One of them may be classified as a thermodynamic one, in which a thermodynamical equilibrium reigns a chain folding and a structureless polypeptide chain seeks for successively local minima in the whole of the free energy hyperspace to find out the global minimum. The other aspect contains the idea that a polypeptide chain proceeds a definite pathway formed by connecting local minima with the lowest pass on the potential energy surface, and is called as a kinetic one, since a kinetic consideration was also taken into account. A kinetic aspect does not always means that the pathway for protein folding is limited to one, nevertheless the number of pathways might be much smaller than that expected in a thermodynamic equilibrium model where all possible potential energy minima should be tested. An existence of a definite pathway, if established, will throw much light upon understanding on the nature of protein folding. There are, however, very little evidence available today as to the folding pathway problem.

On account of the fact that we cannot see directly the local conformation of a polypeptide chain during folding, a kind of probe is necessary to make the process to be experimentally traced. Disulfide bond present in a protein should serve as a probe of this kind as the bond is formed by an oxidation of cysteine-thiol groups in the course of the chain folding. The fact that crosslinkages such as cystine, desmosine, *etc.*, found in proteins are not present in a nascent polypeptide chain but a product of subsequent reactions is well established with evidences. The presence of multinumber of disulfide bonds in a protein will afford a chance to study the sequence of formation of those disulfide bonds during a chain folding. Formation of a disulfide bond between two cysteinyl residues suggests that a close proximity of the regions containing these residues is reached during the folding. If a definite sequence of disulfide bonds formation was found to be present, it would yield a possible pathway of the folding at least partially, and further insight into the nature of polypeptide conformations could surely be obtained. Three proteins, hen egg-white lysozyme (129 residues with 4 disulfides),[5] bovine pancreatic ribonuclease A (124 residues with 4 disulfides),[6] and bovine pancreatic trypsin inhibitor (58 residues with 3 disulfides),[7] have been investigated in this respect. Only the last protein, which is the smallest member among three, has been proved to have a definite folding pathway fairly conclusively. The studies on the other two protein have stayed in preliminary stage. In this report, we will present a biochemical study on the sequence of formation of the four disulfide bonds during the renaturation of the reduced and denatured bovine pancreatic RNase A.

EXPERIMENTAL

Materials. RNase A (purchased from Miles, England) was used after purification by chromatography on IRC–50.[8] Proteolytic enzymes were purchased as: pepsin from Sigma, chymotrypsin from Worthington, trypsin (grade B) from Calbiochem (suppliers all in U. S. A). Iodoacetic acid was recrystallized from chloroform-carbon tetrachloride and stored at −20°C until use under protection from light. Solutions of urea and guanidinium chloride were prepared according to the methods described by Haber[9] and by us,[10] respectively. 2-Mercaptoethanol (specially prepared

for biochemical assays, Nakarai Chemicals), which was supplied in a sealed ampoule, was used as soon as possible after opening an ampoule and stored frozen while not in use. Other reagents were of commercial products of analytical grade and used without further purification.

Amino Acid Analysis. Amino acid analyses were carried out on a Jeol liquid chromatographic system under the condition described by Spackman *et al.*[11]

Titration of Thiol Groups. Titration of thiol groups in protein has been known to be sensitive to the presence of a denaturant. Therefore, throughout this work, the titration was performed at pH 8 in the presence of approximately 6 M guanidinium chloride. 2 g of guanidinium chloride, 0.2 ml of 2 N tris buffer at pH 8.0, and 0.1 ml of 2×10^{-3} M DTNB in 0.1 N tris buffer at pH 6.5 were added in this sequence to 2 ml of a solution to be titrated. The content of thiol groups were calculated from the absorbance at 412 nm for this mixture.[12]

Measurement of Enzyme Activity. The method described by Anfinsen *et al.*[13] was exclusively employed throughout this work. Yeast RNA was used as a substrate.

Reduction and Renaturation of RNase A. RNase A was reduced with 2-mercaptoethanol in the presence of urea.[14] The reduced protein which was shown to have 7.8–8.2 SH groups per mol of protein by DTNB titration was kept in 0.1 N acetic acid and utilized for renaturation experiments within 2 days.

Renaturation of reduced RNase A was carried out at 25°C in the presence of cupric nitrate (10^{-6} M)[15] under slow agitation with a magnetic stirrer. In analytical runs to establish the relation between concentration of protein in a renaturation medium and a rate of renaturation or to see an effect of oxygen saturation on renaturation, 10 to 20 mg of the reduced protein was added to a required amount of 0.1 N tris buffer at pH 8.0, and aliquots were removed at appropriate time intervals to measure amounts of thiol groups and the enzyme activity. A renaturation under oxygen or nitrogen was started by adding the reduced protein to the buffer which was contained in a closed vessel and was preliminarily equilibrated with the desired gas. The vessel had a side tube to add or subtract a sample and an inlet tube, which was kept just above the solution, for oxygen or nitrogen. To avoid evaporation of the solvent, the gas was moistened by passing a wash bottle which contained the same buffer used for renaturation.

Preparative runs for determination of the location of disulfide bonds were carried out with 200–300 mg of reduced RNase A at a concentration of 0.10–0.13 mg/ml in tris buffer at pH 8.0 which was covered with oxygen. Therefore, the reaction was performed in a 3 or 5 l flask to enlarge an open surface of the solution exposed to oxygen. Developments of the reaction was monitored by an activity measurement and by a thiol titration, then quenched with iodoacetic acid at the time when the desired amount of disulfide bonds was formed.

Reaction of Protein Thiols with Iodoacetic Acid. Fully reduced RNase A

(approximately 4 mg) was reacted with iodoacetic acid (0.15 g, ca. 1000-fold molar excess for thiol groups present) at room temperature for 1 hr in 6 M urea —0.1 N sodium phosphate buffer. The reaction was carried out at several values of pH adjusted with 6 N HCl. Partially or completely S-carboxymethylated protein was obtained by desalting the reaction product on a column of Sephadex G–25. The amount of thiol groups left unreacted in protein was titrated with DTNB, and found as a function of pH at which the reaction was carried out; less than 0.1 SH and 2.4 SH per mole of protein after the reaction carried out at pH above 6.4 and 5.9, respectively.

Quenching of a Renaturation Reaction and Analysis of the Product. Quenching of renaturation was achieved by adjusting the pH of a renaturation mixture to 6.5 with conc. HCl and by addition of iodoacetic acid and urea to the mixture. Since it was found that a 6 M urea solution could be obtained by dissolving 490 g of urea in 1 l of water, a renaturation mixture to be quenched was mixed with urea by the amount calculated on the basis of this ratio from its volume to make the solution nearly 6 M of urea. The solution was added to iodoacetic acid (5 g per 100 mg protein) and 1 M phosphate buffer at pH 6.5 to make the solution 0.1 M in phosphate, warmed to 25°C to dissolve urea, and kept at this temperature for 1 hr with stirring. All manipulations were carried out under protection from light during the presence of iodoacetic acid. The mixture was then thoroughly dialysed at 5°C against 0.05 N acetic acid to remove iodoacetic acid, concentrated by ultrafiltration (Diafilter, G–05T, Nihon Shinku K. K.), and lyophilized. A lyophilized material was applied on a Sephadex G–75 column, and eluted with 0.1 M ammonium formate-0.1 N acetic acid (Fig. 3). Fractions containing monomeric protein were pooled and freed of ammonium formate by lyophilization. The product was chromatographed on an IRC–50 column and the main component eluted from the column most rapidly was collected, desalted by a gel filtration with Sephadex G–25, and digested by pepsin, trypsin, and chymotrypsin.

Assay of Disulfide Bonds in Intermediate Species Appeared in Renaturation. Solutions containing 2–3 mg of quenched materials were made 6 M in urea, adjusted to pH 8, and reacted with 0.1 ml of 2-mercaptethanol at 25°C for 1 hr. The reaction mixture was desalted on a small column of Sephadex G–25 and titrated with DTNB.

Enzymic Digestion and Chromatography of the Peptides. Enzymic digestion of partially renaturated-carboxymethylated protein with pepsin, trypsin, and chymotrypsin, in this order, was achieved as described by Spackman *et al.*[16] The entire hydrolyzate was chromatographed[16] on a column of Dowex 50–X2 with non-volatile or volatile buffers.[17] Native RNase A was similarly treated to give a chromatogram which was used as a reference standard.

All chromatographic fractions were analyzed by the ninhydrin method after alkaline hydrolysis[18] and for cystine-containing peptides in the manner described below. Cystine-containing peptides were analyzed for their amino acid compositions after hydrolysis with 6 N HCl.

Analysis for Cystine-Containing Peptides. The presence of disulfide bonds were determined with DTNB after their reduction to thiols. The method is fundamentally identical to the one employed by Cavallini *et al.*,[19] in which a peptide is first reduced with $NaBH_4$, then assayed for liberated thiol groups by DTNB. An aliquot was diluted to 2 ml after adjustment of pH about 10 with 10 N NaOH, added to 0.5 ml of 5% $NaBH_4$ solution containing 0.01 ml of 1 M EDTA per ml, and kept at 37°C for 1 hr. The reaction time for $NaBH_4$ was determined by preliminary experiments using cystine as a model compound: the yield of thiol groups and a reaction time were, 43% at 2 min., 46% at 10 min., 48% at 30 min., and 54% after 50 min., respectively. Excess $NaBH_4$ was then decomposed by incubation of the mixture with 0.1 ml of acetic acid at 38°C for 30 min. with occasional shaking. The resultant solution was mixed with 0.5 ml of 4 M tris and 0.1 ml of 2×10^{-3} M DTNB. The reading of absorbance at 412 nm gave the content of disulfide in the aliquot after comparison with the standard curve which was obtained with cystine as a reference material and shown in Fig. 1.

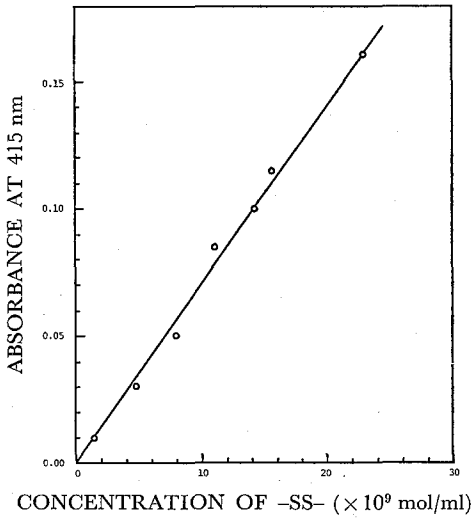

Fig. 1. Titration of disulfides with DTNB after reduction with $NaBH_4$. Compound: cystine.

RESULTS AND DISCUSSIONS

1. Method of Renaturation

Renaturation of reduced RNase A have been carried out by direct oxidation of thiols into disulfide with molecular oxygen in the presence of heavy metal ions as a catalyst,[20] or by disulfide interchange with compounds of low molecular weights having disulfide group.[6] The former procedure was chosen in the present study and cupric nitrate was used as a catalyst.[15] Renaturation with molecular oxygen is usually sluggish and takes longer hours for completion even in the presence of a catalyst. On the contrary, interchange reaction has the advantage[21] that the regeneration of protein activity is rapid, and is considered to reflect the reaction *in vivo* more faithfully than does the former procedure. Recent progress in renaturation experiments were

achieved with the extensive use of disulfide interchange reaction,[5-7] however, the method inevitably introduced another disulfide compounds not related to protein itself and inconvenience in analysis of the intermediates which appeared during a renaturation reaction. Reaction of disulfide-reduced protein with disulfide-compounds such as oxidized glutathione or cystine affords as intermediates a large amount of mixed disulfide between protein thiols and those of low molecular weight compounds, which might introduce ambiguity to establish the location of disulfide bonds proper to protein in renaturation-intermediates. This is the reason why we did not prefer the interchange reaction but oxidation with molecular oxygen as the renaturation method.

Restrictions in experimental techniques required unavoidably a fairly large amount of materials (hundred mg or more was desirable) if we attempted a determination of disulfide bonds in the protein species as intermediates in renaturation. On the other hand, concentration exerted a significant effect on renaturation of reduced RNase A, i.e., the higher yield of renaturation was obtained when the lower concentration of protein was used.[22] If we used a very dilute protein solution, the volume of a renaturation mixture became too much to handle the reaction products. These two restrictions lead us to find the maximum concentration which could hold the yield of renaturated protein still high without much elongation of reaction time and allow handling of maximum amount of protein in the minimum volume of renaturation medium. Several concentrations were tested and satisfactions were obtained when the renaturation was carried out at about 0.1 mg/ml. A representative kinetics of the renaturation reaction at this level of concentration is shown in Fig. 2.

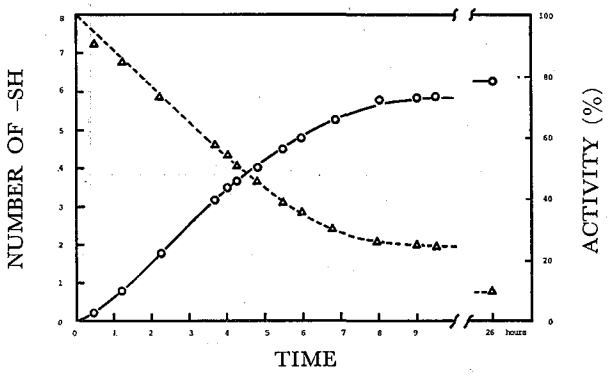

Fig. 2. Renaturation of reduced RNase A. Protein concentration: 0.138 mg/ml. Renaturation was carried out at pH 8.0 (0.1 M tris-HCl buffer), 25°C. Reaction medium was saturated with oxygen and contained 10^{-6} M Cu^{++} Activity is relative to native RNase A and toward RNA.

Saturation of the renaturation medium with oxygen was shown to enhance the reaction rate nearly twice,[10] all preparative experiments including the one represented in Fig. 2 were carried out under this condition. Effect of oxygen saturation were not only limited to accelerate the renaturation but also resulted in reduction of aggregates probably caused by intermolecular disulfide formation.[23] Presence of a sufficient

amount of the oxidant was considered to complete the oxidation within a molecule and to reduce a possibility of intermolecular reaction. Even when the condition was optimized, the fact that the renaturation of reduced RNase A did not reach 100% after 24 or more hours suggested the possible production of intermolecular aggregates which could not enter a further disulfide equilibrium reaction. Precipitates visually observed in preparative runs was probably the products of intermolecular disulfide formation. Almost complete regain of enzymic activity within 12 hr was observed when the renaturation was carried out at the concentration below 0.01 mg/ml.

2. Pathway of Protein Folding during Renaturation

Three proteins have been studied on their folding scheme during renaturation; that is, lysozyme,[5] RNase A,[6] and bovine pancreatic trypsin inhibitor.[7] Preliminary report on lysozyme[5] suggested the presence of a limited number of pathway of folding, while complete studies have not been published yet. The most extensive studies were given to trypsin inhibitor,[7] which showed also the presence of a unique folding pathway. The study on RNase A[6] has suggested, on the contrary, the absence of a definite pathway of folding.

There are four disulfide bonds present in RNase A. When half cystinyl residues are numbered from I to VIII for cys 26, 40, 58, 65, 72, 84, 95, and 110 according to the sequence, these four cystines are I–VI, II–VII, III–VIII, and IV–V, respectively.[16] As a random combination of 8 cystinyl groups to make 4 cystines gives 105 possibilities, the forming order of cystinyl residues during renaturation might be used to distinguish a definite or random pathway of peptide chain folding. If the folding occurs without any particular path, there would not be predominant population of a certain cysteine pair (or pairs) as cystine in the intermediate protein species found in renaturation process. Our strategy to study the folding of RNase A is then to analyze the positions of cystines generated with a progress of renaturation reaction. Renaturation reaction was quenched at an appropriate time interval and the trapped intermediates were digested with proteolytic enzymes. Peptide fragments which were proved for the presence of cystine were analyzed for their amino acid composition. The position of cystine in the primary structure would be determined by a comparison of the amino acid composition of an SS-peptide with the amino acid sequence of the original protein.

(1) Quenching of Renaturation. Three samples called 1, 2, and 3 were obtained by quenching a renaturation reaction of reduced RNase A at the moment when 1.5, 2.2, and 3.1 disulfide bonds were formed, respectively. As it was necessary to use a large volume of renaturation medium for the preparation of each samples, 1, 2, and 3 were the products of three separate runs.

Quenching was performed in the presence of 6 M urea by trapping thiols with iodoacetic acid. The reaction should be carried out at pH as possible as low to eliminate disulfide interchange during the reaction, while a possibility of carboxymethylation of histidyl residues increased. The value of pH was chosen at 6.5, which was the lowest limit to complete the carboxymethylation within 30 min. according to the preliminary experiments with fully reduced RNase A. Two histidines in RNase A (His 12 and His 119) have been known to be highly reactive toward iodoacetic acid and

protected from the reagent by the addition of phosphate ion.[24] Although such precaution was questionable in the present case as the protective effect of phosphate might be expected only for enzymatically active species, we carried out the carboxymethylation in the presence of 0.1 M phosphate. The fact that the carboxymethylation decreased significantly the bulk activity of the material to about 1/3 might be partially ascribed to a formation of carboxymethylhistidines. Unfortunately our system of amino acid analyzer could not distinguish the presence of both of carboxymethylhistidines in hydrolyzates, the amount of modified histidines were left unknown.

(2) Chromatography of the Quenched Products. After completion of carboxymethylation, the reaction mixture was lyophilized, concentrated, and chromatographed on a column of Sephadex G–75. The chromatogram revealed the presence of polymeric protein (Fig. 3) which must be derived from the formation of intermolecular disulfide bonds. Figure 3 also shows that a fraction of polymeric forms decreases with an advance of renaturation. Although the polymeric fractions were not subjected to further analysis, it will be interesting to see whether preferential cysteines are included in these intermolecular disulfide bonds.

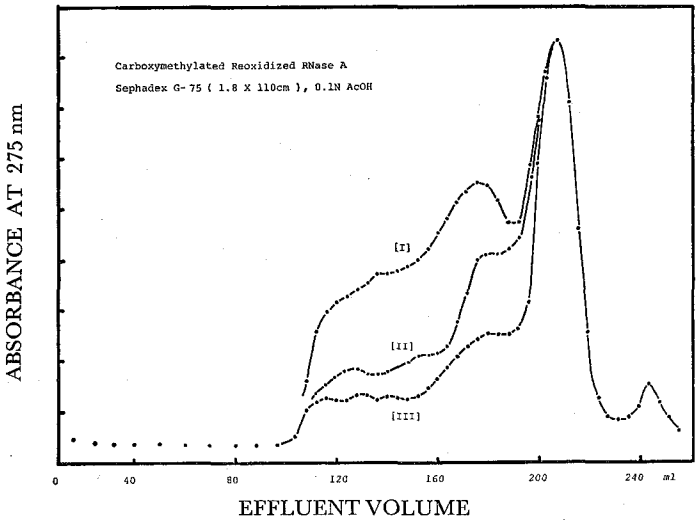

Fig. 3. Sephadex G–75 (1.8×110 cm, 0.1 N AcOH) chromatography of intermediate 1, 2, and 3. Three chromatograms are normalized to the same value of monomeric protein fraction.

The monomeric protein, partially renaturated and carboxymethylated RNase A, was further chromatographed on IRC–50. There were usually three protein fractions in the chromatogram. Figure 4 illustrates the chromatogram of the monomeric fraction of sample 2. The first fraction, only weakly adsorbed on the resin, had no RNase activity toward RNA, the second one about 1/4–1/3 of the native RNase A, and the last almost the same level of the native protein and thus considered to be completely renaturated. The proteins in the first fractions from samples 1, 2, and 3 were named intermediates 1, 2, and 3, respectively. Table I shows the disulfide contents of samples 1, 2, 3, and intermediates 1, 2, and 3.

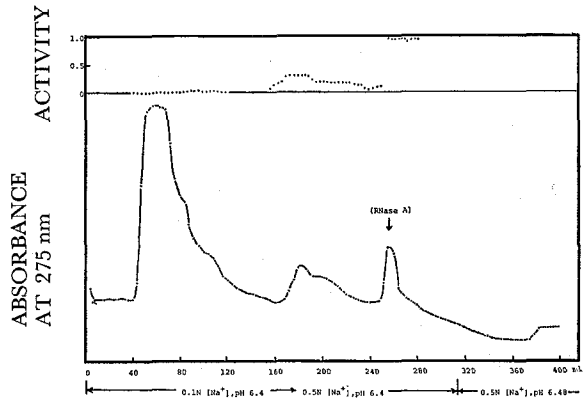

Fig. 4. Chromatogram of monomeric protein fraction of intermediate 2. An arrow shows the elution position of native RNase A. Activity is relative to native protein and toward RNA.

Table I. Disulfide Contents of Protein Species

	disulfide/protein[a)]
sample 1	1.5
2	2.2
3	3.1
intermediate 1	0.9
2	2.0
3	2.8

a) Disulfide contents were obtained by DTNB titration after reduction with 2-mercaptoethanol.

The slightly active second fraction is consisted probably of protein species which lack one or two cystinyl bonds while the other three or two cystines were formed in the same combination of cysteinyl residues as in the native protein. With respect to this slightly active species, presence of free cysteines before the quenching was demonstrated by an appearance of carboxymethylcysteine in a total acid hydrolyzate. A modified RNase A with two specific disulfide bonds (III–VIII and IV–V) opened have been shown to be active.[25] Each of intermediates 1, 2, and 3 was digested with pepsin at pH 2, trypsin at pH 6.5, and cymotrypsin at pH 6.5, in this order.[16] In the reported studies[5~7] on renaturation of lysozyme, bovine trypsin inhibitor, and RNase A, a diagonal paper electrophoresis[26] has been exclusively used to separate and analyze partial hydrolyzates. In spite of the method being convenient, rapid, and applicable on the smallest amount of peptides, it is always accompanied with a possibility of uninvited disulfide interchange. Even at a low pH, it is reported by Cha *et al.*[27] that an erroneous result with paper electrophoresis was yielded during their studies on modified RNase A. As we had not enough knowledge to rely upon paper electrophoresis, a rather classical column chromatographic separation method was adopted for safety. Column chromatography has been considered to afford much reliable and reproducible results, but the old conditions described by Hirs *et al.*[16] was too tedious as the method used so-called non-volatile buffers and required an additional procedure to remove salts in chromatographic fractions. Chromatography

with volatile buffers[17] may avoid these difficulties and used in some of the present study. We intended to use a volatile buffer system throughout the work, but poor separation in some cases forced us to return to the system of Hirs *et al.*

As a model experiment, RNase A was digested with pepsin, trypsin, and chymotrypsin. The partial hydrolyzate was chromatographed on Dowex 50–X2 to test a buffer system. Most clear chromatogram was obtained with the buffer system of Hirs *et al.* Content of cystinyl peptides in each fractions was analyzed by a method commented below.

Among various method available for analysis of cystine, reduction of cystine into cysteine followed by a DTNB titration of thiol was employed for its high sensitivity. The method of Cavallini *et al.*[19] which employed $NaBH_4$ as a reducing reagent for disulfides was used. The reaction condition was modified so as to fit in the present purpose, and Fig. 1 shows the reliability and sensitivity of the method applied for cystine as a model compound. These analyses gave 4 cystine-containing peptide fractions which were shown from their amino acid compositions to represent respectively 4 cystines in native RNase A.

As the experiments with native RNase A was proved to give satisfactory results on determination of the location of cystines in the primary structure, analysis of intermediates 1, 2, and 3 were performed.

Intermediate 1 Intermediate 1 is considered to be protein species appeared in initial phase of renaturation. Dowex 50–X2 chromatography with volatile buffers under the same condition as in Fig. 5 and analysis of the chromatographic fractions showed no evident cystine-containing fractions. Since the parent protein contained 0.9 disulfide per mole of protein (Table I), the result suggested the absence of a specific cysteine pair in intermediate 1. If cystines made of specific cysteine pairs was not present but of nonspecific pairs, cystine-containing peptides would appear in diverse fractions and would not be detected. The amount of intermediate 1 submitted to analysis was small (about 50 mg) because of the presence of a large amount of polymeric protein in sample 1, therefore, a possibility that not a random but a limited number of pair of cysteine was present as cystine in intermediate 1 could not be excluded.

Intermediate 2 Enzymic hydrolyzate of intermediate 2 was chromatographed on Dowex 50–X2 with a volatile buffer system. One fraction clearly positive to cystine-peptides was obtained in this case. Amino acid analysis of the crude peptides suggested half-cystine III, IV, and V were present (Table II). If we assumed that enzymic cleavage occurred as same as reported by Spackman *et al.*,[16] no detection of arginine and proline indicates that the peptide must not include half-cystine VI, II, VII, and VIII. Also absence of half-cystine I was suggested by low yield of methionine. Table II listed the calculated amount of amino acids for two cases that the peptides were composed of a mixture, half-cystine pair IV–V and 1/4 amount of III–IV or III–V, on the same assumption.

Intermediate 3 The chromatogram obtained with Dowex 50–X2 showed the presence of several cystine-containing peptides fractions (Fig. 5). Fraction 1, which was most positive to cystine reaction, was shown to contain half-cystine III, IV, and V (Table II). In fraction 2, presence of half-cystine VI was supported by occurrence of arginine in the amino acid composition, which also suggested the pair V–VI.

Table II. Amino Acid Composition of Cystine-Containing Peptides

	cystine-containing peptides from intermediates[a]			calculated[b]		
	intermediate-2	intermediate-3		(IV–V) +1/4 (III–IV)	(IV–V) +1/4 (III–V)	(V–VI)
		fraction-1	fraction-2			
Lys	1.6	1.8	0.3	1.5	1.25	0
His	0.1	0.1	0.0	0	0	0
Arg	0.0	0.0	0.8	0	0	1
NH_3	5.1	—	2.9	3.5	3.75	2
Asp	2.5	3.3	1.4	3.25	3.5	3
Thr	1.3	0.8	1.1	1	1.25	2
Ser	1.1	1.1	0.6	1.25	1.25	1
Glu	1.9	2.5	1.1	1.25	1.5	1
Pro	0.0	0.0	0.0	0	0	0
Gly	1.4	0.7	0.6	1	1.25	1
Ala	1.1	1.1	0.2	1.5	1.25	0
1/2Cys	2.5	2.5	2.0	2.5	2.5	2
Val	0.6	0.6	0.5	1.5	1.25	0
Met	0.3	0.2	0.2	0	0	0
Ile	0.1	0.1	0.7	0	0	1
Leu	0.1	0.1	0.0	0	0	0
Tyr	0.6	0.6	1.1	1	1.25	1
Phe	0.1	0.1	0.1	0	0	0

a) The values for 1/2cystine were arbitrary selected and those of the other amino acids were normalized to them.

b) On the assumption that the same peptides had been obtained as in Spackman *et al.*[16]

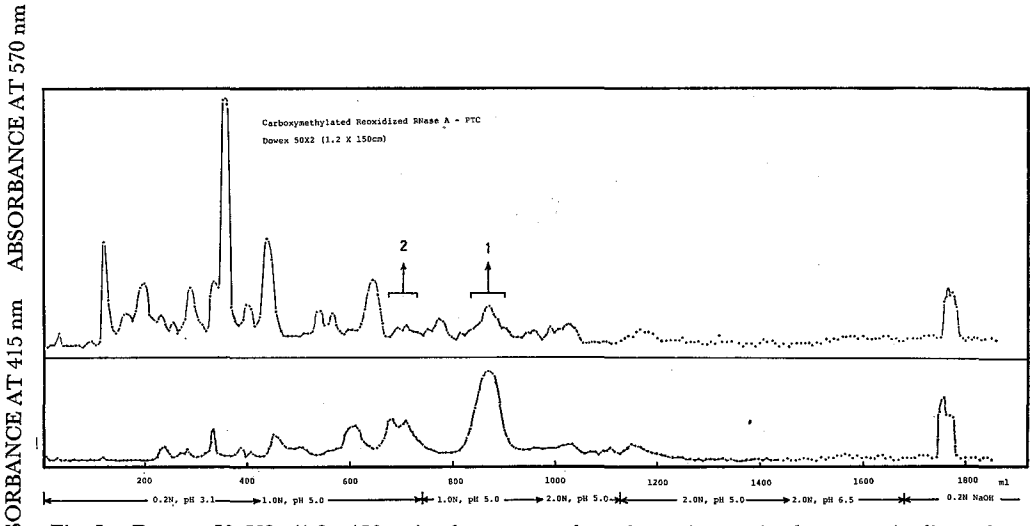

Fig. 5. Dowex 50–X2 (1.2×150 cm) chromatography of pepsin-trypsin-chymotrypsin-digested carboxymethylated intermediate 3. Pyridine — formic acid or acetic acid was used for linear gradient elution. Upper curve is for ninhydrin color values after alkaline hydrolysis and lower one for cystine content. Arrows show fraction 1 and 2 described in the text.

(3) Possible Stages of folding.

The presence of half-cystinyl residues IV and V in both of the intermediates 2 and 3 obtained in the course of renaturation suggests the formation of the correct disulfide

bond IV–V at the initial stage of folding. At the same time the half-cystinyl residue III is contained in the intermediates. That is, it is presumed that these three residues gather together in the primary folding of the nascent polypeptide chain. Of course, another local folding region might be present, since we are looking only for the location of disulfide bonds formed.

For the purpose to trace a folding pathway, comparison with the real three dimensional structure revealed by X-ray crystallography may be helpful.[28,29] According to the analysis of the structure by the use of atomic coordinates of RNase S,[29] there are three candidates for regions of initial folding nucleus. The distance map shown in Fig. 6 may illustrate the location of such regions, *i.e.*, from Glu 55 to Ser 75, from Thr 70 to Cys 110, and from Ala 102 to Val 124. The energy map made by calculating pairwise energies of i-th residue and j-th residue instead of relative distance between the respective C^{α} atoms, r_{ij}, in the map has very similar pattern to that illustrated in Fig. 6, and the regions identified above are energetically stable one. The last two regions take anti-parallel β-conformations with chain reversal regions of Ser^{90}–Lys^{91}–Tyr^{92}–Pro^{93}–Asn^{94}(–Cys^{95}) and His^{105}–Ile^{106}–Ile^{107}–Val^{108}–Ala^{109}(–Cys^{110}), respectively. Interestingly each of them has a half-cystinyl residue at the C-terminal end of the turn.

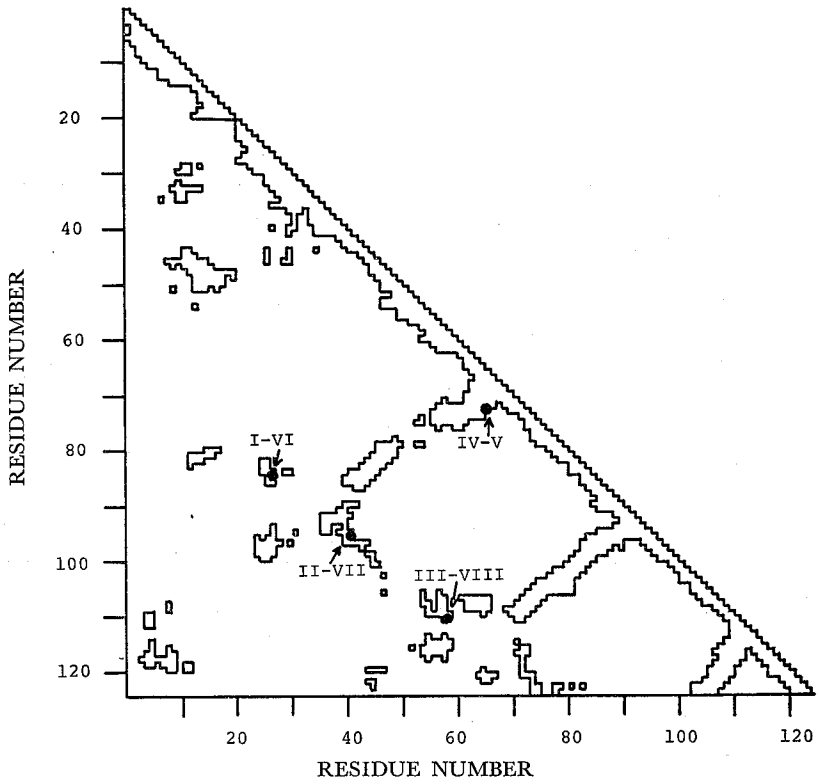

Fig. 6. The distance map for RNase S is illustrated by separating regions where mutual distances r_{ij} between ith and jth residue are less than 10 A. Circles indicate the location of disulfide bonds.

The formation of the disulfide bond IV–V at the initial step implies that the possible nucleus would be the first one from Gln 55 to Ser 75, in which half-cystinyl residues, III, IV, and V detected experimentally are all included. In the native structure, these three residues are located within 10 Å except from the residues III to IV. In addition it seems to be reasonable that the reaction between any two of the residues III, IV, and V occurs first, since the residues are near each other along the primary structure.

When the disulfide bond IV–V has been formed, the probability to select correct pairs reduces from 1/105 to 1/15. Among 15 possibilities a probable next step would be a reaction involving the half-cystinyl residue III, for the residue is left unreacted or its disulfide bond is unshaffled near the nucleus already formed. Presence of several hydrophobic residues in the neighborhood of Cys 58 suggests proximity of some portion of the polypeptide chain contained hydrophobic groups to form a new disulfide bond. Cys 110 is the only residue that satisfies the above situation, and therefore, the disulfide bond III–VIII would be the possible pair in the second step of the course. As a result, one of the two wings in the native structure would be formed.

The final stage of folding, then, is the construction of the other wing by fixing β-structures with the disulfide bonds, II–VII and I–VI, the formation of which is much easier than before as the only two ways are left for the combination. The correct pair II–VII is considered to be formed in the first place of this stage, since an extended β-sheet composed of three portions of the chain, from Pro 42 to His 48, from Met 79 to Thr 87, and from Ala 96 to Lys 104 is stabilized by the disulfide bond. Now that all the correct disulfide bonds are established, the native three dimensional structure is generated.

3. Discussions on the Pathway.

The present proposal of the folding pathway of RNase A based on the experimental results on renaturation and the analysis of the three dimensional structure is summarized as follows: At the initial stage, the reduced polypeptide chain wobbles to initiate folding cores, and particular disulfide bonds are not formed as indicated by the results on intermediate 1. Then, the region containing half-cystinyl residues, III, IV, and V becomes nucleus forming the particular disulfide bond IV–V as detected in intermediates 2 and 3. The next stage is to constitute a hydrophobic core by making a bridge between III and VIII, resulting in completion of one of two wings. Finally the other wing rich in β-structure is fixed through formation of disulfide bonds II–VII and I–VI, giving rise to the native structure.

The postulated pathway must be examined whether it is plausible or not with respect to other experimental results on renaturation obtained so far. First, restoration of secondary structure is not so rapid[23] for the renaturation of RNase A in contrast to quick formation for lysozyme[30] or other proteins.[31] Since the nucleus in the present pathway does not contain an appreciable amount of α-helix nor β-structure, this slow formation of a secondary structure is reasonable. On the other hand, quick development of the structure would occur in parallel with the formation of the other wing and with attachment of α-helical S-peptide part, which has been random coil, to the nucleus. Also, sigmoidal appearance of enzymic activity against time is ex-

plained in the same way.

Second, some chemical or enzymatical derivatives of RNase A cannot be renaturated depending on sites of modification, e.g., diiodo-Tyr-115-RNase A,[27] des (121–124)-RNase A[32] and dansylated RNase A.[33] All the sites are in the neighborhood of the present nucleus, and it is probable that such modifications interfere the nucleation of the core. Since the role of disulfide bonds, IV–V and III–VIII is to initiate the nucleus, it is not contradictory that a derivative, in which phosphorothioate groups are attached to the sulfur atoms of the above two disulfide bonds, is active.[25] According to our scheme, this derivative seems to have no capacity of renaturation.

Third, the nucleus is included in hard cores which were deduced to be unable to unfold in the thermal denaturation of RNase A.[34] Although the pathway of unfolding with intact disulfide bonds might not correspond directly to the opposite direction of folding pathway, the coincidence of the core would support validity of the present scheme. The only experiment difficult to explain is formation of an active material of a 70-residue analogue of RNase A synthesized chemically,[35] since the model compound lacks the portion of the nucleus. Because of absence of knowledges on the three dimensional structure of the analogue which differs in the primary structure, the result may not be comparable with the present folding pathway.

REFERENCES

(1) C. J. Epstein, in "Aspects of Protein Biosynthesis", Part A, Ed. C. B. Anfinsen, Jr., Academic Press, New York, 1970, p. 367.
(2) S. Takahashi, *Tampakushitsu, Kakusan, Koso*, **18**, 410 (1973).
(3) C. B. Anfinsen and H. A. Scheraga, *Adv. Protein Chem.*, **29**, 205 (1975).
(4) D. B. Wetlaufer and S. Ristow, *Ann. Rev. Biochem.*, **42**, 135 (1973).
(5) S. Ristow and D. B. Wetlaufer, *Biochem. Biophys. Res. Comm.*, **50**, 544 (1973).
(6) R. R. Hantgan, G. G. Hammes, and H. A. Scheraga, *Biochemistry*, **13**, 3421 (1974).
(7) T. E. Creighton, *J. Mol. Biol.*, **95**, 167 (1975).
(8) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).
(9) E. Haber, *Proc. Natl. Acad. Sci. U. S. A.*, **52**, 1099 (1964).
(10) S. Takahashi and T. Ooi, *Bull. Inst. Chem. Res., Kyoto Univ.*, **51**, 329 (1973).
(11) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(12) R. F. Colman, *Biochemistry*, **8**, 888 (1969).
(13) C. B. Anfinsen, R. B. Redfield, W. L. Choate, J. Page, and W. R. Carroll, *J. Biol. Chem.*, **207**, 2011 (1954).
(14) F. H. White, Jr., *Methods in Enzymology*, **11**, 481 (1967).
(15) T. Takagi and T. Isemura, *J. Biochem.*, **56**, 344 (1964).
(16) D. H. Spackman, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **235**, 648 (1960).
(17) W. A. Schroeder, R. T. Jones, J. Cormick, and K. McCalla, *Anal. Chem.*, **34**, 1570 (1962).
(18) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).
(19) D. Cavallini, M. T. Graziani, and S. Dupre, *Nature*, **212**, 294 (1966).
(20) F. H. White, Jr., *J. Biol. Chem.*, **236**, 1353 (1961).
(21) V. P. Saxena and D. B. Wetlaufer, *Biochemistry*, **9**, 5015 (1973).
(22) C. J. Epstein, R. F. Goldberger, D. M. Yound, and C. B. Anfinsen, *Arch. Biochem. Biophys., Suppl. No. 1*, 223 (1962).
(23) C. B. Anfinsen, E. Haber, M. Sela, and F. H. White, Jr., *Proc. Natl. Acad. Sci. U. S. A.*, **47**, 1309 (1961).
(24) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2413 (1963).

(25) H. Neumann, I. Z. Steinberg, J. R. Brown, R. F. Goldberger, and M. Sela, *Eur. J. Biochem.*, **3**, 171 (1967).
(26) J. R. Brown and B. S. Hartley, *Biochem. J.*, **101**, 214 (1966).
(27) C.-Y. Cha and H. A. Scheraga, *J. Biol. Chem.*, **238**, 2965 (1963).
(28) G. Kartha, J. Bello, and D. Harker, *Nature*, **213**, 862 (1967).
(29) H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, and F. M. Richards, *J. Biol. Chem.*, **245**, 305 (1970).
(30) K. Yutani, A. Yutani, A. Imanishi, and T. Isemura, *J. Biochem.*, **64**, 449 (1968).
(31) J. W. Teipel and D. E. Koshland, Jr., *Biochemistry*, **10**, 798 (1971).
(32) H. Taniuchi, *J. Biol. Chem.*, **245**, 5459 (1970).
(33) F. H. White, Jr., *J. Biol. Chem.*, **239**, 1032 (1964).
(34) A. W. Burgess and H. A. Scheraga, *J. Theor. Biol.*, **53**, 403 (1975).
(35) B. Gutte, *J. Biol. Chem.*, **250**, 889 (1975).