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SOME RELATIONSHIPS OF STRUCTURE TO FUNCTION IN RIBONUCLEASE

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One of our basic objects in the study of ribonuclease (RNase) has been determination of the structural features essential for its enzymatic activity. The preliminary approach has involved a study of chemical modifications of the enzyme in relation to activity, with conclusions being drawn as to the parts of the molecule affected and the likelihood that these parts were associated with enzymatic activity. There are numerous reports of this kind in the literature on many enzymes; RNase is one of the more thoroughly studied in this respect. As indicated in TABLE 1, some treatments destroy RNase activity while others do not. It can be inferred from such results that only a part of the enzyme molecule (the "active center") is essential for catalysis.

Physical Studies on RNase

The changes in certain physicochemical properties have been studied to gain further insight into the nature of the chemical changes that accompany inactivation. The ultraviolet (U. V.) spectrum of acid hydrolyzates of RNase contains the expected peak for tyrosine with a maximum at 2760 Å. However, the corresponding peak in native RNase is displaced about 15 Å toward higher wave lengths and shows a higher maximum (ϵ = 9800 for native RNase and 9390 for the hydrolyzate).[1-3] There is an abundance of evidence[4-7] that suggests that this effect is due to hydrogen bonds between tyrosine hydroxyl groups and carboxylate groupings. Most procedures examined thus far that cause the inactivation of RNase also eliminate this "native" spectral displacement. FIGURE 1 demonstrates the effects of various conditions on the U. V. spectrum of RNase. The displacement is abolished by limited pepsin digestion, the product of which apparently differs from native RNase only insofar as the C-terminal tetrapeptide is missing.[8] This product has little or no RNase activity. The displacement is also eliminated by 8 M urea, but the native U. V. spectrum is restored by addition of 0.15 M phosphate.[2] It has been suggested that restoration of the native spectrum by orthophosphate, polymetaphosphate, and certain other anions is duplicated by the substrate, ribonucleic acid (RNA), and that this effect accounts for the undiminished activity which RNase displays in 8 M urea despite elimination of the spectral displacement in the absence of these anions.[3, 9]

That it is possible to destroy enzymatic activity without abolishing the spectral shift was shown recently by Klee and Richards.[18] The 95 per cent guanidination of RNase resulted in a product with only 33 per cent of the specific activity of native RNase, but it exhibited only a lowering of the extinction, without the shift to a lower wave length.

F. M. Richards[10] has reported that limited digestion of RNase with the bacterial protease subtilisin yields an active product that consists of the N-terminal "tail" of twenty amino acids attached to the larger moiety, apparently

through hydrogen bonds. This product also exhibits the spectral displacement that is eliminated by urea and restored by phosphate. Separation of the N-terminal tail and the macromolecular component causes elimination of the native displacement that occurs together with complete loss of activity. Recombination of these components completely restores activity, as well as the spectral displacement. Neither part has the ability to regenerate activity on recombination after photo-oxidation. This reaction appears to affect only

TABLE 1
QUALITATIVE SUMMARY OF CHANGES IN ENZYMATIC ACTIVITY AND PHYSICAL PROPERTIES OF RIBONUCLEASE

Reaction	Changes from "native" properties			
	Activity destroyed*	Spectral shift**	Negative rotatory increase†	Viscosity increase†
Oxidation with performic acid[19]	yes	yes	yes	yes
Reduction to 1.5 SH[3]	slight	no	no	no
Reduction to 8 SH[3]	yes	yes	yes	yes
Limited pepsin digestion[2]	yes	yes	slight	slight
Complete pepsin digestion[2]	yes	yes	yes	–
Methylation[3]	yes	yes	yes	–
95 Per cent guanidination[18]	yes	no‡	no	–
8 M Urea[2, 30]	no	yes	yes	yes
8 M Urea + 0.15 M phosphate[2]	no	no	no	no
2 M Guanidine[3]	no	yes	yes	yes
2 M Guanidine + 0.15 M phosphate[3]	no	no	no	no
6 M Guanidine[3, 9]	yes	yes	yes	yes
6 M Guanidine + 0.15 M phosphate[3, 9]	yes	yes	yes	yes
pH > 12.7[2]	yes	yes	–	–
pH < 2[1, 2]	no	yes§	–	–
100° C. for 2 hours[2]	yes	yes	–	–
Liquid NH_3[2]	no	no	–	–
Aqueous NH_3 (28 per cent)[2]	no	no	–	–

* Complete or appreciable destruction, unless otherwise indicated.
** Shift of ultraviolet spectrum of native ribonuclease to that of the total acid hydrolyzate, whereby the maximum of the "tyrosine" peak moves 15 Å toward the ultraviolet and decreases in extinction.
† Taken as indication of unfolding of secondary structure.
‡ No shift in maximum, but decrease in extinction.
§ This shift is reversible; when the pH is increased for the purpose of enzyme assay, the "native" spectrum is restored, and the product exhibits full activity.

histidine, which has been postulated frequently to be part of the active centers of certain esterases and proteases;[11-17] therefore the N-terminal tail may play a direct role in the active center.

Occurring with the denaturation of proteins is an unfolding of the secondary structure, which results in increases in negative optical rotation and viscosity. It is noteworthy that limited pepsin digestion of RNase, while it abolished activity and the native spectral displacement, had little effect on rotation.[2] It has been concluded that the secondary structure of RNase under these conditions of digestion remains nearly intact, but that essentially only the tertiary structure necessary for activity has been disrupted.

In contrast to the effect of limited pepsin digestion, cleavage of the disulfide

bonds of RNase (whether by oxidation with performic acid[19] or by reduction with sulfhydryl-containing reagents)[20, 21] causes marked increases in the negative optical rotation and in viscosity, in addition to abolishing the native spectral displacement. These effects constitute evidence that cleavage of disulfide bonds causes disorientation of the secondary structure, in addition to disrupting the tertiary configuration.

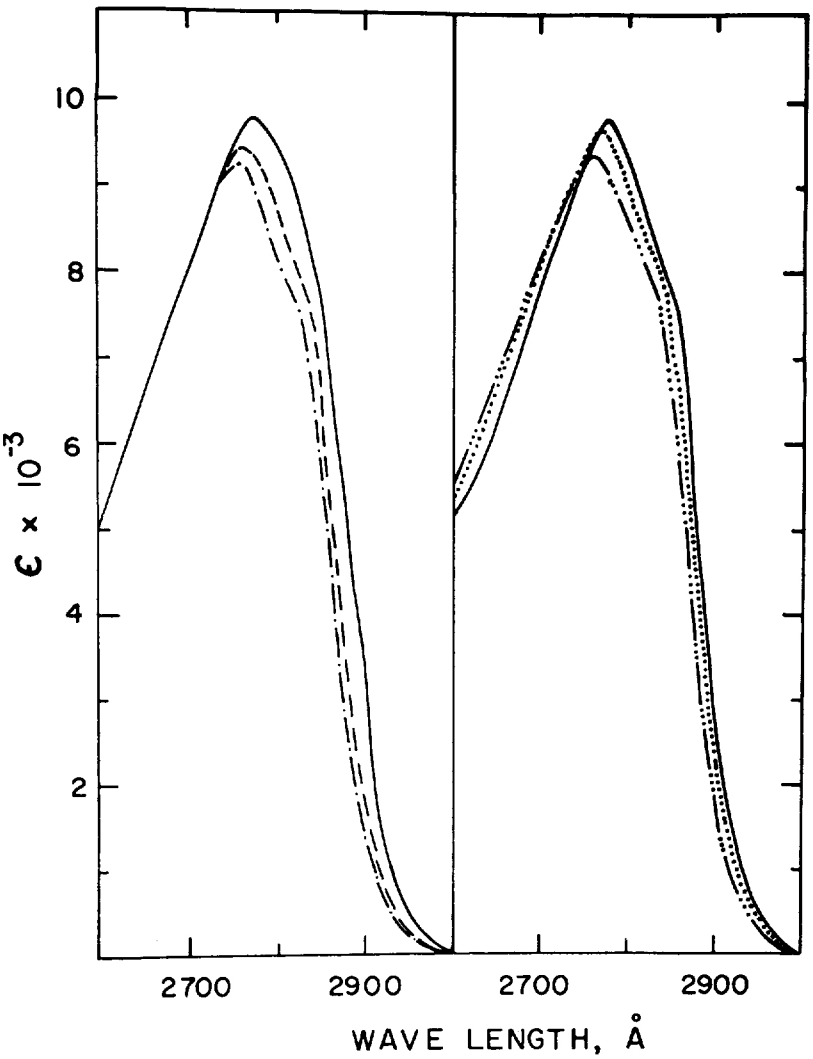

FIGURE 1. Curves represent absorption spectra as follows: —, native RNase in water, phosphate buffer, or a solution of 8 M urea and 0.15 M phosphate buffer of *p*H 6.5; – – – –, pepsin-inactivated RNase in water; —·—·, RNase or pepsin-inactivated RNase at *p*H 6.5 after complete digestion with pepsin at *p*H 1.8; —···—, RNase in 8 M urea; ····, RNase in 8 M urea and 0.003 M phosphate buffer of *p*H 6.5.

For all reactions of RNase thus far studied, experimental conditions that produced increase in the negative optical rotation also resulted in an increase in viscosity. Another physical property, rotatory dispersion, has not been studied as thoroughly, but has served as additional confirmation of the uncoiling of the secondary structure of RNase in urea and guanidine.[3] In every situation where physical properties were affected in such a manner as to indicate secondary unfolding, there was also an elimination of the native spectral displacement and a destruction of activity. In all cases, except that in which RNase was dissolved in 8 M urea, activity was lessened or destroyed by a treatment that modified the native spectral displacement, although changes in

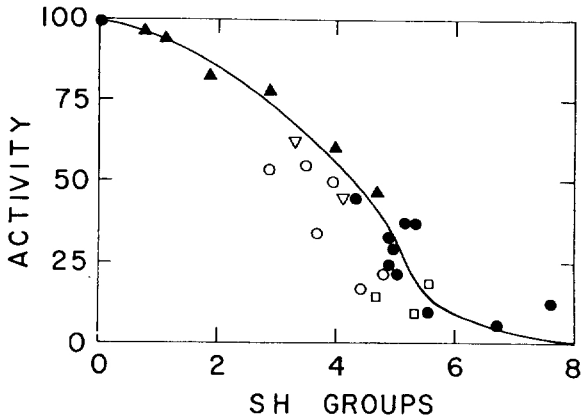

FIGURE 2. Enzyme activity (expressed as percentages of the specific activity of native RNase) as a function of the number of moles of SH per mole of enzyme. A *closed triangle* represents reduction in absence of urea; a *closed circle*, reduction in 8 M urea; an *open square*, reoxidation of RNase that had been reduced for 4.5 hours in 8 M urea and contained 8 SH groups per mole; an *open circle*, reoxidation of samples containing more than 6 SH groups per mole; an *open triangle*, reoxidation of samples containing about 4 SH groups per mole. The curve is drawn through the black points to indicate approximately the course of reduction.

secondary structure, as indicated by rotatory or viscosimetric changes, did not necessarily follow. A summary of these studies is given in TABLE 1. Experimental results thus far indicate that the active center of RNase involves folding of the peptide chain to create a specific tertiary structure.

Reduction and Reoxidation of RNase

The reductive cleavage of disulfide bonds in RNase results in loss of enzymatic activity, and a study of this reaction is therefore of interest in the investigation of the active center. Reduction of RNase has been achieved in this laboratory with thioglycolate at *p*H 8.5 (adjusted with trimethylamine) in the presence or absence of urea; further details are given elsewhere.[20, 21] FIGURE 2 summarizes a number of experiments undertaken to correlate enzymatic activity with sulfhydryl (SH) groups per mole of protein appearing on reduction of native RNase and reoxidation of reduced RNase; the curve is drawn through the black points to indicate the path of reduction, and the remaining points

represent some early experiments performed on the reoxidation of reduced RNase. It is noteworthy that these latter points tend to follow the same curve. It may be seen that inactivation proceeds at a relatively slow rate to about the half-reduced level, and then increases markedly. Thus it appears that the 2 disulfide bonds initially cleaved have little effect on enzymatic activity, while the final 2 are more intimately associated with the active center.

The locations of the disulfide bonds have been virtually established* by Spackman *et al.*[22] and Anfinsen and Ryle.[23] They appear to connect half-cystine residues 1–6, 2–8, 3–7, and 4–5. It has been reported[24] that the 1–6 bond is somewhat more labile than the others under the conditions of reduction employed in our laboratory. Since it appears to be absent at a level of reduction where considerable RNase activity remains, it has been concluded tentatively that this disulfide bond is not associated with the active center. However, the possibility that the function of disulfide bonds in catalysis is an entirely indirect one cannot be eliminated at present. It is possible that any disulfide bond, if cleaved while the others remained intact, similarly would be found to be unessential for activity.

According to Kern,[25] reduction of two of the three disulfide bonds in pepsin yields a partially active product that continues to lose activity with no further change in the extent of reduction. This observation was interpreted to mean that a secondary change was responsible for at least some of the activity loss, and that the absence of two of the three disulfide bonds permitted eventual denaturation and inactivation. These results would suggest that the function of disulfide bonds in pepsin is at least partially protective in nature. Similar results, however, have not been observed with RNase. The disulfide bonds in RNase seem more directly concerned with the active center than those in pepsin, and the question may be raised as to whether these bonds in RNase form a part of the essential structure of the active center or function in a protective manner. Upon reduction of trypsin, Liener,[26] found a complete loss of enzymatic activity with disappearance of only one of its three disulfide bonds. Here, also, a close connection is indicated between disulfide bonds and the active center.

Enzymatic activity lost on reduction of RNase may be regenerated by oxidation with molecular oxygen. Such reoxidation has yielded complete reactivation of reduced RNase, starting with samples having specific activities of 5 to 10 per cent that of native RNase.

Methods. Reduction and determination of the resulting SH content by spectrophotometric titration with *p*-chloromercuribenzoate were carried out as previously described.[20, 21] The reoxidation procedure involves aeration of solutions of reduced RNase. Phosphate, citrate, and barbital buffers have been used for maintenance of *p*H, with no significant differences noted in the extent of reoxidation or reactivation. Results of a preliminary study of the effects of *p*H on activity and SH content are shown in FIGURE 3. The disappearance of SH groups was markedly less rapid at low *p*H values, and the *p*H range for maximum regeneration of activity was quite broad, ranging from 6.1

* Because of the possibility of some chemical rearrangement during isolation and characterization of cysteic acid peptides, some uncertainty remains in regard to the 2-8 and 3-7 bonds.

to 8.6. A tendency toward precipitation of protein was noted in some experiments, which possibly was the result of intermolecular coupling of SH groups to form polymers. Precipitation generally was minimized by reducing the rate of aeration to 1 bubble every 5 sec. At *p*H 8.6, precipitation was more marked, hence such a high *p*H was considered undesirable. The *p*H range for oxidation was limited further by the solubility of reduced RNase. At advanced levels of reduction (higher than 7 SH groups per mole) RNase is only slightly soluble between *p*H 6 and 7. Therefore most of the oxidation studies were performed between *p*H 7 and 8, with 0.01 M sodium phosphate buffer. The protein concentration was routinely 1 mg./ml. Reoxidized RNase was pre-

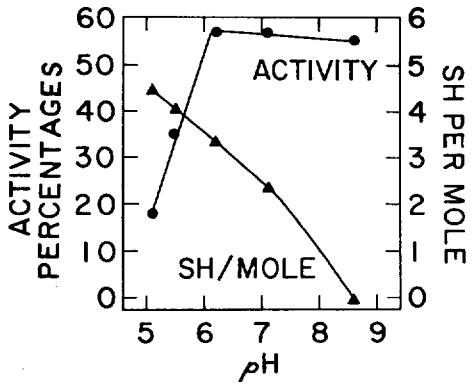

FIGURE 3. SH groups per mole of protein, and enzyme activity (expressed as percentages of the specific activity of native RNase) on reoxidation of reduced ribonuclease as functions of *p*H. Enzyme activities were determined using ribonucleic acid as the substrate.[19] A *closed circle* represents variation of activity with *p*H; a *closed triangle*, variation of SH groups per mole of protein according to *p*H. The RNase had been reduced for 3 hours in 8 M urea as previously described[20, 21] and possessed 7.5 SH groups per mole (by titration with *p*-chloromercuribenzoate) at the start of reoxidation, which was carried out for 68 hours as described in the text. The *p*H values were maintained throughout oxidation by 0.01 M phosphate buffers.

pared for physical studies by dialysis against distilled water and by lyophilization.

Results and discussion. FIGURES 4 and 5 summarize oxidation studies on RNase at 3 levels of reduction. Insofar as we have been able to ascertain, there is no difference between the levels of reduction of RNase reduced for 4.5 hours and that reduced for 24 hours, both reactions being carried out in 8 M urea. However, the results on oxidation of RNase that had been reduced for 24 hours indicated much less elimination of SH and regeneration of activity than for material reduced only 4.5 hours. A possible explanation is that the protein structure is further modified, nonspecifically and irreversibly, after all of the disulfide bonds have been broken. As controls, 4.5-hour-reduced carboxymethylated RNase and native RNase were oxidized in the same manner; activity, however, was not altered by this procedure.

Preliminary physical studies have been conducted with RNase that had been reduced for 60 min. in 8 M urea to yield 6.6 SH groups per mole (activity, 5 to

10 per cent of native RNase), and then reoxidized for 40 hours. Enzymatic activity was completely regenerated by this procedure. A decrease in negative rotation resulted from reoxidation; this finding is consistent with the conclusion

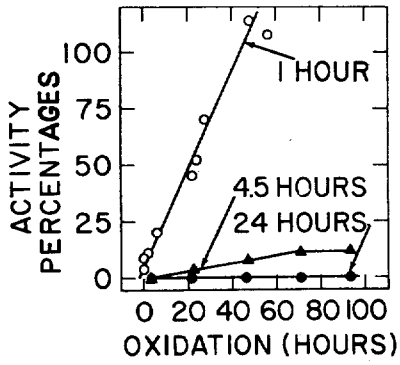

FIGURE 4. Changes in enzyme activity (expressed as percentages of the specific activity of native RNase during reoxidation of reduced RNase. Enzyme activities were determined using ribonucleic acid as the substrate.[19] All protein samples had been reduced in 8 M urea prior to oxidation, as previously described.[20, 21] An *open circle* represents RNase reduced 1 hour; a *closed triangle*, RNase reduced 4.5 hours; a *closed circle*, RNase reduced 24 hours. The *p*H of the reaction mixtures was maintained at 8 with 0.01 M phosphate buffer.

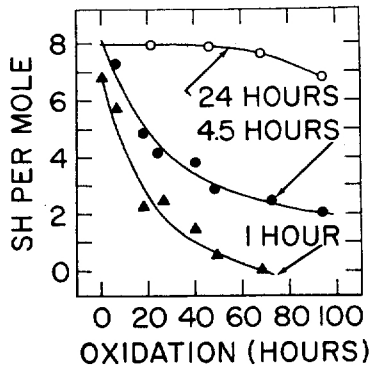

FIGURE 5. Changes in SH groups per mole of protein during reoxidation of reduced RNase. SH groups were determined by spectrophotometric titration with *p*-chloromercuribenzoate.[21, 31] All protein samples had been reduced in 8 M urea, as previously described,[20, 21] prior to oxidation. A *closed triangle* represents RNase reduced 1 hour; a *closed circle*, RNase reduced 4.5 hours; an *open circle*, RNase reduced 24 hours. The *p*H of the reaction mixtures was maintained at 8 with 0.01 M phosphate buffer.

that the reoxidized product possesses at least as much helical coiling as the native molecule. Further support for this conclusion comes from the observation that viscosities of the native and reoxidized enzymes are similar, and that both are significantly lower than that of the 60-min.-reduced carboxymethylated RNase. Finally, as a result of reoxidation, there is a nearly complete return to the U. V. spectrum of native RNase. These preliminary studies strongly suggest a re-formation of secondary and tertiary structure that, al-

though possibly not identical to that of native RNase, is sufficiently similar to it to permit complete regeneration of enzymatic activity.

Species Differences

Investigation of species differences in molecular structure of RNase may be expected to yield some valuable information on relationships between structure and function in this enzyme. Such work was initiated in our laboratory by Stig Åqvist.[28, 29] Serine is substituted for threonine at position 3, and glutamic acid for lysine at position 37 in ovine pancreatic RNase as compared to the enzyme from beef pancreas. In addition to these variations, there is evidence for at least 1 more difference, but the details must await further investigation. It should be noted that Residue 3 represents a difference of only 1 methylene group, which might be insufficient to produce a difference in specific activity even if this residue were involved directly in the active center. Aside from this precaution, the inference may be made that, since these positions are interchangeable from beef to sheep without change in specific activity, they are not involved in the active center. Work is in progress in our laboratory (under the direction of Trygve Tuve) on the purification of spinach RNase. Further comparative structural studies are planned with this enzyme and with porcine ribonuclease, which is now available in pure form.

Summary

RNase activity depends upon a variety of structural features. Some of these may be functionally involved in the binding of substrate, and others may form a structural part of the active center or may act as determinants of its proper 3-dimensional orientation. It would appear that at least 2 histidine residues, 1 of which is located in the N-terminal tail of 20 amino acids, participate directly in the process of catalysis. From the evidence at hand, hydrogen bonds between tyrosine hydroxyl groups and unidentified carboxylate groups play a role in the tertiary structure of the active center. There is evidence that 1 lysine residue[18] is essential for RNase activity.

On the basis of studies on the reduction of disulfide bonds, certain of them appear to be obligatory for activity. The importance of disulfide bonds to activity is further substantiated by the reappearance of activity upon reoxidation of the reduced enzyme. Preliminary physical studies on the reoxidized product suggest a refolding to a structure similar to that of the native enzyme.

Acknowledgment

We thank W. F. Harrington, who performed some of the preliminary physical studies on reoxidized RNase.

Addendum

It has been observed[31] that thiolesters, present as impurities in thioglycolic acid, may undergo a "thiolation" reaction[32] with protein amino groups during reduction. This difficulty can be eliminated by vacuum distillation of thioglycolic acid before it is used in reduction. Recent unpublished results on reduction and reoxidation of RNase indicate that thiolation may account in large part for the nonspecific alteration (discussed above) of RNase during reduction. RNase was devoid of enzymatic activity when treated under conditions similar to those described[20, 21] for complete reduction (except that vacuum-distilled thioglycolic acid was used as the reducing agent). However, when this protein was reoxidized by the methods

given above, values for regenerated RNase activity were observed to approach those of the native enzyme.

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