Structure and Enzymic Activity of Ribonuclease-A Esterified at Glutamic Acid-49 and Aspartic Acid-53

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The dimethyl ester of bovine pancreatic ribonuclease-A (dimethyl RNAase-A), the initial product of esterification of RNAase-A in anhydrous methanolic HCl, was isolated in a homogeneous form. The two carboxy functions esterified in this derivative are those of glutamic acid-49 and aspartic acid-53. There were no changes in the u.v.-absorption spectral characteristics, the accessibility of the methionine residues, the resistance of the protein to proteolysis by trypsin and the antigenic behaviour of RNAase-A as a result of the esterification of these two carboxy groups. Dimethyl RNAase-A exhibited only 65% of the specific activity of RNAase-A, but still had the same K_m value for both RNA and 2':3'-cyclic CMP. However, the V_{max} was decreased by about 35%. On careful hydrolysis of the methyl ester groups at pH 9.5, dimethyl RNAase-A was converted back into RNAase-A. Limited proteolysis of dimethyl RNAase-A by subtilisin resulted in the formation of an active RNAase-S-type derivative, namely dimethyl RNAase-S, which was chromatographically distinct from dimethyl RNAase-A and had very nearly the same enzymic activity as dimethyl RNAase-A. Fractionation of dimethyl RNAase-S by trichloroacetic acid yielded dimethyl RNAase-S-protein and dimethyl RNAase-S-peptide, both of which were inactive by themselves but regenerated dimethyl RNAase-S when mixed together. Dimethyl RNAase-A-peptide was identical with RNAase-S-peptide. RNAase-S-protein could be generated from dimethyl RNAase-S-protein by careful hydrolysis of the methyl ester groups at pH 9.5. The interaction of dimethyl RNAase-S-protein with RNAase-S-peptide appears to be about 4-fold weaker than that between the RNAase-S-protein and RNAase-S-peptide. Conceivably, the binding of the S-peptide 'tail' of dimethyl RNAase-A with the remainder of the molecule is similarly weaker than that in RNAase-A, and this brings about subtle changes in the geometrical orientation of the active-site amino acid residues of these modified methyl ester derivatives. It is suggested that these changes could be responsible for the generation of the catalytically less-efficient RNAase-A and RNAase-S molecules (dimethyl RNAase-A and dimethyl RNAase-S respectively).

The predominant role of the interpeptide interactions in the formation of the catalytically active structure of an enzyme is apparent from studies on the structure and activity changes of proteins when

Abbreviations used: RNAase-A, ribonuclease-A (the major chromatographic component of bovine pancreatic ribonuclease); RNAase-S, S-protein and S-peptide are subtilisin modified derivatives of RNAase-A (Richards & Vithayathil, 1959).

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subjected to denaturing environments, such as urea and guanidine hydrochloride, or chemical modifications (Richards & Wyckoff, 1971; Anfinsen & Scheraga, 1975). Chemical-modification studies resulting in complete inactivation of enzymes have been very useful in locating the active-site amino acid residues (Cohen, 1968). However, it is now recognized that various functional groups of proteins in regions other than the active site are intimately involved in the catalysis by a well-defined sequence of co-operative contributions during the interaction of the enzyme with its substrate. Modification of some of these functional groups has no influence on the enzymic activity, whereas modifications of some other functional groups results in the formation of derivatives with lower specific activity. A structural aspect of protein catalysis which, however, is not clear at present, is what makes an enzyme partially

active. Therefore, a detailed structural analysis of partially active derivatives of an enzyme (with known three-dimensional structure) may be expected to provide information about the structural rearrangements of the molecule that are responsible for a decrease in the specific activity. The present study deals with the structural analysis of partially active methyl ester derivatives of bovine pancreatic RNAase-A.

The initial product of esterification of RNAase-A in anhydrous methanolic HCl (0.1M) contains five partially active methyl ester derivatives (Acharya & Vithayathil, 1975). One dimethyl and two trimethyl derivatives have been isolated in a homogeneous form. In the dimethyl RNAase-A the two carboxy groups esterified have been identified as those of glutamic acid-49 and aspartic acid-53. The two trimethyl ester derivatives of RNAase-A also had these two carboxy groups esterified. The third carboxy group esterified in both of these derivatives was present in the C-terminal tryptic fragment representing residues 105-124 of RNAase-A. This fragment contains three free carboxy functions, namely those of glutamic acid-111, aspartic acid-121 and the C-terminal valine. The exact location of the third carboxy group modified in these two trimethyl ester derivatives is not known at present (Acharya & Vithayathil, 1975).

The specific activities of dimethyl RNAase-A and the two trimethyl esters of RNAase-A (RNAase trimethyl esters III and IV) against yeast RNA were 65, 56 and 30% respectively of that of native RNAase-A (Acharya & Vithayathil, 1975). The partial activity of these methyl ester derivatives of RNAase-A suggested that the carboxy groups esterified in these derivatives are not directly involved in the catalytic function of the enzyme. More definite information about the involvement of the modified carboxy groups in the biological function of RNAase-A could be derived by a comparative study of the overall conformation as well as the kinetic parameters of these derivatives. The present paper reports such a study with a special emphasis on dimethyl RNAase-A.

Materials and Methods

Purified RNAase-A obtained by chromatography of RNAase-A (type Ia, lot 65B-0350; Sigma Chemical Co., St. Louis, MO, U.S.A.) on CM-cellulose (Taborsky, 1959) was used in this study. 2':3'-Cyclic CMP was also a product of Sigma. The details of the procedures for chromatography of the protein samples on Amberlite XE-64, amino acid analyses, disc electrophoreses and analyses of the tryptic peptides have been described previously (Acharya & Vithayathil, 1975). Esterification was carried out in anhydrous methanolic 0.1M-HCl at 20°C and the individual methyl ester derivatives were isolated by

ion-exchange chromatography on Amberlite XE-64 in phosphate buffer (Acharya, 1968; Acharya & Vithayathil, 1975). Reaction of the protein samples with *o*-benzoquinone was carried out as described by Acharya *et al.* (1977). Procedures for activity determination against cyclic phosphates and immunochemical analysis by Ouchterlony and precipitin reaction were described by Manjula *et al.* (1976). Subtilisin modification of dimethyl RNAase-A was carried out as described by Manjula *et al.* (1977). Alkali-denatured RNAase-A was prepared by keeping RNAase-A in 0.1M-NaOH for 2h and the protein was isolated by dialysis against water followed by lyophilization.

Results

Enzymic activity of RNAase-A methyl esters

The specific activity of the methyl ester derivatives was measured with RNA and 2':3'-cyclic CMP as the substrates (Table 1). Activity against RNA was measured by the trichloroacetic acid-uranyl acetate procedure (Klee & Richards, 1957), as well as by the Kunitz (1946) method as described by Frensdorff & Sela (1967). Activity against 2':3'-cyclic CMP was followed by the spectrophotometric method (Crook *et al.*, 1960). The specific activity of each of the methyl ester derivatives as measured by the three different procedures was nearly the same (Table 1). Apparently both the depolymerization of RNA by the *trans*-esterification reaction and the hydrolytic reaction of the cyclic phosphate are influenced to more or less the same extent as observed with dimethyl RNAase-A and RNAase-A methyl esters III and IV.

Activity measurements of dimethyl RNAase-A against RNA as a function of pH showed that the pH-activity profiles of dimethyl RNAase-A and RNAase-A are very similar and the pH optimum of both is around pH 7.8 (Fig. 1). Furthermore, the specific activity of dimethyl RNAase-A at each pH value was nearly 65% of that of native RNAase-A.

The activity of the methyl esters against cyclic phosphate was also measured at different substrate concentrations and the results were treated by the method of Lineweaver & Burk (1934) (Fig. 2). The apparent K_m of RNAase-A, dimethyl RNAase-A and RNAase-A methyl esters III and IV are all the same and the V_{max} values are different. The calculated value for K_m is 2.56mM (22°C). The reported value for RNAase-A at 26°C is 3.3mM (Witzel & Barnard, 1962). The apparent K_m for dimethyl RNAase-A with yeast RNA as the substrate was determined by the procedure described by Frensdorff & Sela (1967). Again the apparent K_m of RNAase-A and dimethyl RNAase-A for the RNA substrate is the same and the two preparations differ only in their V_{max} values. The V_{max} of dimethyl RNAase-A is about 60% of that of RNAase-A.

Table 1. *Enzymic activity of the methyl ester derivatives of RNAase-A*

For calculation of the percentage activity of these derivatives, the concentration of the protein was determined from the A_{278} measurements, with a molar extinction coefficient of 9840 at 278 nm (Saroff & Carroll, 1962). Abbreviation: n.d., not determined.

Derivative	Number of carboxy groups modified	Activity (% of native RNAase-A)		
		Against RNA as substrate		
		Trichloroacetic acid-uranyl acetate procedure (Klee & Richards, 1957)	Depolymerization assay (Kunitz, 1946)*	Against 2':3'-cyclic CMP†
RNAase-A	0	100	100	100
Dimethyl RNAase-A	2	65	55	66
RNAase-A methyl ester III	3	56	50	55
RNAase-A methyl ester IV	3	30	—	32
RNAase-A methyl ester V	3	20	—	—
RNAase-A methyl ester VI	n.d.	>2	—	—

* Activity determined by the change in the spectrum of RNA that occurs during RNAase-catalysed depolymerization (Frensdorff & Sela, 1967).
† Activity determined by the changes in the spectrum of 2':3'-cyclic CMP (Crook *et al.*, 1960).

Fig. 1. *Optimal pH for activity of RNAase-A (○) and dimethyl RNAase-A (●)*
The enzymic activity was measured against yeast RNA by the acid-soluble nucleotide assay (Klee & Richards, 1957). The ordinate gives the measured increase in A_{260} of the acid-soluble nucleotides produced compared with the blank, at various pH values.

Structural features of dimethyl RNAase-A

U.v.-absorption spectra of dimethyl RNAase-A and three trimethyl RNAase-A samples in 0.2M-phosphate buffer, pH6.4, were essentially similar to that of RNAase-A. All of them gave an absorption maximum at 278nm and the molar absorption coefficients of all these derivatives were nearly the same, and close to that reported for RNAase-A (Saroff & Carroll, 1962). As for RNAase-A (Rupley &

Fig. 2. *K_m determination for the partially active methyl ester derivatives of RNAase-A*
The activity of RNAase-A and the methyl ester derivatives was measured against cytidine 2':3'-cyclic phosphate in 0.1M-Tris/HCl buffer, pH7.0, containing 0.2M-NaCl. A substrate concentration ranging from 0.1 to 1.2mg/ml was used. ΔA_{292} values were measured (Anderson *et al.*, 1968). The initial velocity (v) at each substrate concentration was determined. The reciprocal of this initial velocity was plotted against the reciprocal of substrate concentration to obtain the Lineweaver-Burk (1934) plot. All activity measurements were carried out at 22°C. Symbols: ○, RNAase-A; ●, dimethyl RNAase-A; △, RNAase-A methyl ester III; ▲, RNAase-A methyl ester IV.

Scheraga, 1963), dimethyl RNAase-A and RNAase-A methyl ester III were resistant to inactivation by trypsin at pH8.0 and 25°C.

Fig. 3. Antigenic structure of partially active methyl ester derivatives of RNAase-A

The quantitative precipitin reactions were carried out in 0.01M-sodium phosphate buffer, pH7.5, containing 0.9% NaCl. Symbols: ○, RNAase-A; ●, dimethyl RNAase-A; △, RNAase-A methyl ester III; ▲, RNAase-A methyl ester IV.

Antigenic structure of dimethyl RNAase-A. The similarities in the antigenic structures of RNAase-A, dimethyl RNAase-A and RNAase-A methyl esters III and IV were ascertained by a comparison of their immunochemical behaviour towards RNAase-A antiserum. Double diffusion in agar showed that all the three methyl ester derivatives cross react with RNAase-A antiserum with complete identity. A comparison of the quantitative precipitin curves (Fig. 3) of RNAase-A and the methyl esters with RNAase-A antiserum revealed that at equivalence the amounts of the precipitate produced by dimethyl RNAase-A and RNAase-A methyl ester III are very nearly the same as that produced by RNAase-A, whereas the amount of precipitate produced by RNAase methyl ester IV was slightly lower than that of RNAase-A. By the above two criteria, the antigenic conformations of dimethyl RNAase-A and RNAase-A methyl ester III appeared to be similar to that of RNAase-A.

Reactivity of the methionine residues of dimethyl RNAase-A. The four methionine residues of RNAase-A are unavailable for reaction with iodoacetate at physiological pH, and become accessible for reaction on denaturation in 8.0M-urea or on partial unfolding of the molecule at acidic pH (Neumann *et al.*, 1962; Stark & Stein, 1964). The influence of esterification of carboxy groups of a protein could be compared in part with acidification of a protein solution. Hence a study of the accessibility of the methionine residues of methyl ester derivatives of RNAase-A

Fig. 4. Accessibility of the methionine residues of RNAase-A derivatives for reaction with *o*-benzoquinone

The figure shows the difference spectra of: *o*-benzoquinone-treated RNAase-A (—); dimethyl RNAase-A (---); RNAase-A methyl ester III (···); octamethyl RNAase-A (-·-·-), and alkali-denatured RNAase-A (----) against corresponding unreacted proteins. The reaction was carried out at pH3.0±0.05 for 2min in the presence of 3.5mM-*o*-benzoquinone.

can provide information about any partial unfolding of the molecule. The accessibility of the methionine residues of dimethyl RNAase-A, and RNAase-A methyl esters III and IV for reaction with *o*-benzoquinone at pH3.0, was measured as described earlier (Acharya *et al.*, 1977). The difference spectra of *o*-benzoquinone-treated samples of RNAase-A, dimethyl RNAase-A, RNAase-A methyl ester III and alkali-denatured RNAase-A against the corresponding untreated blanks are shown in Fig. 4. The methionine residues of RNAase-A, as well as dimethyl RNAase-A, are quite inert for reaction with *o*-benzoquinone. On the other hand nearly 0.3 methionine residue of RNAase-A methyl ester III/molecule was reactive. Under similar conditions, nearly 2.0 methionine residues of RNAase-S/molecule are accessible for reaction with *o*-benzoquinone (Murthy & Vithayathil, 1970; Acharya *et al.*, 1977).

Thus the resistance of dimethyl RNAase-A to proteolysis by trypsin, its antigenic behaviour and the unavailability of its methionine residues for reaction with *o*-benzoquinone suggests the close similarity in the overall structures of RNAase-A and dimethyl RNAase-A. Apparently, esterification of the carboxy groups of glutamic acid-49 and aspartic acid-53 has very limited influence on the non-covalent interactions responsible for stabilizing the native structure of RNAase-A.

Fig. 5. Regeneration of RNAase-A from dimethyl RNAase-A

The methyl ester groups of dimethyl RNAase-A were hydrolysed by incubation at pH 10.5 or 9.5 for 6h. This protein sample was then chromatographed, after adjusting the pH to 5.0 on Amberlite XE-64 columns (0.6cm × 25cm) at pH 6.0. (a) Elution profile of 2mg of RNAase-A (measured at A_{280}). (b) Chromatographic pattern of 2.5mg of dimethyl RNAase-A after 6h of hydrolytic reaction at pH 10.5 and 22°C (measured at A_{280}). Curve (c) represents the activity profile of the regenerated material when assayed directly and in the presence of S-protein (measured at A_{260}). Curve (d) represents the activity of the regenerated material when assayed with added S-peptide (measured at A_{260}). (e) Chromatographic pattern of the regenerated RNAase-A obtained on hydrolysis of the methyl ester groups of dimethyl RNAase-A by incubating the enzyme at pH 9.5 and 10°C for 12h (measured at A_{280}). The inset shows the recovery of the enzymic activity from dimethyl RNAase-A at pH 10.5 and 22°C (○) and at pH 9.5 and 22°C (●).

Regeneration of RNAase-A from dimethyl RNAase-A

Attempts were made to regenerate RNAase-A from dimethyl RNAase-A by careful hydrolysis of the methyl ester groups in it. On incubation in carbonate-bicarbonate buffers at 23°C and at pH values of 9.5 and 10.5, dimethyl RNAase-A regenerated the specific activity of RNAase-A after about 5 and 3h respectively (Fig. 5, inset). A control experiment with a 50% active methyl ester preparation (containing on average 2.6 carboxy groups esterified/molecule) showed that at pH 10.5 nearly 95% of the methyl ester groups are hydrolysed in about 4h. Dimethyl RNAase-A incubated at pH 10.5 (carbonate-bicarbonate buffer) for 4h was isolated by dialysis and lyophilization. Chromatography of this fully active regenerated material on Amberlite XE-64 (Fig. 5) showed that its elution pattern (curve b) is different

from that of RNAase-A (curve a). The protein was eluted as an unsymmetrical peak, indicating the presence of two or more components in it, one of the components under the peak corresponding to RNAase-A and the other component(s) as a broad shoulder on the descending side of the peak. Activity measurements of the chromatographic fractions with S-protein and S-peptide (curves c and d) showed that S-protein does not change the activity of the fractions, whereas S-peptide increased the specific activity of the fractions eluted after peak corresponding to the RNAase-A. The chromatographic behaviour of this regenerated RNAase-A preparation is very similar to that of the product obtained when RNAase-A is maintained at pH 11.0 and 25°C for 20min (Vithayathil *et al.*, 1967). Thus it appears that on incubation of dimethyl RNAase-A at pH 10.5, in addition to the hydrolysis of the methyl ester groups, the protein undergoes structural alterations similar to those observed at pH 11.0 with RNAase-A.

To overcome this 'alkali-reaction' during the regeneration of RNAase-A from dimethyl RNAase-A, the hydrolysis was carried out in 0.05M-carbonate-bicarbonate buffer, pH 9.5, at 10°C. After 12h of incubation, the specific activity of the protein was nearly the same as that of native RNAase-A. On chromatography of this material on Amberlite XE-64 the protein eluted as a symmetrical peak at a position corresponding to that of RNAase-A (Fig. 5), with an overall recovery of 90%. The elution profile indicated the absence of any 'alkali-reaction' under this hydrolytic condition. Thus, the partially active dimethyl RNAase-A is converted into RNAase-A on careful hydrolysis of its two methyl ester groups. Therefore, the loss in catalytic efficiency of the protein is clearly due to the esterification of the carboxy group of aspartic acid-53 and glutamic acid-49, and not due to any other side reactions under the conditions used for esterification.

Subtilisin modification of dimethyl RNAase-A

Incubation of dimethyl RNAase-A with subtilisin at pH 8.0 and 2°C did not show any loss in the enzymic activity at least up to 12h (Fig. 6). However, when the activity of the subtilisin digest was measured after treatment with trypsin, a gradual accumulation of active trypsin-sensitive material was apparent, indicating the presence of RNAase-S-type species in the subtilisin digest. A 12h subtilisin digest of dimethyl RNAase-A was chromatographed on Amberlite XE-64 (Fig. 7) by elution with a stepwise gradient (Acharya & Vithayathil, 1975). All the protein eluted with 0.3M-sodium phosphate buffer, pH 6.4, as a single peak at a position slightly later than that expected for dimethyl RNAase-A. Activity determinations of the chromatographic fractions before and after tryptic digestion revealed the pre-

Fig. 6. *Subtilisin modification of dimethyl RNAase-A* Subtilisin digestion of RNAase-A and dimethyl RNAase-A was carried out at pH 8.0 and 2°C. Protein concentration was 10 mg/ml, and the enzyme/substrate ratio was 1:250 (w/w). Activity of RNAase-A and dimethyl RNAase-A after subtilisin digestion is shown by curves (a) and (c) respectively. The activity of the same subtilisin-digests of RNAase-A and dimethyl RNAase-A after tryptic digestion is shown by curves (b) and (d) respectively. Curve (e) represents the activity of the subtilisin-digest of dimethyl RNAase-A when assayed in the presence of S-protein. S-peptide had no influence on the activity of these subtilisin-digests.

sence of a small amount of trypsin-resistant material in the ascending side of the protein peak, whereas the rest of the material was trypsin sensitive. The trypsin-sensitive fractions were isolated by dialysis and lyophilization. The relative positions of the trypsin-resistant material (presumably dimethyl RNAase-A) and the trypsin sensitive material are comparable with the change in the chromatographic position of RNAase-A on its conversion to RNAase-S (Richards & Vithayathil, 1959). Hence, the trypsin-sensitive fractions of the subtilisin-digest of dimethyl RNAase-A were designated dimethyl RNAase-S. Dimethyl RNAase-S had the same specific activity as dimethyl RNAase-A.

Fractionation of dimethyl RNAase-S by trichloroacetic acid and characterization of the protein and the peptide components. Dimethyl RNAase-S was subjected to fractionation by trichloroacetic acid. The precipitate and the supernatant thus obtained were inactive when assayed separately, but reconstituted nearly full activity of dimethyl RNAase-S when they were present together. Thus the trichloroacetic acid fractions of dimethyl RNAase-S appear to be similar to the trichloroacetic acid fractions of RNAase-A (Richards & Vithayathil, 1959). Hence the precipitate and the supernatant obtained on trichloroacetic acid fractionation of dimethyl RNAase-S are designated as dimethyl RNAase-S protein, and dimethyl RNAase-S peptide respectively.

Fig. 7. *Chromatographic behaviour of subtilisin-digest of dimethyl RNAase-A*

A 12h subtilisin-digest of dimethyl RNAase-A was chromatographed on an Amberlite XE-64 column (0.6cm×25cm) equilibrated and eluted with 0.2M-sodium phosphate buffer, pH 6.4, with 2mg of protein applied to the column. The column was first eluted with 30ml of 0.2M-sodium phosphate buffer, pH 6.4, and then with 0.3M-sodium phosphate buffer, pH 6.4 (indicated by the arrow) (Acharya & Vithayathil, 1975). Symbols: ●, A_{280} (protein concentration); △, enzymic activity of the chromatographic fractions assayed directly; ○, enzymic activity after tryptic digestion; ▲, activity of the fractions when assayed with S-protein.

The amino acid composition of the S-peptide fractions of RNAase-S and dimethyl RNAase-S were nearly the same. Also, both peptides had the same electrophoretic mobility on paper at pH 6.5. The identity of the S-peptide part of dimethyl RNAase-A as RNAase-S-peptide was confirmed by reconstitution experiments in which the former was mixed with RNAase-S-protein at pH 6.05 and chromatographed on Amberlite XE-64 columns (Fig. 8a). The active reconstituted material was eluted at the position expected for RNAase-S'. This demonstrates that the peptide portions of RNAase-S and dimethyl RNAase-S are the same. Thus it is clear that dimethyl RNAase-A undergoes limited proteolysis when digested with subtilisin, in much the same way as RNAase-A, resulting in the formation of a two-fragment complementing system, namely dimethyl RNAase-S.

Attempts were also made to regenerate S-protein from dimethyl RNAase-S-protein by careful hydrolysis of the methyl ester groups. Dimethyl RNAase-S-protein was incubated in 0.05M-carbonate-bicarbonate buffer of pH 9.5 and at 10°C for 12h. At the

Fig. 8. Chromatographic behaviour of the active material formed on mixing dimethyl RNAase-S-peptide and dimethyl RNAase-S-protein, after hydrolysis of the methyl ester groups, with RNAase-S-protein and RNAase-S-peptide respectively

The chromatography of the protein samples was carried out on columns (0.6 cm $\times$ 25 cm) of Amberlite XE-64 resin at pH 6.06. (a) Chromatography of the active material formed on mixing the peptide part of dimethyl RNAase-S (approx. 200 μ g) with RNAase-S-protein (approx. 800 μ g) at pH 6.0. (b) Chromatography of the active material formed on mixing dimethyl RNAase-S-protein (approx. 850 μ g) that had been incubated at pH 9.5 and 10°C for 12 h with RNAase-S-peptide (approx. 220 μ g). The A_{220} measurements (●) represent protein concentration. The activities of the fractions have been measured at 260 nm before (○) and after (△) tryptic digestion. The chromatographic position of RNAase-S' on these columns is indicated by a vertical arrow. The trypsin-resistant material eluted at a position before that of RNAase-S' (peak position approx. 15 ml, Fig. 8b) is presumably the regenerated RNAase-A. The trichloroacetic acid precipitate (dimethyl RNAase-S-protein) used in this study had some trypsin-resistant activity, which is apparently the original undigested dimethyl RNAase-A present in the subtilisin-digest of dimethyl RNAase-A.

end of this period the pH was adjusted to 6.0 with 0.1 M-acetic acid, RNAase-S-peptide was added to the sample and the mixture was chromatographed on Amberlite XE-64 (Fig. 8b). The reconstituted protein was eluted at the position of RNAase-S' and exhibited the same specific activity as RNAase-S'. It is therefore clear that at pH 9.5 dimethyl RNAase-S-protein is converted into S-protein, which in the presence of RNAase-S-peptide chromatographs at the RNAase-S' position.

Protein-peptide interactions in dimethyl RNAase-S and RNAase-A methyl esters

Subtilisin digestion of dimethyl RNAase-A did not affect the original enzymic activity of the protein (see above). However, when the activity of the digest was measured in the presence of added S-protein (about a 2-fold excess), the mixture exhibited nearly 100% activity (Fig. 6); S-peptide, however, had no influence. Similarly, the chromatographically purified dimethyl RNAase-S also exhibited a higher activity when assayed in the presence of S-protein (Fig. 7). Presumably, the interaction between dimethyl RNAase-S-protein and S-peptide is weaker than that between RNAase-S-protein and S-peptide. When S-protein is added to dimethyl RNAase-S, exchange of the

S-protein moiety occurs giving rise to RNAase-S and dimethyl S-protein. To elucidate this weakening of the interactions in the S-peptide-S-protein system as a result of modification of the carboxy groups of aspartic acid-53 and glutamic acid-49, the decrease in the activity of RNAase-S in the presence of dimethyl S-protein was measured. The activity expressed by a mixture of RNAase-S and dimethyl S-protein will depend on the association constants of S-peptide with dimethyl S-protein and RNAase-S-protein and hence the activity expressed will be a function of the amounts of dimethyl RNAase-S and RNAase-S at equilibrium (Table 2). If the strength of interaction of dimethyl RNAase-S-protein with its peptide tail is the same as that of RNAase-S-protein with S-peptide, an equimolar mixture of dimethyl RNAase-S and RNAase-S-protein should exhibit an activity of about 82% (Table 2). On the other hand, if the peptide-protein interactions in dimethyl RNAase-S are weaker than that in RNAase-S a higher activity should be observed. In fact an equimolar mixture of dimethyl RNAase-S and RNAase-S-protein exhibited an enzymic activity of about 92%, showing that the peptide-protein interaction in dimethyl RNAase-S is indeed weaker than that in RNAase-S.

Though the interaction between the peptide and

Table 2. *Calculated activity of a mixture of RNAase-S and dimethyl RNAase-S*

The activity of a mixture of RNAase-S and dimethyl RNAase-S was calculated assuming that these proteins do not show any interactions affecting their activity. The activities of RNAase-S and dimethyl RNAase-S were taken as 100 and 65% respectively of that of RNAase-A.

Amount of protein in the mixture*

RNAase-S	Dimethyl RNAase-S	Calculated activity (%)†
100	0	100.0
90	10	96.5
90	20	93.0
70	30	89.5
60	40	86.0
50	50	82.5

* The amount of the RNAase-S and dimethyl RNAase-S present in the mixture was expressed as the percentage of the total protein present.

† These activities are presented as the percentage of the activity expected if all the protein present in the mixture was RNAase-A.

the protein parts in dimethyl RNAase-S appears to be weaker than that in RNAase-S, this is not detected in dimethyl RNAase-A by a study of its interactions with S-peptide and S-protein. RNAase-A methyl esters III, IV and V also did not show any noticeable change in their activities in the presence of either S-peptide or S-protein (Table 1). On the other hand, RNAase-A methyl ester VI (original activity less than 2%) showed an increase in activity in the presence of S-protein. However, S-peptide did not significantly increase the low activity of RNAase-A methyl ester VI. An equimolar mixture of RNAase-A methyl ester VI and RNAase-S-protein exhibited nearly 20% activity, and showed a progressive increase in activity with increasing amounts of S-protein. At a molar ratio of 5:1 for RNAase-S-protein/RNAase-A methyl ester VI, the latter generated nearly 75% of RNAase-A activity. This indicates that in RNAase methyl ester VI, the interaction of the peptide 'tail' region with the remainder of the molecule is sufficiently weakened so that the external S-protein could compete with the S-protein part of the molecule for the peptide tail. Such behaviour has been observed previously with the fully methylated RNAase-A (Vithayathil & Richards, 1961; Acharya *et al.*, 1977).

Discussion

The present study deals with the influence of esterification of the carboxy groups of RNAase-A on its overall structure. Our earlier studies showed that modification of 4 of the 11 free carboxy groups of

RNAase-A resulted in nearly 80% loss of enzymic activity, with limited influence on the overall conformation (Acharya *et al.*, 1977). The initial products of esterification of RNAase-A contained five chromatographically distinct partially active derivatives (Acharya & Vithayathil, 1975). These partially active methyl ester derivatives of RNAase-A appeared to be formed in a sequential order and dimethyl RNAase-A is apparently the first product exhibiting lower specific activity (65%). The 2 carboxy groups esterified in dimethyl RNAase-A have been shown to be those of glutamic acid-49 and aspartic acid-53. Riehm & Scheraga (1965) have reported that modification of the carboxy group of aspartic acid-53 with diazoacetyl glycineamide has no influence on the enzymic activity of RNAase-A. Hence, the lower specific activity of dimethyl RNAase-A is indicative of a structural role for the carboxy group of glutamic acid-49 in maintaining the biologically active structure of RNAase-A.

The modification of the carboxy groups of glutamic acid-49 and aspartic acid-53 of RNAase-A lowered the specific activity of the protein only by about 35%. Modification of these 2 carboxy groups did not affect the pH optimum of the protein. There was no difference in K_m between RNAase-A and dimethyl RNAase-A. If it is assumed that the Michaelis constant reflects the stability of the enzyme-substrate complex, the results suggest that the modified carboxy groups, i.e. those of glutamic acid-49 and aspartic acid-53, do not influence markedly the formation of the complex between the low-molecular-weight substrate 2':3'-cyclic CMP and the enzyme. The esterification has only decreased the V_{max} of the protein. The catalytic efficiency of the protein in the *trans*-esterification as well as the hydrolytic step appeared to be decreased to approximately the same extent. Presumably the carboxy groups esterified are involved in the maintenance of the optimal topography of the active-site amino acid residues of the protein.

Though the specific activity of dimethyl RNAase-A is lower than that of native RNAase-A, it appears to have a conformation very similar to that of the native protein. The antigenic behaviour of this derivative is indistinguishable from that of the native protein. Just as in native RNAase-A, none of its methionine residues are accessible for reaction with *o*-benzoquinone at pH 3.0, indicating the absence of any partial unfolding of the molecule as a result of esterification. The resistance of dimethyl RNAase-A towards tryptic digestion also points to its compact, native-like conformation.

The similarity in the overall structure of RNAase-A and dimethyl RNAase-A is also clear from the results of the subtilisin-modification studies. As for RNAase-A, dimethyl RNAase-A is also acted on by subtilisin at the peptide bond linking residues 20 and 21 of the molecule to yield enzymically active dimethyl

RNAase-S. Dimethyl RNAase-S has the same specific activity as dimethyl RNAase-A. This activity is not significantly influenced by the presence of excess S-peptide, but in the presence of excess of S-protein, dimethyl RNAase-S exhibited the same specific activity as that of RNAase-S. By competition experiments, it was apparent that the binding of S-peptide 'tail' to dimethyl S-protein is slightly, but noticeably, weaker than the peptide-protein interactions in RNAase-S. If a mixture of dimethyl RNAase-S and S-protein is in a quasi-equilibrium state, i.e.

then it would follow from the activity expressed by an equimolar mixture (see the Results section and Table 2) that the esterification of the carboxy groups of glutamic acid-49 and aspartic acid-53 results in about a 4-fold weaker binding of the S-peptide to the modified S-protein (compared with that in the native system).

Vithayathil & Richards (1961) have observed that the inactive fully methylated RNAase-A (11 carboxy groups modified) exhibited nearly 30% of the activity of RNAase-A when assayed in the presence of unmodified S-protein, showing that the interaction of the methylated 'peptide' with the methylated 'protein' portion of the molecule is weaker than the 'peptide-protein' interaction in RNAase-A as a result of esterification was apparent much before all the carboxy groups of the protein are modified (Acharya *et al.*, 1977). A preparation that had on an average 5.3 carboxy groups/esterified molecule (5% enzymic activity) exhibited 30% activity in the presence of S-protein. Although dimethyl RNAase-A did not show any changes in activity in the presence of added S-protein, RNAase-A methyl ester VI (containing only 4 to 5 methyl ester groups) could be activated by S-protein, suggesting that the interaction between the 'peptide tail' and the protein part of the molecule is weaker than that in dimethyl RNAase-A and native RNAase-A. In the RNAase-S system, it has been shown that the interaction of the tetramethyl S-peptide with the unmodified S-protein is about an order of magnitude weaker than that of the unmodified S-peptide with S-protein (Vithayathil & Richards, 1961). However, this interaction was strong enough to show a sharp break in the 'peptide-protein' titration curve at a molar ratio of 1:1 for RNAase-S-protein/methylated S-peptide, and the complex exhibited an activity nearly 30% of that of RNAase-S. The results of the present study suggest that the interaction of the dimethyl S-protein with the unmodified S-peptide in the dimethyl RNAase-S system is about 4-fold weaker than the 'protein-peptide' interactions in the RNAase-S system. In view of these observations, it is reasonable to assume

that the binding of the S-peptide tail to the dimethyl S-protein part of dimethyl RNAase-A is similarly not as strong as that in RNAase-A. A perturbation in the tightness of the binding of S-peptide to S-protein region could bring out subtle changes in the geometrical orientation of the active-site amino acids, for example histidine-12 and histidine-119. The esterification of the carboxy groups of glutamic acid-49 and aspartic acid-53 probably permits a shift in polypeptide chain that may result in a shift in the pK_a value of the histidine residues. It is noteworthy that Lin (1970) has shown that the removal of aspartic acid-121 (in pepsin-inactivated RNAase-A, residues 1-120) results in an increase in the originally low pK_a value of histidine-119, as evidenced by the change in the rate of its alkylation by iodoacetic acid at pH 5.5. If the weaker binding of S-peptide to the S-protein part in dimethyl RNAase-A brings about a small increase in the pK_a value of the active site histidine-119, this could render this histidine residue less efficient in proton transfer during the catalytic process. Further chemical studies (such as carboxymethylation), as well as spectroscopic studies (such as n.m.r.), are needed to probe in detail the geometry of the active-site histidine residues of dimethyl RNAase-A.

Riehm & Scheraga (1966) have shown that modification of the carboxy group of aspartic acid-53 by carbodi-imide caused a decrease of approx. 5°C in the transition temperature of RNAase-A and modification of the carboxy groups of glutamic acid-49 and aspartic acid-53 and glutamic acid-111 of RNAase-A lowered its transition temperature by about 10 to 15°C. Therefore it is likely that the esterification of carboxy groups of aspartic acid-53 and glutamic acid-49 has resulted in a similar perturbation of the co-operative non-covalent interactions of the native protein. Such a perturbation of the native non-covalent interactions could generate a less favourable topology of the active-centre amino acid residues. However, a change in the geometrical orientation of the active-centre amino acid residues of RNAase-A as a result of modification of the carboxy groups of glutamic acid-49 and aspartic acid-53 is not readily conceivable from the crystal structure of RNAase-S (Wyckoff *et al.*, 1970). But the result of the present study clearly suggests that this modification has influenced the 'peptide-protein' interactions in dimethyl RNAase-S. The crystal structure of RNAase-S shows that aspartic acid-53 is in the middle of the helical segment, whereas glutamic acid-49 is in the beginning of this helical segment. Apparently, the carboxy group of glutamic acid-49 is hydrogen-bonded to serine-80. A study of the bonding interactions between S-peptide and S-protein shows that many residues in the segment residues 45-50 are involved in this interaction; there are main chain hydrogen-bonds between histidine-12

and valine-47, aspartic acid-14 and valine-47, serine-16 and histidine-48 (Wyckoff *et al.*, 1970) and between histidine-12 and threonine-45 (Patel *et al.*, 1975). The carboxy group of glutamic acid-49 does not appear to be directly involved in any non-covalent interactions with the amino acid residues in the S-peptide. However, the esterification of glutamic acid-49 can result in the loss of original interaction between the glutamic acid-49 and serine-80, which in turn has apparently weakened the S-peptide-S-protein interaction.

Alterations in the geometrical orientation of the amino acid residues at the active centre have been invoked previously to explain the lower activity (45%) of the [Orn¹⁰]-RNAase-S (Marchiori *et al.*, 1972; Valle *et al.*, 1977). It has been suggested that interaction between glutamic acid-2 and arginine-10 probably acts by 'freezing' the S-peptide conformation and the amino acid residues directly involved in catalysis, and this helps to maintain the geometry of the active-site histidine residues that is optimum for catalysis. Recent crystallographic studies on [Orn¹⁰]-RNAase-S have suggested that small changes in the molecule have occurred to accommodate the new atomic interactions and charge equilibria; e.g. the interactions between the δ -nitrogen atom of Orn¹⁰ and a carboxylate oxygen atom of glutamic acid-2. Similar small changes in RNAase-A to accommodate the modification of the carboxy groups of aspartic acid-53 and glutamic acid-49 and a resulting loss of interaction between glutamic acid-49 and serine-80 could be responsible for the generation of a catalytically less efficient RNAase-A molecule. Slight changes in atomic positions and charge distribution (and possibly in bound solvent molecules) can possibly affect the enzymic activity, both in binding of the substrate and in the catalytic reaction (Richards & Wyckoff, 1973).

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