Stacey, K. A. (1956) *Light Scattering in Physical Chemistry*, Butterworth, London.
Verger, R., Mieras, M. C. E., & Haas, G. H. de (1973) *J. Biol. Chem. 248*, 4023–4034.
Verger, R., & Haas, G. H. de (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.
Wells, M. A. (1973) *Biochemistry 12*, 1086–1093.
Wells, M. A. (1974) *Biochemistry 13*, 2248–2257.

Enzymatic Resynthesis of the Hydrolyzed Peptide Bond(s) in Ribonuclease S†

Gene A. Homandberg‡ and Michael Laskowski, Jr.*

ABSTRACT: Bovine pancreatic ribonuclease S was incubated with subtilisin at pH 6.2 in partially nonaqueous solutions of 0–95% (v/v) glycerol. The solutions were monitored at various times for the presence of ribonuclease A‡ (a mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and possibly Ser^{21A}-ribonuclease A) by enzymatic assay in the presence of 40% (v/v) dioxane (where ribonuclease S is inactive) and by sodium dodecyl sulfate gel electrophoresis. In all cases, the concentration of ribonuclease A‡ first increased and then declined. In water the maximal amount of synthesis was only 4.3%; it smoothly increased with increasing glycerol concentration until in 90% (v/v) glycerol 50% synthesis was attained. The increase in synthesis with glycerol concentration is in rough quantitative agreement with model studies on peptide bond hydrolysis equilibria in simple peptides [Homandberg, G. A., Mattis, J. A., & Laskowski, M., Jr. (1978) *Biochemistry 17*, 5220]. From the data a very rough value of $K_{hyd}°$ for the Ala^{20}–Ser^{21} peptide bond in water can be estimated as 20; it is roughly unity in 90% (v/v) glycerol. Addition of organic cosolvents other than glycerol was ineffective in promoting synthesis. The ribonuclease A‡ obtained from the synthesis in 90% (v/v) glycerol was isolated and subjected to 25 cycles of Edman degradation in an automatic sequencer. The results showed it to be a mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and possibly Ser^{21A}-ribonuclease A.

The discovery by Richards & Vithayathil (1959) that subtilisin specifically hydrolyzes the Ala^{20}–Ser^{21} bond in bovine pancreatic ribonuclease A is one of the milestones in protein chemistry. The product of this hydrolysis, ribonuclease S,[1] is a noncovalent complex of the S-peptide and S-protein and retains enzymatic activity. However, after low pH dissociation both isolated components are devoid of activity but regain it when they are allowed to recombine. These observations had important implications for our understanding of the nature of enzymatic active sites and of the effect of noncovalent interactions upon protein conformation and folding. Furthermore, they allowed for a large number of investigations of the functional and structural role of individual amino acid residues in ribonuclease since S-peptide or S-protein analogues could be prepared and their recombination with the other component and the resultant enzymatic activity of the complex could be studied. Many such studies are still going on. Earlier work was reviewed by Richards & Wyckoff (1971). However, to the best of our knowledge all such studies were conducted with noncovalently bound complexes. No attempt to resynthesize the hydrolyzed peptide bond between the S-peptide and S-protein has been reported.

In a recent paper (Homandberg et al., 1978) we have shown that addition of large concentrations of organic cosolvents significantly raises the value of the equilibrium constant for peptide bond synthesis and yet does not abolish the catalytic activity of proteinases. We have also shown that the reactive site peptide bond in modified soybean trypsin inhibitor (Kunitz) can be more efficiently resynthesized in the presence of large amounts of glycerol. It appeared that enzymatic resynthesis of the split peptide bond in ribonuclease S would serve as a test of the applicability of the enzymatic resynthesis to proteins other than proteinase inhibitors.

However, the problem was more complex than that of dealing with soybean trypsin inhibitor (Kunitz), where the limited proteolysis is very strictly limited and no other bonds aside from Arg^{63}–Ile are hydrolyzed even after prolonged exposure to massive amounts of trypsin and where significant resynthesis can be carried out even in the absence of organic cosolvents (Mattis & Laskowski, 1973). For ribonuclease S the value of K_{syn} in water was not known,[2] and therefore we could not calculate a priori how large an increase in K_{syn} was required in order to carry out efficient synthesis. More importantly the proteolysis giving rise to ribonuclease S is not strictly limited.

First, subtilisin hydrolyzes not only the Ala^{20}–Ser^{21} but alternatively also the Ser^{21}–Ser^{22} bond (Gross & Witkop, 1967). Therefore, ribonuclease S may well be a mixture of four components: (1–20, 21–124), (1–20, 22–124), (1–21, 20–124), and (1–21, 22–124) (Doscher & Hirs, 1967). This

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received August 17, 1978.* Supported by Grant GM 10831 from the National Institute of General Medical Sciences, National Institutes of Health, and by Grant PCM 77-17554 from the National Science Foundation.

‡ Recipient of U.S. Public Health Service Postdoctoral Fellowship 1F32 GM 06475-01. Present address: Laboratory of Chemical Biology, NIAMDD, National Institutes of Health, Bethesda, MD 20014.

[1] Nomenclature used: ribonuclease S [presumed mixture of noncovalent complexes (1–20, 21–124), (1–20, 22–124), (1–21, 21–124), (1–21, 22–124)] obtained by incubation of bovine pancreatic ribonuclease S with subtilisin; ribonuclease A‡ (presumed mixture of ribonuclease A, des-Ser^{21}-ribonuclease A, and Ser^{21A}-ribonuclease A) obtained by enzymatic resynthesis of the hydrolyzed peptide bond(s) in ribonuclease S; ribonuclease S′ obtained by recombining S-peptide and S-protein.

[2] However, in a personal communication Dr. I. M. Chaiken suggested that at equilibrium in water we may well have 5% of ribonuclease A, a value very close to the one reported here. In an earlier report on this work (Homandberg & Laskowski, 1978) we have mistakenly concluded, by examining the data at too long a time of incubation (see Figure 1), that the equilibrium amount of ribonuclease A would be much lower (0.1%), and thus we believed that the added glycerol had a much larger effect on K_{syn} than reported here.

0006-2960/79/0418-0586$01.00/0

possibility complicates the nature of the expected synthetic product, which could be a mixture (ribonuclease $A^{\ddagger}$) of ribonuclease A, des-Ser^{21}-ribonuclease A, and Ser^{21A}-ribonuclease, and greatly complicates the appropriate equilibrium expressions.

More importantly it is well known that subtilisin is not strictly limited to hydrolysis in the 15–25 loop of ribonuclease. Upon extended exposure to subtilisin as many as 30 peptide bonds in ribonuclease can be hydrolyzed (Richards, 1955). As a result we cannot expect a long-term stable equilibrium between ribonuclease $A^{\ddagger}$ and ribonuclease S. Rather, as is shown in the paper, the regeneration of ribonuclease $A^{\ddagger}$ is only a transient phenomenon.

For the reasons given above and detailed below we are quite cautious[3] about emphasizing in this paper values of K_{syn}. Instead we prefer to use the amount of synthesis attained, although we believe that these values are quite close to the equilibrium values.

The complications of the ribonuclease system, while severe, appear to us to be rather typical. Most "limited" proteolyses are not as strictly limited as that of soybean trypsin inhibitor (Kunitz), and therefore if enzymatic resynthesis of peptide bonds is to become an important protein semisynthetic tool, these complications must be dealt with in many other situations as well.

Experimental Procedures

Materials

Bovine pancreatic ribonuclease A (type XII-A) and S (grade XII-S), bovine pancreatic ribonuclease S-peptide (grade XII-PE from lot 17C-8102), bovine pancreatic S-protein (grade XII-PR from lot 29B-8130), subtilisin BPN′ (type VII) (EC 3.4.4.16), and cytidine cyclic 2′,3′-monophosphate were obtained from Sigma Chemical Co. Spectral grade dioxane was from Mallinckrodt. Since one of the criteria for enzymatic resynthesis of the hydrolyzed peptide bond(s) in ribonuclease S was the amino acid composition of the product, the amino acid compositions of the various ribonuclease protein samples used in these studies were determined and closely agreed with the results obtained in the original studies of the ribonuclease S system (Richards & Vithayathil, 1959). Bio-Phore precast gels (12% total monomer) were used because maximum uniformity and reproducibility were necessary for the resolution of sodium dodecyl sulfate treated ribonuclease A and S-protein. These gels and Bio-Rex 70 were obtained from Bio-Rad Laboratories. Sephadex G-50 (superfine) and PD-10 disposable columns were from Pharmacia. All other chemicals were reagent grade. None of the organic cosolvents were subjected to further purification. It should be noted that artifacts caused by contaminants in spectral grades of glycerol have been observed by Bello (1969), who was able to eliminate them by repurification. Anhydrous glycerol was not used in these studies. The concentrations of glycerol (v/v) refer to glycerol as supplied, with no corrections for water contamination.

Methods

Assays for the interconversion of ribonuclease A and S were performed by two independent methods. In the first method the cytidine cyclic 2′,3′-monophosphate assay of Crook et al. (1960) was used to determine the ribonuclease activity at 25 °C. Ribonuclease A (or $A^{\ddagger}$) was differentiated from ribonuclease S by adjusting the substrate solution to 40% (v/v) dioxane to dissociate and inactivate ribonuclease S (Cowgill, 1966). The remaining activity is due to ribonuclease A ($A^{\ddagger}$). Assays of stock solutions of ribonuclease S showed a residual activity representing 1.2% of that expected for ribonuclease A. No preincubation of ribonuclease S in dioxane was necessary prior to mixing of substrate and enzyme. The rate of absorbance change at 284 nm was proportional to enzyme concentration up to at least 15 μg/mL.

The alternate method of differentiating ribonuclease A and S was based on the slight difference of migration of their products of sodium dodecyl sulfate denaturation on sodium dodecyl sulfate gels. Samples of either protein in water or organic cosolvent [0–95% (v/v)] were first treated with phenylmethanesulfonyl fluoride (Barel & Glazer, 1968) to eliminate proteolytic artifacts. The samples were then heated at 100 °C for 30 s and one volume of denaturation buffer (Fairbanks et al., 1971) was added. After 5 min the samples were allowed to cool to 60 °C and incubation was continued for 1 h. This harsh treatment was necessary because of the stabilizing effect of glycerol on ribonuclease (Bello, 1969). The precast gels were equilibrated in the 0.205 M Tris-acetate buffer at pH 6.6, containing 0.1% sodium dodecyl sulfate as recommended by Bio-Rad. Aliquots (10–50 μL) containing 25–125 μg of protein were layered under the buffer on top of the gels. Electrophoresis was performed at 6 mA/gel of 100 V at 7 °C for about 6 h or until the bromphenol blue band passed out of the gel. The tubes were then eliminated from the gels, the gels were stained with Coomassie R 250 and destained according to Weber & Osborn (1969), and the bands were quantitated by scanning at 545 nm.

Effect of organic cosolvents on conversion of ribonuclease A to S or on conversion of ribonuclease S to ribonuclease $A^{\ddagger}$ was investigated by replacing a portion of the water in a ribonuclease–subtilisin mixture with different organic cosolvents such as the polyols which can promote synthesis of small peptides (Homandberg et al., 1978). For most of the data to be shown, 5 mg of ribonuclease A or S was first dissolved in water or buffer (minimally 0.05 mL) and organic cosolvent (maximally 0.95 mL) was slowly added. The additional precautions often necessary for the introduction of an organic cosolvent into a protein solution (Singer, 1962) were unnecessary with ribonuclease A or S. The solution was then adjusted to an apparent pH of 6.2 (except where the pH dependence was studied) for reasons which will become apparent later. An aliquot (10 μL) of a subtilisin BPN′ solution (5 mg/mL) was added to the organic cosolvent solution last, and the reactions were allowed to proceed at 25 °C. Small aliquots were withdrawn as a function of time and subjected to assay in 40% (v/v) dioxane. Although subtilisin BPN′ has been reported to be less specific for the Ala^{20}–Ser^{21} bond of ribonuclease A than is subtilisin Carlsberg (Doscher & Hirs, 1967), the BPN′ enzyme was used because it is a more efficient catalyst of synthesis in glycerol solutions.

Isolation and characterization of ribonuclease $A^{\ddagger}$ was attempted on a preparative scale after the optimal conditions for synthesis were found. Ribonuclease S (50 mg) was dissolved in water (1.0 mL) and glycerol (9.0 mL) was slowly added with stirring. Subtilisin BPN′ (100 μL of a 5 mg/mL solution) was added last. After two weeks at 25 °C the subtilisin was inactivated by addition of phenylmethanesulfonyl fluoride. To decrease the amount of any possible proteolysis by trace quantities of uninhibited subtilisin, aliquots (100 μL) of the cosolvent solution were diluted one at a time with nine

[3] One of the reviewers of this manuscript believes that we "...have thrown caution to the winds." He continues "...the thermodynamic discussion is an impressive weakness in an otherwise interesting paper.... I suggest the results be presented unadorned." We did not take the advice but the reviewer may be right. Let the reader beware.

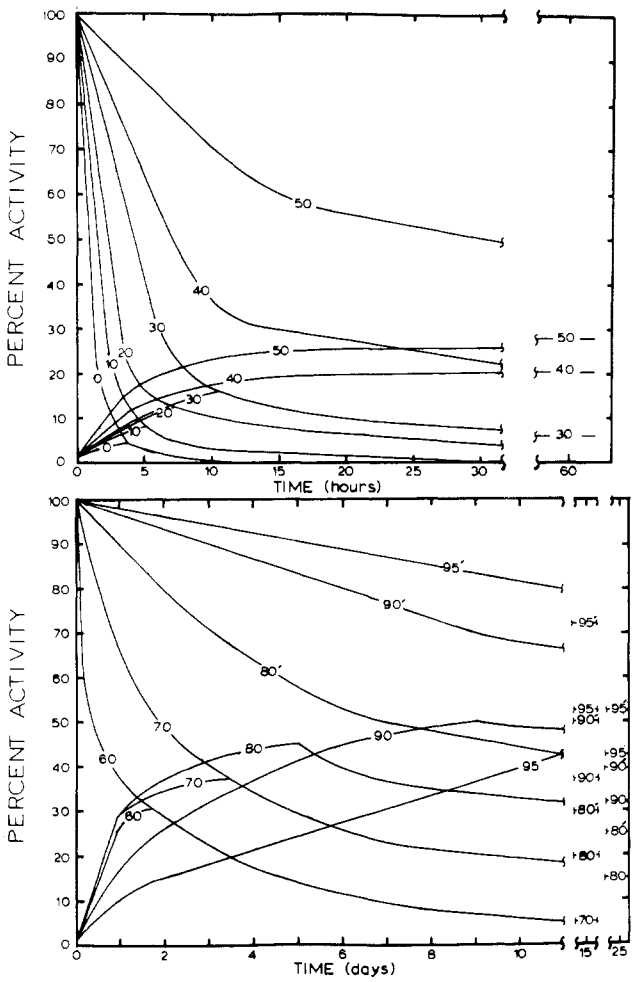


FIGURE 1: The dependence of ribonuclease activity in 40% (v/v) dioxane upon the time of incubation with subtilisin BPN'. The reactions at 25 °C and at apparent pH 6.2 in 0–50% (v/v) glycerol (top panel) were catalyzed by 20 μg/mL of subtilisin or in 60–95% (v/v) glycerol (bottom panel) by 100 μg/mL of subtilisin. The numbers on the curves correspond to percentage (v/v) glycerol. The substrate was either 2 mg/mL of ribonuclease A (curves descending) or 2 mg/mL of ribonuclease S (curves ascending initially). The percent activities were determined by measuring the activity of aliquots of the reaction mixture against cytidine cyclic 2',3'-monophosphate in 40% (v/v) dioxane and comparing to that of a standard solution of ribonuclease A. Assays were conducted (top panel) every 2 h until maximal synthesis was attained and every fifth hour subsequently or (bottom panel) at every 12 h until maximal synthesis was attained and every second day subsequently. Smooth curves were drawn to connect all points; to simplify the figure only the curves are shown.

volumes of 0.1 M sodium phosphate buffer, pH 6.47, and quickly applied to a column of Bio-Rex 70 (1.5 × 90 cm), which had been equilibrated with the same buffer. The column was then washed with 0.2 M sodium phosphate buffer, pH 6.47, to separate ribonuclease A‡ and S (Richards & Vithayathil, 1959). Ribonuclease A‡ eluted with 0.7–1.0 column volume and ribonuclease S with 1.1–1.5 column volumes. The peaks were assigned on the basis of activity toward cytidine cyclic 2',3'-monophosphate in 40% (v/v) dioxane (ribonuclease S is inactive) and by migration of the samples on sodium dodecyl sulfate gels. Each peak represented 46% of the material applied to the column. The ribonuclease A‡ peak was concentrated 10-fold on a Flash evaporator to avoid the aggregation observed after lyophilization (Craig et al., 1963) and then desalted on a Pharmacia PD-10 column (Sephadex G-25) which had been equilibrated with 0.1 M ammonium bicarbonate. The ribonuclease A‡ was then compared to ribonuclease A in terms of elution volumes on Sephadex G-50

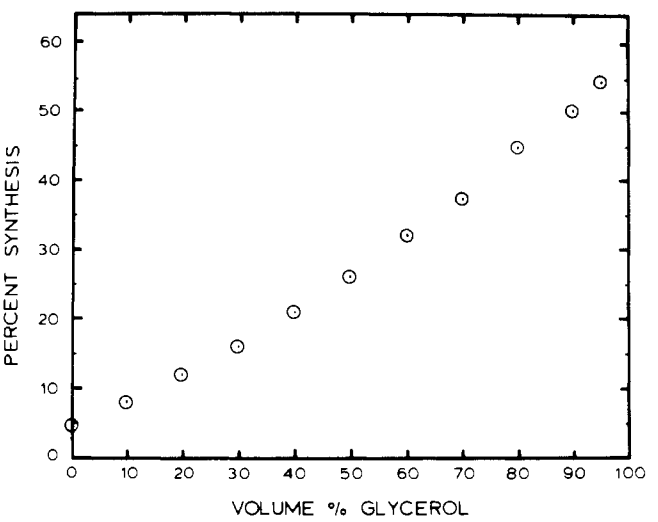


FIGURE 2: The dependence of the maximal synthesis observed as a function of the volume percent of glycerol. The data correspond to the maxima in the curves corresponding to the ribonuclease S incubations of Figure 1 (curves ascending initially).

(superfine) and Bio-Rex 70, mobility on disc gels and sodium dodecyl sulfate gels, amino acid composition before and after dialysis against 0.2 M acetic acid, and activity toward cytidine cyclic 2',3'-monophosphate in 40% (v/v) dioxane. Samples (2 mg) of the ribonuclease A‡ and of the ribonuclease S starting material were sequenced in the presence of 4 mg of Polybrene (Klapper et al., 1978) for 25 or 5 cycles, respectively, of Edman degradation on a Beckman Model 890C sequencer.

Results

Enzymatic Synthesis of Ribonuclease A‡ in Water. Our ribonuclease S sample was subjected to kinetic assay in the presence of 40% (v/v) dioxane, which dissociates the S-peptide from S-protein (Cowgill, 1966). A residual activity corresponding to 1.2% of ribonuclease A was consistently observed. This residual activity may be due to an inherent property of ribonuclease S in this assay system or a result of small contamination by ribonuclease A‡ or a combination of both of these. Since we did not resolve this complication this residual activity is included in the results of Figures 1 and 2.

Upon incubation of ribonuclease S with subtilisin in aqueous solution the ribonuclease A activity rises from 1.2 to 4.3% in 3 h (bottom curve, top panel, Figure 1) and then declines to less than 0.1% in 12 h. Incubation of ribonuclease A with subtilisin leads to a monotonic decrease in activity. The two activity curves come into coincidence after 3 h of incubation (the time of maximal synthesis in the ribonuclease S curve) and remain coincident thereafter. We interpret these results as showing that during the first 3 h of the reaction the principal kinetic process is the establishment of the ribonuclease A‡ ⇄ ribonuclease S equilibrium; the subsequent decline in activity is the result of subsequent proteolysis of ribonuclease S at many sites (Richards, 1955). In order to interpret the data quantitatively we must further assume that ribonuclease with several peptide bonds hydrolyzed is inactive in our assay system.

The interpretation provided here would be far from convincing were we to use the data in pure aqueous solvent alone. However, it is further supported by the data obtained in the presence of glycerol.

Effect of Added Glycerol. In a recent paper (Homandberg et al., 1978) we have shown that addition of large amounts of organic cosolvents to the reaction medium considerably increases the value of the peptide bond synthesis equilibrium

constants and allows for moderately efficient enzymatic synthesis of peptide bonds, where such a synthesis is only marginal in absence of added cosolvent. Therefore, if the interpretation of the activity rise described in water is correct, larger increases in activity should be observed in the presence of added cosolvent.

Figure 1 summarizes the data obtained on incubation of ribonuclease S and ribonuclease A with subtilisin in various water–glycerol mixtures. Qualitatively the results are closely similar to those described in water. On incubation of ribonuclease S the activity first rises and then declines, while the ribonuclease A curves come into coincidence with the ribonuclease S curves at the point of maximal synthesis except at the highest glycerol concentrations.

Quantitatively, however, the curves are strikingly different. The most important of the differences is the increase in the maximal activity obtained upon ribonuclease S incubations with rising glycerol concentration. These maximal activities are plotted in Figure 2 as a function of added glycerol concentration. The maximal activity rises from 4.3% in water to 50% in 90% (v/v) glycerol. Assuming that these results correspond to attainment of ribonuclease S $\rightleftarrows$ ribonuclease A‡ equilibria the values can be converted to K_{syn} values by writing

$$K_{syn} = \frac{\%\ \text{maximal synthesis}}{100 - \%\ \text{maximal synthesis}} \qquad (1)$$

The values of K_{syn} rise from 0.05 in water to 1 in 90% (v/v) glycerol, i.e., a 20-fold increase. Over the same glycerol concentration range the equilibrium constant for synthesis of benzyloxycarbonyltryptophanylglycinamide from benzyloxycarbonyltryptophan and glycinamide increases 10-fold (Homandberg et al., 1978). At 60% (v/v) glycerol K_{syn} for ribonuclease A‡ is 0.42, an eightfold increase over the water value, in accord with the eightfold increase in K_{syn} for the reactive site Arg^{63}–Ile peptide bond in soybean trypsin inhibitor (Kunitz) (Homandberg et al., 1978). Thus the increase in the extent of observed synthesis is in fair accord with expectations, subject to the obviously large experimental errors in K_{syn}, especially in water.

The other quantitative difference between the curves in water and in glycerol is the extreme slowing of all proteolytic processes as glycerol is added.

The results were monitored not only by enzymatic activity but also occasionally by sodium dodecyl sulfate gel electrophoresis. Ribonuclease S showed only a single band but after incubation with subtilisin a slower moving band at the position of ribonuclease A (corresponding to ribonuclease A‡) appeared. The ratio of the A‡ band area to that of the sum of the A‡ and S band areas roughly corresponds to the percent activity in Figures 1 and 2 until the maximum activity is reached. After this point this ratio (with large experimental error) remains the same but the intensity of both bands declines as ribonuclease S is converted to smaller products.

In order to eliminate several complicated explanations of the data two more experiments were carried out in 90% (v/v) glycerol. In one ribonuclease S′,[1] rather than S, was used as a starting material. The ribonuclease S′ was prepared by mixing 5 mg of S-protein with 2 mg of S-peptide (excess) in 0.1 mL of water, incubating for 5 h to ensure complete association, and then adding 0.9 mL of glycerol and 50 μg of subtilisin BPN′. The same limiting value of 50% activity was obtained as in incubation of ribonuclease S. In another experiment ribonuclease S and ribonuclease A‡ were separated from the 90% (v/v) glycerol reaction and then allowed to equilibrate again. Both reached the same 50% activity. This last experiment eliminates the possibility that the driving force for synthesis was transpeptidation, i.e., joining of fragments 1–21 and 20–124 by eliminating Ser^{21}.

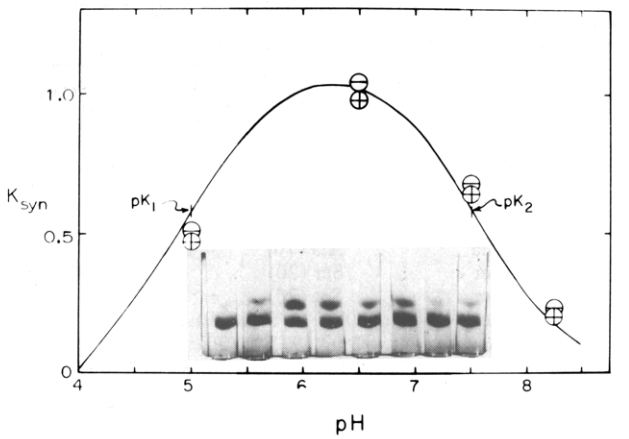


FIGURE 3: The pH dependence of K_{syn} in 90% (v/v) glycerol. The curve is the best fit of the data to eq 2. The data are based on scans of the bands on the sodium dodecyl sulfate electrophoresis gels shown in the inset. Solutions of ribonuclease A (⊖) and S (⊕), all at 5 mg/mL in 90% (v/v) glycerol, were adjusted to pH 5.0 (buffered with 3 mM sodium acetate), to pH 6.5 (3 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate), to pH 7.5 (3 mM sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate), or to pH 8.2 (3 mM Tris) and subtilisin BPN′ was added to each solution for a final concentration of 50 μg/mL. After 3 weeks aliquots were subjected to sodium dodecyl sulfate electrophoresis (see Methods). The first and second gels from left to right correspond to aliquots of solutions at pH 5.0 where ribonuclease A and S, respectively, were the starting materials. Correspondingly the third and fourth gels were ribonucleases A and S at pH 7.5, the fifth and sixth gels, pH 7.5, and the last two gels, pH 8.2.

Effect of pH upon the maximal extent of synthesis in 90% (v/v) glycerol was monitored by sodium dodecyl sulfate disc gel electrophoresis. The results are presented in Figure 3. The maximal synthesis values were converted to K_{syn} according to eq 1. The pH range was severely limited since subtilisin is inactivated at low pH and also ribonuclease S dissociates (Gross & Witkop, 1966). The few available points were fitted to the Dobry et al. (1952) equation for pH dependence of K_{syn} for a peptide bond

$$K_{syn} = \frac{K_{syn}^{\circ}}{1 + H^+/K_1 + K_2/H^+} \qquad (2)$$

where K_{syn}° in the pH-independent value of K_{syn} and K_1 and K_2 are respectively the ionization constants of the carboxyl group (of Ala^{20} and/or Ser^{21}) and of the amino group (of Ser^{21} and/or Ser^{22}) of the reactants (ribonuclease S). It should be noted (Mattis & Laskowski, 1973) that eq 2 applies to synthesis of a peptide bond only if the synthesis does not perturb the pK values of any other ionizable groups in the protein. Nonetheless, the fit is quite good. The best values for $K_{syn}^{\circ} = 1$ are $pK_1 = 5.0$ and $pK_2 = 7.4$. These are highly reasonable values. Richards (1955) found $pK_2 = 7.4$ for the several amino groups liberated in *extensive* digestion of ribonuclease by subtilisin. Homandberg et al. (1978) and references cited therein found that organic cosolvents exert only a slight effect upon pK values of amino groups. On the other hand the effects upon carboxyl groups are large. In a separate experiment we determined that the pK of acetylglycine is 1.1 units higher in 90% (v/v) glycerol than in water. The expected pK of a COOH-terminal group in a protein is approximately 3.8 (Nozaki & Tanford, 1967) so that the expected pK_1 in 90% (v/v) glycerol is 4.9, in excellent

Table I: Edman Degradation Products from Residues Surrounding Resynthesis Site(s) in Ribonuclease A‡

cycle	ribonuclease A sequence	yield (nmol) of PTH-amino acids from ribonuclease A‡		
		ribonuclease A	des-Ser[21]-	Ser[21A]- and/or carry-over
18	Ser			
19	Ala	Ala (60)		Ser (6)
20	Ala	Ala (70)		
21	Ser	Ser (20)		Ala (15)
22	Ser	Ser (20)		
23	Ser	Ser (10)	Asx (20)	
24	Asn	Asx (20)	Tyr (20)	Ser (8)
25	Tyr	Tyr (20)		
26	Cys			

agreement with the results of Figure 3.

The large shift of the carboxyl pK is the major cause of the increase in K_{syn} in 90% (v/v) glycerol as already outlined by Homandberg et al. (1978).

Isolation and Characterization of Ribonuclease A‡. Ribonuclease A‡ was isolated from the reaction mixture in 90% (v/v) glycerol by ion exchange chromatography as described under Methods. The isolated product shared the following properties with authentic ribonuclease A: elution volume on a Sephadex G-50 gel exclusion column and on a Bio-Rex 70 ion exchange column, electrophoretic mobility on high and low pH disc gels and on sodium dodecyl sulfate disc gels, amino acid analysis, and specific activity towards cytidine cyclic 2′,3′-monophosphate. The sample was also subjected to 25 cycles of Edman degradation in a Beckman 890C sequencer. The results are shown in Table I. The first 22 cycles were in excellent accord with the sequence of ribonuclease A. However, at cycle 23 both the expected Ser[23] and Asn (residue 24) were found. Similarly at cycle 24 we find both the expected Asn[24] and Tyr (residue 25). This result is consistent with the product being the expected mixture of des-Ser[21]-ribonuclease A, ribonuclease A, and Ser[21A]-ribonuclease A. The last of these materials is difficult to detect in a sequencer run because its partial presence produces the same effect as carry-over (due to incomplete reactions) of the parent sequence. The starting material, ribonuclease S, was sequenced for five cycles. Its S-protein component was clearly a mixture of 20–124 and 21–124. Thus the synthetic material, ribonuclease A‡, is not ribonuclease A but a mixture of ribonuclease A and des-Ser[21]-ribonuclease A. We expect that Ser[21A]-ribonuclease A is also present in this mixture but we cannot offer definite proof.

Preliminary Work on "Deletion Mutants". The results given above already show that des-Ser[21]-ribonuclease can be prepared, albeit as a component of a mixture. Therefore an attempt was made to synthesize covalent des-Ala[20]-ribonuclease A by incubation of ribonuclease E (Klee, 1965) in 90% (v/v) glycerol. After 8 weeks of incubation 24% yield of a synthetic product was detected by sodium dodecyl sulfate gel electrophoresis.

On the other hand no synthesis was detected upon incubation of des-Ala[19]-Ala[20]-ribonuclease S monitored both by electrophoresis and by activity assays. Whether such a negative conclusion has any important conformational implications is very difficult to judge.

Other Organic Cosolvents. Glycerol is relatively ineffective in promoting the synthesis of benzyloxycarbonyltryptophanglycinamide compared to other organic cosolvents because it raises the pK of carboxyl groups less than the other cosolvents do. Therefore several other cosolvents—ethylene glycol, 1,3-propanediol, 1,4-butanediol, 1,5-pentanediol, and triethylene glycol—were screened at concentrations ranging from 40 to 80% (v/v). In none of these cases was synthesis any greater than in water alone. An attempt to force the synthesis in 80% (v/v) 1,4-butanediol (an optimal solvent for synthesis of small peptides) by using a 100-fold excess of S-peptide also failed. While we are reasonably confident that these negative results are correct, it is possible that solvent concentrations or sampling times were not chosen correctly and a more extensive study might show that some of these solvents could be used successfully.

Discussion

The ability to resynthesize the hydrolyzed peptide bond(s) in ribonuclease S in the presence of glycerol is of interest for two principal reasons. First it shows the general applicability of the enzymatic method for protein semisynthesis. Secondly it suggests the possibility of obtaining at least approximate values of the equilibrium constants for the formation or hydrolysis of specified peptide bonds in globular proteins.

It is clear that the most desirable method of investigating the structural and functional role of amino acid residues in globular proteins is the comparison of properties between the parent proteins and analogues in which the residue of interest is specifically replaced. The major problem is obtaining desirable analogues. This problem can be attacked by obtaining mutant proteins, homologous proteins from various species, specific modification of residues of interest, or total synthesis and semisynthesis. At the present stage of technology semisynthesis (see reports in Offord & DiBello, 1978) appears quite attractive. The protein of interest is cleaved (generally enzymatically) into fragments, the fragments are separated, one of them is replaced by an analogue, and then they are mixed and coupled. However, the organic chemical coupling of fragments has the strong disadvantage that protection and deprotection of various functional groups are required to assure specificity. If the coupling could be carried out by enzymes the specificity could be largely provided by the enzyme and most (or all) of the protection and deprotection steps could be omitted. However, the enzymatic coupling suffers from two major disadvantages. One of these is that equilibrium constants for peptide bond synthesis are generally quite small (although much larger than the prevailing belief that the hydrolysis reaction is generally essentially irreversible). It is this disadvantage that was largely remedied in this paper by the use of 90% (v/v) glycerol rather than water as a solvent in which to carry out the synthesis. The second disadvantage is that few proteolyses are truly limited. Generally, aside from the critical bond of interest, many other bonds are hydrolyzed as well. Such subsequent hydrolysis may in many cases prevent efficient resynthesis of the critical bond of interest. In our case it clearly manifested itself by a decline in the amount of ribonuclease A‡ after the synthetic "equilibrium" was reached, and therefore it imposes an important requirement upon further attempts to apply enzymatic resynthesis to other systems. It is clear that when such attempts are made in the future the assay for the putative synthetic product must be carried out at frequent time intervals lest the time of maximal synthesis be missed. Indeed at the initial stages of this work we believed that the synthesis in water and in dilute glycerol solutions was much less than finally determined since we examined only incubation mixtures well beyond the time of maximal synthesis. An incidental problem important in any attempt to carry out such an assay or to isolate the synthetic product is the need to inhibit the catalyzing enzyme prior to the assay or isolation. Otherwise, since most assays or isolation procedures involve exposure to media which favor peptide bond

hydrolysis rather than synthesis, the synthetic product may well be hydrolyzed.

Glycerol, the only cosolvent found successful in this study, is a relatively poor promoter of peptide bond synthesis since it perturbs the pK_1 of carboxyl groups relatively weakly compared to other cosolvents (Homandberg et al., 1978). The surprising success of glycerol is probably rather easy to explain. Bello (1969) found that, in contrast to other related cosolvents, glycerol is a ribonuclease A stabilizer, while the others are destabilizers. It is quite likely that the other cosolvents caused dissociation of ribonuclease S into S-peptide and S-protein and thus changed the problem from one of synthesis of a "cyclic" to one of synthesis of a "noncyclic" peptide bond. However, this is not a sufficient explanation of the failure to force the synthesis in 80% (v/v) 1,4-butanediol in the presence of a 100-fold (~30 mM) excess of S-peptide. In this system the equilibrium constant for synthesis of a noncyclic peptide bond is 40 M^{-1} (Homandberg et al., 1978), and therefore 50% synthesis should be expected, provided the synthetic equilibrium between S-peptide and S-protein was obtained. However, if there is little or no association of S-protein and S-peptide in this system it is quite likely that the nonspecific proteolysis of S-protein did not allow the equilibrium of the bond of interest to be established.

In applications to other protein systems we recommend that several cosolvents should be screened again in order to show whether only glycerol will be successful or whether other cosolvents can be used as well.

A number of objections can be raised concerning the conversion of the 4.3% maximal synthesis of ribonuclease A in water to a $K_{syn}°$ of 0.05 ($K_{hyd}° = 20$). First and rather trivially ribonuclease S is a mixture of the expected four components and ribonuclease A[‡], of two or three. However, this introduces only trivial errors into the reported value. The more serious objections that can be raised are that 4.3% synthesis is too small to be believable experimentally and even more importantly that insufficient proof of attainment of equilibrium was provided.[3] These objections are largely but not completely answered by the facts that the ribonuclease A– and ribonuclease S–subtilisin incubation curves come into coincidence at the point of maximal synthesis, that the values of the maximal synthesis obtained in various glycerol solutions smoothly depend upon the added glycerol concentration, and that the effect of glycerol upon these maximal synthesis values is quantitatively similar, although not identical, to that of the effect of glycerol upon $K_{syn}°$ of benzyloxycarbonyltryptophanylglycinamide and of the reactive site in soybean trypsin inhibitor (Kunitz) (Homandberg et al., 1978). Furthermore, the pH dependence of the maximal synthesis value in 90% glycerol is the simplest possible predicted for peptide bond hydrolysis equilibria (Dobry et al., 1952). Thus, treating maximal synthesis values obtained in various glycerol solutions and at various pH values as if they were equilibrium amounts of synthesis introduces no inconsistencies. It is entirely possible that the maximal synthesis values may be slightly smaller than the true equilibrium values, but the difference is not likely to be great. It appears to us that a similar treatment of cosolvent data for resynthesis of peptide bonds in other globular proteins may also allow the determination of rough values of $K_{syn}°$ in water for these proteins.

We have repeatedly pointed out (e.g., Mattis & Laskowski, 1973; Sealock & Laskowski, 1973; Finkenstadt et al., 1974; D. A. Estell and M. Laskowski, Jr., manuscript in preparation) that the values of the equilibrium constant for the hydrolysis of a single specified peptide bond in globular proteins may provide important information about the strength of conformational interactions surrounding the cleaved peptide bond. Unfortunately, very few such equilibrium constants have been measured. The few that are known are all for the reactive site peptide bonds in protein proteinase inhibitors. The reason for that limitation is in part that for protein proteinase inhibitors the reactive site hydrolysis equilibrium constants are near unity at neutral pH. Thus at equilibrium about half of the peptide bonds are intact and about half are hydrolyzed—the easiest situation for exact measurements. The additional reason for the relative ease of determination of K_{hyd} of protein proteinase inhibitors is that the hydrolysis of their reactive site peptide bonds is a very limited proteolysis as in the case of the soybean trypsin inhibitor (Kunitz).

We believe that the results presented in this paper can be used to assert that $K_{syn}°$ for the Ala^{20}-Ser^{21} bound in ribonuclease A in water is about 0.05 or $K_{hyd}°$ ($1/K_{syn}°$) is 20. This should be regarded only as a rough statement for a variety of reasons, but we believe this value to be correct to within a factor of two or three. In view of the widely held belief that the 16–24 loop in ribonuclease A and S is floppy (Richards & Wyckoff, 1971) this is a surprisingly low value of $K_{hyd}°$, only one to two orders of magnitude higher than $K_{hyd}°$ for the reactive sites of protein proteinase inhibitors, where the low values of $K_{hyd}°$ were interpreted by us as indicative of great rigidity of the surrounding residues. Before this result can be properly interpreted $K_{syn}°$ values for several other peptide bonds in proteins of known structure should be determined and compared.

Acknowledgments

We are grateful to William Kohr, without whose technical expertise in amino acid analyses and protein sequencing the heterogeneity of our synthetic product could have gone undetected, and to Irwin M. Chaiken for suggesting the dioxane assay system.

References

Barel, A. O., & Glazer, A. N. (1968) *J. Biol. Chem. 243*, 1344.

Bello, J. (1969) *Biochemistry 8*, 4535.

Cowgill, R. M. (1966) *Biochim. Biophys. Acta 120*, 196.

Craig, L. T., King, T. P., & Crestfield, A. M. (1963) *Biopolymers 1*, 231.

Crook, E. M., Mathias, A. P., & Rabin, B. R. (1960) *Biochem. J. 74*, 234.

Dobry, A., Fruton, J. S., & Sturtevant, J. M. (1952) *J. Biol. Chem. 195*, 149.

Doscher, M. S., & Hirs, C. H. W. (1967) *Biochemistry 6*, 304.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Finkenstadt, W. R., Hamid, M. A., Mattis, J. A., Schrode, J., Sealock, R. W., Wang, D., & Laskowski, M., Jr. (1974) in *Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J., & Truescheit, E., Eds.) pp 389–411, Springer-Verlag, New York.

Gross, E., & Witkop, B. (1966) *Biochem. Biophys. Res. Commun. 23*, 720.

Gross, E., & Witkop, B. (1967) *Biochemistry 6*, 745.

Homandberg, G. A., & Laskowski, M., Jr. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1813.

Homandberg, G. A., Mattis, J. A., & Laskowski, M., Jr. (1978) *Biochemistry 17*, 5220.

Klapper, D. G., Wilde, C. E., III, & Capra, J. D. (1978) *Anal. Biochem. 85*, 126.

Klee, W. A. (1965) *J. Biol. Chem. 240*, 2900.

Mattis, J. A., & Laskowski, M., Jr. (1973) *Biochemistry 12*, 2239.
Nozaki, Y., & Tanford, C. (1967) *Methods Enzymol. 11*, 715.
Offord, R. E., & DiBello, C. (1978) *Semisynthetic Peptides and Proteins*, Academic Press, New York and San Francisco.
Richards, F. M. (1955) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 29*, 322.
Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647.
Sealock, R. W., & Laskowski, M., Jr. (1973) *Biochemistry 12*, 3139.
Singer, S. J. (1962) *Adv. Protein Chem. 17*, 1.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Diastereomers of 5′-*O*-Adenosyl 3′-*O*-Uridyl Phosphorothioate: Chemical Synthesis and Enzymatic Properties†

Peter M. J. Burgers and Fritz Eckstein*

ABSTRACT: A procedure is described for the synthesis of the title compounds via phosphotriester intermediates. The 2-cyanoethyl group is used to protect the P–SH function during the course of the synthesis. Resolution of the phosphorus diastereomers is accomplished at the phosphotriester stage. Removal of the 2-cyanoethyl group without racemization, followed by removal of the other protective groups, affords the optically pure diastereomers of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate. Their designation as *Rp* and *Sp* follows from the stereospecificity in the hydrolysis catalyzed by RNase A. These diastereomers are useful for the investigation of the stereospecificity as well as of the stereochemical course of action of nucleases. Snake venom exonuclease hydrolyses only the *Rp* diastereomer, whereas both diastereomers are substrates for RNases A and T_2. The results with the latter indicate that RNase T_2 also operates by an in-line mechanism.

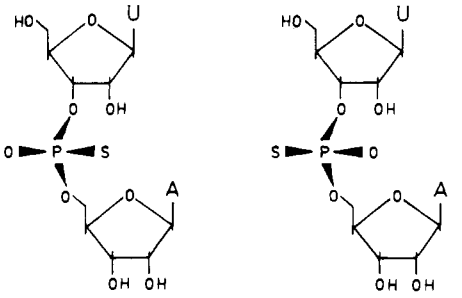
Diastereomeric phosphorothioate analogues of nucleotides are important tools for the elucidation of the stereochemistry of action of different classes of enzymes. For instance, the stereochemical course of action of RNase A has been established by its reaction with the diastereomers of cyclic uridine 2′,3′-*O*,*O*-phosphorothioate (Eckstein, 1975).

To obtain a more complete insight into enzyme–substrate interactions of exo- and endonucleases in general, one needs the two diastereomeric phosphorothioate analogues of an appropriate diribonucleoside monophosphate in their optically pure form. In addition, the study of these diastereomers by CD and NMR spectroscopy could give important information about the influence of the phosphodiester function on the conformation of dinucleoside monophosphates in general and on intramolecular base-stacking interactions in particular.

In this publication we describe the synthesis and separation of the diastereomers of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate [Up(S)A][1] and their enzymatic hydrolysis by the ribonucleases A and T_2 and by the exonucleolytic phosphodiesterase from snake venom. An alternative method of preparation of Up(S)A did not allow the separation of the diastereomers at any stage of the synthesis (Burgers & Eckstein, 1978a).

General Methods and Materials

Thin-layer chromatography was performed on Merck Kieselgel 60 F_{254} plates in system A ($CHCl_3$–MeOH, 92:8 v/v) and on Merck DC Alufolien Cellulose F_{254} plates in system B (1 M NH_4OAc–EtOH, 3:7 v/v). Merck Kieselgel 60H was used for column chromatography.



Up(S)A (R_p) Up(S)A (S_p)

A Packard-Becker 8200 chromatograph, equipped with a Packard 1170 UV detector operating at 254 nm and a Servogor RE 511 recorder, was employed for the high-performance liquid chromatography. The strong anion-exchanger Nucleosil 10 SB from Macherey & Nagel was stirred with three lots of 0.5 M NH_4OAc (pH 4.5), decanted to remove the fine particles and then packed into a stainless steel column (40 cm × 2 mm) according to the slurry method. Isocratic elution of the column was performed with a varying range of buffers, all containing 0.05 M KH_2PO_4 and 0.2–0.5 M KCl at pH 4.5. The flow rate was 1.0 mL/min at pressures of 160–220 atm.

Ultraviolet absorption spectra were measured on a Shimadzu UV-200 spectrophotometer. Kinetic measurements were carried out using a Zeiss PMQ II spectrophotometer equipped with an automatic cuvette changer.

The ^1H and ^{31}P nuclear magnetic resonance spectra were recorded with a Bruker-Physics HFX 60 spectrometer

† From Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Germany. *Received August 25, 1978.* This work was supported by the Deutsche Forschungsgemeinschaft.

[1] Abbreviations used: Up(S)A, 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate; *Rp* and *Sp*, diastereomers of Up(S)A; U>pS, cyclic uridine 2′,3′-*O*,*O*-phosphorothioate; LC, high-performance liquid chromatography.

0006-2960/79/0418-0592$01.00/0