Studies on Polypeptides. XXXIV. Enzymic Properties of Partially Synthetic De(16–20)- and De(15–20)-ribonucleases S'[1-3]

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Abstract: Syntheses are described of two peptides which correspond to positions 1–14 and 1–15, respectively, of the amino acid sequence of bovine pancreatic ribonuclease A. The stereochemical homogeneity of these peptides, *i.e.*, lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid and lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. A practical nomenclature is proposed for enzymes which result from combination of fragments or analogs of S-peptide with S-protein (partially synthetic ribonucleases S'). The enzymic properties of de(15–20)- and de(16–20)-RNAases S' with both RNA and cytidine 2',3'-phosphate were essentially identical with those of RNAase S'. The catalytic efficiency of the methionine-13 *d*-sulfoxides of these enzymes is markedly lower especially toward the cyclic phosphate. The increase in activity of the de(15–20) enzyme over that of the previously described de(14–20) enzyme locates aspartic acid 14 as a "binding site" in S-peptide.

The observation[3-5] that the synthetic tridecapeptide (II) has the ability to bring about essentially full activation of S-protein at molar ratios of approximately 30:1 in contrast to S-peptide (I)[6] which activates fully at molar ratios of 1:1 suggested the presence of important "binding sites" located between positions 14 and 20 of the S-peptide molecule. In order to assess the binding contributions of individual amino acid residues in this region, we have initiated the present investigation. Two peptides (III and IV) were synthesized (Chart I) and their ability to activate S-protein was assessed. The results implicate aspartic acid 14 as a strong binding site.

Chart I

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
I, S-peptide[a]

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-OH
II

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-OH
III

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-OH
IV

[a] For determination of the structure of S-peptide see D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **237**, 1845 (1962); J. T. Potts, A. Berger, J. Cooke, and C. B. Anfinsen, *ibid.*, **237**, 1851 (1962); and E. Gross and B. Witkop, *ibid.*, **237**, 1856 (1962).

Peptide Syntheses (Chart II). For reasons outlined in a previous communication[7] methionine was incorporated into the peptides in the form of its *d*-sulfoxide. For the synthesis of III, aspartic acid (VII) in the form of the triethylammonium salt was coupled with the N-hydroxysuccinimide ester of *t*-butoxycarbonylmethionine *d*-sulfoxide (VIII)[7] and the ensuing *t*-butoxycarbonylmethionylaspartic acid *d*-sulfoxide was deblocked with trifluoroacetic acid. Methionylaspartic acid *d*-sulfoxide (IX) was isolated as the crystalline monohydrate. The dipeptide was acylated with the azide of *t*-butoxycarbonylhistidine (X)[8] to give crystalline *t*-butoxycarbonylhistidylmethionylaspartic acid *d*-sulfoxide hydrate. Exposure to trifluoroacetic acid converted the protected tripeptide into histidylmethionylaspartic acid *d*-sulfoxide (XI) which was purified by chromatography on the resin AG 1-X2. Careful analytical evaluation of the amorphous tripeptide dihydrate demonstrated the presence of a stereochemically homogeneous single compound. For conversion to the heptapeptide

(1) The authors wish to express their appreciation to the U. S. Public Health Service and the American Cancer Society for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned are of the L configuration. In the interest of space conservation the customary L designation for individual amino acid residues is omitted. The following abbreviations are used: DMF = dimethylformamide, THF = tetrahydrofuran, TFA = trifluoroacetic acid, OPNP = *p*-nitrophenyl ester, O-*t*-But = *t*-butyl ester, ONHS = N-hydroxysuccinimide ester, Z = benzyloxycarbonyl, *t*-Boc = *t*-butoxycarbonyl, and Met(O) = methionine *d*-sulfoxide.

(3) See F. M. Finn and K. Hofmann, *J. Am. Chem. Soc.*, **87**, 645 (1965), for paper XXXIII in this series.

(4) K. Hofmann, F. M. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaihara, *ibid.*, **85**, 833 (1963).

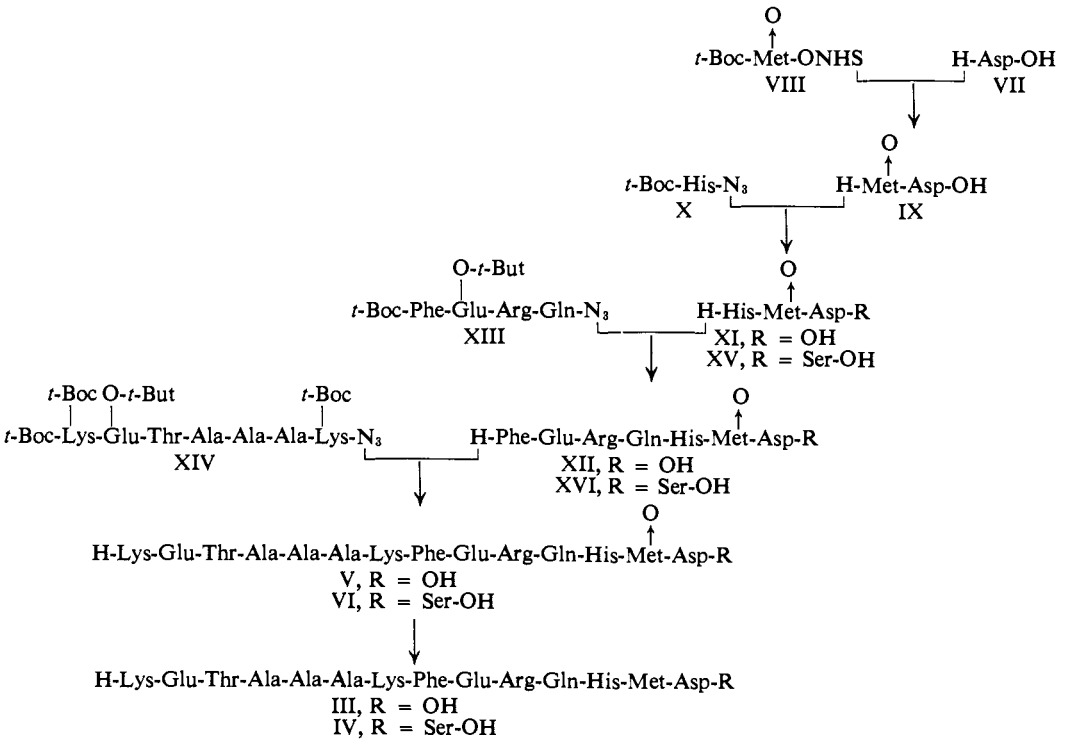
(5) K. Hofmann, R. Schmiechen, M. J. Smithers, R. D. Wells, Y. Wolman, and G. Zanetti, *ibid.*, **87**, 640 (1965).

(6) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958): RNAase S, subtilisin modified beef ribonuclease RNAase A; S-peptide, the eicosapeptide obtained from RNAase S; S-protein, the protein component obtained from RNAase S; RNAase S', the reconstituted enzyme obtained by mixing equimolar proportions of S-protein and S-peptide.

(7) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 631 (1965).

(8) E. Schröder and H. Gibian, *Ann.*, **656**, 190 (1962).

Chart II



XII the tripeptide XI was coupled with the azide XIII[9] essentially as described previously[5] in connection with the synthesis of related peptides. The resulting partially protected heptapeptide was purified and the purified material was exposed to the action of trifluoroacetic acid to afford crude XII. Following chromatography on CMC the heptapeptide XII was obtained in the form of the amorphous, stereochemically homogeneous pentahydrate. Interaction of the azide XIV[5] with the heptapeptide XII gave a partially protected derivative of V from which the protecting groups were removed with trifluoroacetic acid. The ensuing crude tetradecapeptide sulfoxide V was purified by chromatography on CMC. The pentadecapeptide VI was synthesized in an essentially identical manner except that peptide XV[7] was substituted for peptide XI. Conversion of the peptide sulfoxides V and VI into the corresponding peptides III and IV containing methionine was accomplished by repeated exposure of the sulfoxides to 1% aqueous thioglycolic acid according to the conditions described in the Experimental Section. The degree of reduction was followed by quantitative amino acid analyses of aminopeptidase M digests.

Evaluation of Stereochemical Homogeneity of Peptides. Since 1958[10] we have evaluated the stereochemical homogeneity of synthetic peptides by digestion with leucine aminopeptidase (LAP) followed by quantitative amino acid analysis of the digests. A crude enzyme preparation obtained from hog kidney according to the procedure of Spackman, *et al.*,[11] served as the source of enzyme. In 1963 Pfleiderer and Celliers[12] described the isolation from hog kidney ribosomes of aminopeptidase M (AP-M), an enzyme which differs markedly from the "classical" LAP in that it is not activated by heavy metals. Pfleiderer, *et al.*,[13] assessed the action of this new enzmye toward a series of peptides and found that it exhibits a broad side-chain specificity. Aminopeptidase M was employed for evaluation of the stereochemical homogeneity of peptides in the present investigation with excellent results. The enzyme is commercially available as a highly purified powder which appears to be stable indefinitely when stored at 4°.

Experimental Section

Materials. The ion exchangers, carboxymethylcellulose (CMC) and AG 1-X2, were purchased from Bio-Rad Laboratories, Richmond, Calif. The exchanger IRA-400 was supplied by Mallinckrodt Chemical Works, St. Louis, Mo. Aminopeptidase M (AP-M) was obtained from Röhm and Haas GmbH, Darmstadt, West Germany. Yeast RNA (Sigma Chemical Co.) was purified by precipitation from 0.1 *M* sodium acetate as described by Klee and Richards.[14] Cytidine 2′,3′-phosphate barium salt (Schwarz Bio-Research) was used without purification. Ribonuclease A was prepared from bovine pancreatic ribonuclease (Sigma Chemical Co., crystallized five times) by the procedure of Crestfield, *et al.*[15] Ribonuclease S, S-protein, and S-peptide were prepared as described previously.[3]

Enzyme Assays. Ribonuclease assays with RNA as substrate were performed as previously described.[3] With cytidine 2′,3′-phosphate as substrate a 0.2-ml aliquot of a solution of enzyme ($3.5–4.0 \times 10^{-6}$ *M* with respect to S-protein) in pH 6.69 Tris buffer[16] was added to 1.8 ml of a solution of substrate (10^{-3}–10^{-4} *M*) dissolved in the same buffer. Increase in absorbancy was monitored at 284 mμ with a Cary 15 recording spectrophotometer. Each point on Figures 2–5 is based on triplicate assays.

For evaluation of stereochemical homogeneity the peptides (1 to 5 μmoles) were dissolved in 0.1 *M* Tris–HCl buffer pH 7.75[17]

(9) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 620 (1965).
(10) K. Hofmann, M. E. Woolner, G. Spühler, and E. T. Schwartz, *ibid.*, **80**, 1486 (1958).
(11) D. H. Spackman, E. L. Smith, and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).
(12) G. Pfleiderer and P. G. Celliers, *Biochem. Z.*, **339**, 186 (1963).
(13) G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *ibid.*, **340**, 552 (1964).
(14) W. A. Klee and F. M. Richards, *J. Biol. Chem.*, **229**, 489 (1957).
(15) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 618 (1963).
(16) E. M. Crook, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, 74, 234 (1960).
(17) This buffer contained known quantities of norleucine and α-amino-β-guanidopropionic acid as internal standards.

(1.0 ml), an aqueous solution of AP-M (approximately 700 milliunits per μmole of peptide) added, and the volume adjusted to 2 ml with water. The digestion mixtures were incubated under toluene for 20 hr at 37°, diluted to a concentration of 0.5 μmole of peptide per ml with sodium citrate buffer pH 2.2, and suitable aliquots analyzed with a Beckman-Spinco amino acid analyzer.

General Procedures. For removal of *t*-butoxycarbonyl and *t*-butyl ester groups, approximately 15 ml of anhydrous trifluoroacetic acid per gram of protected peptide was employed. The solutions were kept at room temperature for 15–20 min, then ice-cold dry ether, approximately 500 ml per 10 ml of trifluoroacetic acid, was added, and the suspensions were cooled at −10° for 30 min. The precipitates were collected, washed with ether, and dried *in vacuo* over P_2O_5 and KOH pellets. 1-Butanol was equilibrated with the appropriate aqueous phases prior to its use for extractions. Fractions of 20 ml each were collected from the various ion-exchange columns with automatic fraction collectors at flow rates of 2–4 ml/min. General procedures for thin layer and paper chromatography are those described previously.[9,18]

α-Aspartylserine. β-*t*-Butylaspartylserine (0.50 g)[7] was deblocked with trifluoroacetic acid, the hygroscopic residue was dissolved in water (100 ml), and Amberlite IRA-400 (acetate cycle, 50 ml settled in water) was added. The mixture was stirred for 1 hr at room temperature and the resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was recrystallized from water–ethanol as clusters of needles: 0.25 g (63%); mp 179–180° dec; $[\alpha]^{24}D$ + 17.1° (*c* 1.08, water); R_f^1 0.22, R_f^2 0.34; single ninhydrin and chlorine positive spot; amino acid ratios in 20-hr AP-M digest $Asp_{0.99}Ser_{1.01}$.

Anal. Calcd for $C_7H_{12}O_6N_2$: C, 38.2; H, 5.5; N, 12.7; O, 43.6. Found: C, 38.1; H, 5.5; N, 12.9; O, 42.8.

Methionylaspartic Acid *d*-Sulfoxide Hydrate (IX). To a stirred solution, cooled at 0°, of aspartic acid (3.33 g) and triethylamine (6.9 ml) in water (100 ml) was added succinimido *t*-butoxycarbonylmethioninate *d*-sulfoxide[7] (7.25 g). The mixture was stirred for 2 hr at 0° and for 20 hr at room temperature. The solvent was removed *in vacuo*, the residue was dissolved in 1 *N* hydrochloric acid (60 ml), and the solution was concentrated to approximately 20 ml. This solution was extracted with six 60-ml portions of 1-butanol. The butanol phases were washed with three 25-ml portions of 2% acetic acid and then evaporated to dryness, yield 9.6 g. This material was deblocked with trifluoroacetic acid and the residue dissolved in water (100 ml) was applied to an AG 1-X2 column (acetate cycle, 2 × 40 cm), which was eluted with water (500 ml) and 0.1 *N* acetic acid (500 ml). The desired peptide was located in the 0.1 *N* acetic acid eluates by the ninhydrin reaction. These fractions were combined, the solvent was evaporated *in vacuo*, and the residue was recrystallized twice from water–methanol as plates: 3.7 g (62%); mp 150–151° dec; $[\alpha]^{25}D$ + 70.8° (*c* 0.98 water); R_f^1 0.21, R_f^2 0.04; single ninhydrin and chlorine positive spot.

Anal. Calcd for $C_9H_{16}O_6N_2S \cdot H_2O$: C, 36.2; H, 6.1; N, 9.4; O, 37.5; S, 10.7. Found: C, 36.1; H, 6.2; N, 9.5; O, 37.9; S, 10.7.

N^{α}-*t*-Butoxycarbonylhistidylmethionylaspartic Acid *d*-Sulfoxide Hydrate. *t*-Butyl nitrite (2.3 ml) was added to a stirred solution cooled at −25° of N^{α}-*t*-butoxycarbonylhistidine hydrazide[8] (5.4 g) in DMF (35 ml) containing 14.0 ml of 5.7 *N* hydrogen chloride in dioxane. The mixture was stirred at −20° for 30 min, then cooled to −50°, and triethylamine (11.1 ml) was added. To this solution containing the azide of N^{α}-*t*-butoxycarbonylhistidine was added a solution of methionylaspartic acid *d*-sulfoxide hydrate (IX, 2.8 g) in water (25 ml) containing triethylamine (2.75 ml), and the mixture was stirred for 1 hr at −10° and for 40 hr at 4°. The solvents were removed *in vacuo*, the residue was dissolved in water (100 ml), and the solution was added to an AG 1-X2 column (acetate cycle, 3.7 × 40 cm) which was eluted with water (1.2 l.) and 0.1 *N* acetic acid (1.6 l.). The desired peptide was located in the 0.1 *N* acetic acid eluates by the Pauly reaction. These eluates were combined, the solvent was evaporated *in vacuo*, and the residue recrystallized twice from water–ethanol as silky needles: 2.7 g (54%); mp 148–149° dec; $[\alpha]^{27}D$ +11.5° (*c* 1.18, water); R_f^1 0.59, R_f^2 0.25; single ninhydrin negative, Pauly and chlorine positive spot; amino acid ratios in acid hydrolysate $His_{1.00}Met^{19}{}_{0.67}Asp_{1.02}$.

Anal. Calcd for $C_{20}H_{31}O_9N_5S \cdot H_2O$: C, 44.9; H, 6.2; N, 13.1; O, 29.9; S, 6.0. Found: C, 44.6; H, 6.5; N, 12.8; O, 29.8; S, 6.1.

Histidylmethionylaspartic Acid *d*-Sulfoxide Dihydrate (XI). N^{α}-*t*-Butoxycarbonylhistidylmethionylaspartic acid *d*-sulfoxide hydrate (2.2 g) was deblocked with trifluoroacetic acid, and the hygroscopic residue dissolved in water (200 ml) was added to an AG 1-X2 column (acetate cycle, 2 × 36 cm) which was eluted with water (500 ml) and 0.05 *N* acetic acid (500 ml). The desired peptide was located in the water and 0.05 *N* acetic acid eluates by the Pauly reaction. These eluates were combined, the solvent was evaporated *in vacuo*, and the residue was lyophilized from small volumes of water: 1.68 g (90%); $[\alpha]^{26}D$ +42.1° (*c* 1.04, 10% acetic acid); R_f^3 0.66 × His; single ninhydrin, Pauly, and chlorine positive spot;

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amino acid ratios in AP-M digest $His_{0.95}Met_{1.04}Asp_{1.00}$.

Anal. Calcd for $C_{15}H_{23}O_7N_5S \cdot 2H_2O$: C, 39.7; H, 6.0; N, 15.4; O, 31.8; S, 7.1. Found: C, 39.7; H, 6.2; N, 15.1; O, 31.1; S, 7.3.

Phenylalanylglutamylarginylglutaminylhistidylmethionylaspartic Acid *d*-Sulfoxide Pentahydrate (XII). *t*-Butyl nitrite (0.25 ml) was added to a stirred solution cooled at −25° of *t*-butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate[9] (2.02 g) in DMF (25 ml) containing 1.54 ml of 5.7 *N* hydrogen chloride in dioxane. The solution was stirred for 20 min at −20°, then cooled at −40°, and triethylamine (1.20 ml) was added. To this solution of the azide, histidylmethionylaspartic acid *d*-sulfoxide dihydrate (XI, 0.50 g) in water (10 ml) and triethylamine (0.33 ml) was added. The mixture was stirred at −10° for 1 hr and at 4° for 40 hr. The solvents were removed *in vacuo*, and the residue was dissolved in 50 ml of 2% acetic acid and acidified with glacial acetic acid to approximately pH 3. This solution was extracted with six 50-ml portions of 1-butanol, and the butanol phases were washed with eight 50-ml portions of 2% acetic acid. The aqueous phases were combined and concentrated to approximately 100 ml, Amberlite IRA-400 (acetate cycle; 50 ml settled in water) was added, and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was lyophilized three times from water. This material dissolved in water (100 ml) was added to an AG 1-X2 column (acetate cycle; 2 × 44 cm), which was eluted with water (500 ml) and 0.1 *N* acetic acid (500 ml). The desired peptide was located in the water and 0.1 *N* acetic acid eluates by the Pauly reaction. These eluates were combined, the solvent was removed *in vacuo*, and the residue was lyophilized from water; yield 800 mg; R_f^1 0.51, R_f^2 0.59; Pauly, Sakaguchi, and chlorine positive spot, contaminated with histidylmethionylaspartic acid *d*-sulfoxide dihydrate.

The crude protected heptapeptide (770 mg) was deblocked with TFA and the hygroscopic residue was dissolved in water (100 ml). Amberlite IRA-400 (acetate cycle; 50 ml settled in water) was added and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was lyophilized from water. This material dissolved in water (500 ml) was applied to a CMC column (2 × 50 cm) which was eluted with water (1 l.) and 0.005 *M* ammonium acetate (2 l.). The desired peptide was located in the 0.005 *M* eluates by the Pauly reaction. These eluates were pooled, and the solvent was evaporated *in vacuo*. The residue was lyophilized to constant weight from small volumes of water: 470 mg; $[\alpha]^{29}D$ −12.1° (*c* 0.68, 10% acetic acid); R_f^3 0.62 × His; single ninhydrin, Pauly, Sakaguchi, and chlorine positive spot; amino acid ratios in acid hydrolysate $Phe_{1.00}Glu_{2.08}Arg_{0.97}His_{0.94}Met^{19}{}_{0.61}Asp_{1.01}$; amino acid ratios in AP-M digest $Phe_{1.11}$-

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$Glu_{1.12}Arg_{1.08}Gln_{0.88}His_{0.88}Met_{1.08}Asp_{0.89}$.

Anal. Calcd for $C_{40}H_{59}O_{14}N_{13}S \cdot 5H_2O$: C, 45.0; H, 6.5; N, 17.0; O, 28.5; S, 3.0. Found: C, 45.2; H, 6.8; N, 17.4; O, 28.4; S, 3.9.

Phenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine *d*-Sulfoxide Heptahydrate (XVI). *t*-Butyl nitrite (0.34 ml) was added to a stirred solution cooled at −25° of *t*-butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamine hydrazide diacetate trihydrate[9] (2.7 g) in DMF (25 ml) containing 2.04 ml of 5.7 *N* hydrogen chloride in dioxane. The mixture was stirred at −20°

(18) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara *J. Am. Chem. Soc.*, 87, 611 (1965).

(19) Methionine plus homocysteic acid; values not corrected for destruction.

for 20 min, then cooled at −40° and neutralized with triethylamine (1.6 ml). To this solution of the azide histidylmethionylaspartylserine *d*-sulfoxide dihydrate[7] (0.54 g), in water (20 ml), and triethylamine (0.28 ml) were added. The mixture was stirred at −10° for 1 hr and at 4° for 40 hr. The solvents were removed *in vacuo*, and the residue was dissolved in 50 ml of 2% acetic acid and acidified with glacial acetic acid to approximately pH 3. This solution was extracted with six 50-ml portions of 1-butanol and the butanol phases were washed with ten 50-ml portions of 2% acetic acid. The aqueous phases were combined and evaporated to dryness, and the residue was dissolved in water (100 ml). Amberlite IRA-400 (acetate cycle; 40 ml settled in water) was added and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid, and filtrate and washings were combined and evaporated to dryness. The residue was lyophilized four times from water. This material dissolved in water (100 ml) was added to an AG 1-X2 column (acetate cycle; 2 × 40 cm), which was eluted with water (500 ml) and 0.1 *N* acetic acid (750 ml). The desired peptide was located in the water and 0.1 *N* acetic acid eluates by the Pauly reaction. These eluates were combined, the solvent was removed *in vacuo*, and the residue was lyophilized from water: 660 mg; R_f^1 0.66, R_f^2 0.71; Pauly, Sakaguchi, and chlorine positive spot, contaminated with histidylmethionylaspartylserine *d*-sulfoxide dihydrate.

The crude protected octapeptide (630 mg) was deblocked with TFA and the hygroscopic residue was dissolved in water (100 ml). Amberlite IRA-400 (acetate cycle; 50 ml settled in water) was added and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was lyophilized twice from water. This material dissolved in water (400 ml) was applied to a CMC column (2 × 44 cm) which was eluted with water (500 ml) and 0.005 *M* ammonium acetate (2000 ml). The desired peptide was located in the 0.005 *M* eluates by the Pauly reaction. These eluates were pooled and the solvent was evaporated. The residue was lyophilized to constant weight from small volumes of water: 410 mg; $[\alpha]^{26}D$ −22.9° (*c* 1.14, 10% acetic acid); R_f^3 0.42 × His; single ninhydrin, Pauly, Sakaguchi, and chlorine positive spot; amino acid ratios in acid hydrolysate $Phe_{1.03}Glu_{2.10}Arg_{0.90}His_{1.00}Met^{19}{}_{0.81}Asp_{1.03}Ser_{1.00}$; amino acid ratios in AP-M digest $Phe_{0.99}$-

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$Glu_{1.11}Arg_{0.94}Gln_{1.16}His_{0.89}Met_{0.96}Asp_{1.01}Ser_{0.94}$.

Anal. Calcd for $C_{43}H_{64}O_{16}N_{14}S \cdot 7H_2O$: C, 43.4; H, 6.6; N, 16.5; O, 30.9; S, 2.7. Found: C, 43.5; H, 6.6; N, 16.3; O, 31.1; S, 3.4.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic Acid *d*-Sulfoxide Hydrate (V). N^{α},N^{ϵ}-Di-*t*-butoxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-*t*-butoxycarbonyllysine azide,[5] prepared from 1.63 g of the hydrazide,[18] was dissolved in DMF (50 ml) and the solution was added to a solution of phenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid *d*-sulfoxide pentahydrate (XII, 250 mg) in ice-cold 50% aqueous DMF (10 ml) containing triethylamine (0.1 ml). The mixture was stirred for 2 hr at 0° and for 40 hr at 4°. The solvents were removed *in vacuo* and the residue was dissolved in 50 ml of 1-butanol. The solution was acidified with glacial acetic acid to approximately pH 3 and extracted with ten 50-ml portions of 2% acetic acid. The aqueous phases were reextracted with five 50-ml portions of 1-butanol. The organic phases were combined and the solvents were removed *in vacuo*. The residue was dissolved in 100 ml of 2-propanol–methanol–water (1:1:1) and the solution was added to an AG 1-X2 column (acetate cycle; 2 × 44 cm) which was eluted with 400 ml of 2-propanol–methanol–water (1:1:1) and 600 ml of 2-propanol–methanol–0.1 *N* acetic acid (1:1:1). The desired peptide was located in the 2-propanol–methanol–0.1 *N* acetic acid (1:1:1) eluates by the Pauly reaction. These eluates were combined and the solvents were evaporated *in vacuo*. The residue (520 mg), dissolved in 100 ml of methanol–water (9:1), was applied to a CMC column (2 × 40 cm) which was eluted with 500 ml of methanol–water (9:1) and 600 ml of methanol–5% aqueous acetic acid (9:1). The desired peptide was located in the methanol–acetic acid eluates by the Pauly reaction. These eluates were combined and the solvents were removed *in vacuo:* 220 mg; R_f^{VI} 0.35; single ninhydrin negative, Pauly and chlorine positive spot.

The protected tetradecapeptide (220 mg) was deblocked with anhydrous TFA and the hygroscopic powder dissolved in water (100 ml). Amberlite IRA-400 (acetate cycle; 50 ml settled in water) was added and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was lyophilized from water. This material dissolved in water (300 ml) was added to a CMC column which was eluted with the following ammonium acetate solutions: 0.005 *M* (500 ml), 0.01 *M* (1500 ml), and 0.025 *M* (1000 ml). The desired peptide was located in the 0.025 *M* eluates by the Lowry reaction.[20] These eluates were pooled and the solvent was removed *in vacuo*. The residue was lyophilized to constant weight from small volumes of water: 80 mg; $[\alpha]^{26}D$ −48.5° (*c* 0.49, 10% acetic acid); R_f^3 0.35 × His; single ninhydrin, Pauly, and chlorine positive spot; amino acid ratios in acid hydrolysate $Lys_{1.97}Glu_{3.17}Thr_{0.98}Ala_{3.05}Phe_{1.00}Arg_{0.88}His_{0.97}Met^{19}{}_{0.70}Asp_{1.00}$; peptide content of hydrated sample 71% as determined with internal standard.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic Acid Hydrate (III). The tetradecapeptide *d*-sulfoxide (V, 17 mg) was dissolved in 1% aqueous thioglycolic acid (25 ml). The solution was incubated under nitrogen at 45° for 24 hr when the thioglycolic acid was exchanged for acetate on an IRA-400 column (prepared from 35 ml of water-settled resin). The column was eluted with water, Pauly positive eluates were pooled, and freshly distilled thioglycolic acid was added to make a 1% solution. The incubation and ion-exchange steps were carried out three times and the final solution was lyophilized to yield a colorless, fluffy, hygroscopic powder: 17 mg; $[\alpha]^{26}D$ −42.3° (*c* 0.73, 10% acetic acid); R_f^3 0.47 × His; ninhydrin, Pauly, Sakaguchi, and chlorine positive spot; amino acid ratios in AP-M digest $Lys_{2.27}Glu_{2.12}Thr_{0.96}Ala_{2.98}Phe_{0.98}Arg_{0.98}Gln_{0.81}His_{0.96}Met_{0.98}Asp_{0.96}$ (6% of the methionine was present as *d*-sulfoxide); peptide content of hydrated sample 69.1% as determined with internal standard.

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine *d*-Sulfoxide Hydrate (VI). N^{α},N^{ϵ}-Di-*t*-butoxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-*t*-butoxycarbonyllysine azide[5] prepared from 950 mg of the hydrazide[18] was dissolved in DMF (30 ml) and the solution was added to a solution of phenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine *d*-sulfoxide heptahydrate (240 mg) in 50% aqueous DMF (10 ml) containing triethylamine (0.1 ml). The solution was stirred for 2 hr at 0° and for 40 hr at 4°. The solvents were removed *in vacuo* and the residue was dissolved in 70 ml of 1-butanol. The solution was acidified with acetic acid to approximately pH 3 and extracted with ten 50-ml portions of 2% acetic acid. The aqueous phases were reextracted with five 50-ml portions of 1-butanol, the organic phases were combined, and the solvents were removed *in vacuo*. The residue was dissolved in 100 ml of 2-propanol–methanol–water (1:1:1) and the solution added to an AG 1-X2 column (acetate cycle; 2 × 46 cm) which was eluted with 400 ml of 2-propanol–methanol–water (1:1:1) and 600 ml of 2-propanol–methanol–0.1 *N* acetic acid (1:1:1). The desired peptide was located in the 2-propanol–methanol–0.1 *N* acetic acid eluates by the Pauly reaction. These eluates were combined and the solvents were evaporated *in vacuo*. The residue (490 mg) dissolved in 100 ml of methanol–water (9:1) was applied to a CMC column (2 × 42 cm) which was eluted with 400 ml of methanol–water (9:1) and 900 ml of methanol–5% acetic acid (9:1). The desired peptide was located in the methanol–5% acetic acid (9:1) eluates by the Pauly reaction. These eluates were combined and the solvents were removed *in vacuo:* 310 mg, R_f^{VI} 0.45, single Pauly and chlorine positive spot.

The protected pentadecapeptide (310 mg) was deblocked with TFA and the hygroscopic powder was dissolved in water (100 ml). Amberlite IRA-400 (acetate cycle; 50 ml settled in water) was added and the mixture was stirred for 1 hr at room temperature. The resin was removed by filtration and washed with two 100-ml portions of 2% acetic acid. The combined filtrate and washings were evaporated *in vacuo* and the residue was lyophilized. This material was dissolved in water (500 ml) and the solution was added to a CMC column (2 × 50 cm) which was eluted with the following ammonium acetate solutions: 0.005 *M* (500 ml), 0.01 *M* (800 ml), and 0.025 *M* (1000 ml). The desired peptide was located in the 0.025 *M* eluates by the Lowry reaction.[20] These eluates were combined and the solvent was removed *in vacuo*. The

(20) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

residue was lyophilized to constant weight from small volumes of water: 90 mg; $[\alpha]^{26}D$ −48.0° (*c* 0.88, 10% acetic acid); R_f[3] 0.20 × His; single ninhydrin, Pauly, and chlorine positive spot; amino acid ratios in acid hydrolysate $Lys_{2.09}Glu_{3.03}Thr_{0.97}Ala_{3.02}Phe_{1.02}Arg_{1.02}His_{1.01}Met^{19}{}_{0.89}Asp_{1.00}Ser_{0.96}$; amino acid ratios in AP-M digest $Lys_{1.98}Glu_{2.19}Thr_{1.00}Ala_{2.89}Phe_{0.94}Arg_{0.97}Gln_{1.03}His_{0.99}Met_{1.00}Asp_{1.05}Ser_{0.98}$ (peptide content 90% determined with internal standard).

Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine Hydrate (IV). The pentadecapeptide *d*-sulfoxide (VI, 20 mg) was reduced with thioglycolic acid in the manner described for the preparation of III; colorless, fluffy powder; 20 mg; $[\alpha]^{27}D$ −48.0° (*c* 0.34, 10% acetic acid); R_f[3] 0.35 × His; amino acid ratios in AP-M digest $Lys_{2.03}Glu_{2.15}Thr_{1.05}Ala_{3.06}Phe_{1.01}Arg_{0.95}$-Gln + $Ser_{1.84}His_{1.01}Met_{1.00}Asp_{0.93}$ (no sulfoxide was present); peptide content of hydrated sample 82% as determined by internal standard.

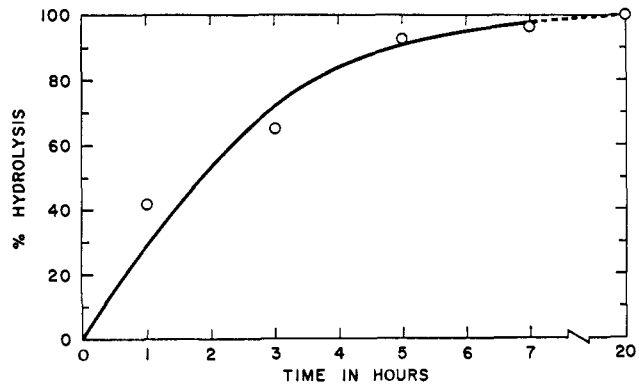

Figure 1. Rate of hydrolysis of aspartylserine (1.04 μmoles) by AP-M (975 milliunits). See text for experimental conditions.

Results and Discussion

In the course of these studies the stereochemical homogeneity of peptides was confirmed by evaluating the amino acid composition of AP-M digests. In some instances AP-M digests of peptides containing the sequence aspartylserine showed less than the theoretical amount of aspartic acid and serine on amino acid analysis, although their total acid hydrolysates contained all of the amino acids in the proper ratios. Since these findings could be due to racemization or α,β rearrangement of the aspartic acid residue, α-aspartylserine was prepared by the procedures employed for synthesis of the more complex peptides, and its behavior toward AP-M was carefully investigated. Under our assay conditions, a 5-hr digestion period was sufficient to bring about complete hydrolysis of such peptides as histidylmethionylaspartic acid *d*-sulfoxide (XI), phenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid *d*-sulfoxide (XII), and lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid (III). However, 5-hr digests of phenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine *d*-sulfoxide (XVI) afforded low recovery of both aspartic acid and serine and showed the presence of a new peak at 194-ml effluent on the amino acid analyzer. The compound corresponding to this peak was identified as aspartylserine. Subsequent studies on the rate of hydrolysis of this dipeptide (Figure 1) showed it to be slowly but completely hydrolyzed. Peptide XVI also underwent complete hydrolysis after 20 hr of incubation.

In addition to the resistance of aspartylserine to AP-M a second difficulty was encountered when digesting peptides containing this sequence. Frequently amino acid analyses of 20-hr digests of such peptides showed theoretical amounts of all the expected amino acids except aspartic acid which was absent. We have traced this observation to growth of a *Pseudomonas* species[21] during the 20-hr digestion time. This organism appears to have the ability to deaminate aspartic acid selectively with liberation of proportional quantities of ammonia. Toluene, employed as a bacteriostatic agent, has not proven effective in all instances in suppressing growth.

(21) We wish to express our appreciation to Dr. A. Braude of the Department of Medicine for identification of this organism.

The advent of partially synthetic ribonucleases,[3,4] *i.e.*, enzymes which result from combination of synthetic peptides with S-protein, prompted adoption of a system of nomenclature allowing their simple, unambiguous description. This is particularly important since an almost unlimited number of modifidations of S-peptide can be produced synthetically. The following terminology will be used in this and future publications on the subject. In accordance with current nomenclature,[6] partially synthetic enzymes which are produced by combining a fragment or analog of S-peptide with S-protein are designated as ribonucleases S′. Amino acid substitutions are treated according to convention. For example, an RNAase S′ in which the glutamine moiety in position 12 is replaced by glutamic acid will become 12-Glu-RNAase S′. Missing portions of the primary structure are indicated by placing the prefix de- in front of the position numbers which characterize the missing section or sections. In accordance with these suggestions the partially synthetic ribonucleases resulting from combination of lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid (III) and lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserine (IV) with S-protein, which are the subject of this communication, are simply de(16–20)-RNAase S′ and de(15–20)-RNAase S′. Recently, Klee[22] obtained a modified ribonuclease A by reacting the enzyme with elastase. This modified enzyme (ribonuclease E) which lacks the alanine residue in position 20 but is otherwise identical with RNAase S′ thus becomes de(20)-RNAase S′.

In order to assess the S-protein activating ability of synthetic peptides, particularly highly active ones, it becomes essential to determine accurately the peptide concentration. Simple weight measurements are not sufficient for this purpose as the synthetic materials are frequently obtained in the form of hydrates or in association with their counterions. In this study the average recovery of amino acids from AP-M digests determined quantitatively on the amino acid analyzer was employed. The internal standards norleucine and α-amino-β-guanidopropionic acid were incorporated into the digest to correct for losses incurred during technical manipulations. Quantitative

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

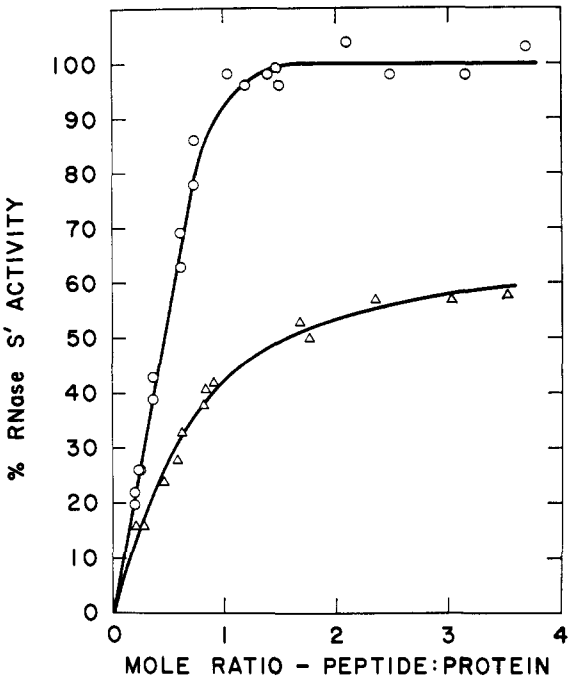

Figure 2. Ability of de(16–20)-RNAase S′ (O) and its *d*-sulfoxide (△) to hydrolyze yeast RNA.

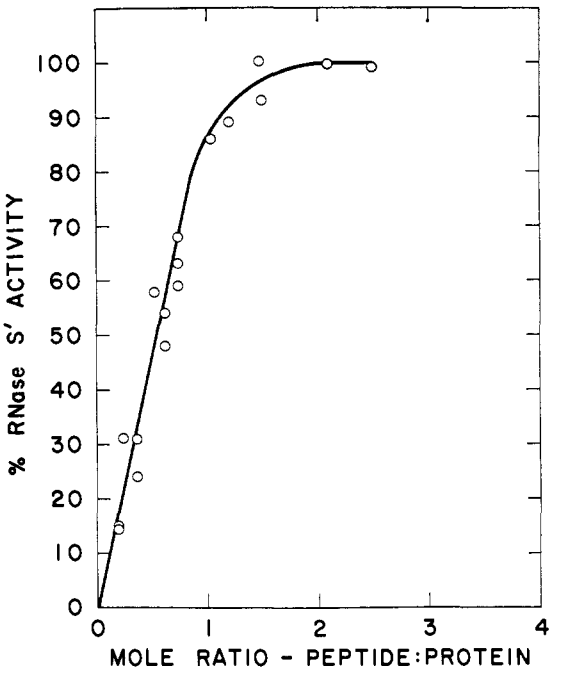

Figure 3. Ability of de(16–20)-RNAase S′ to hydrolyze cytidine 2′,3′-phosphate.

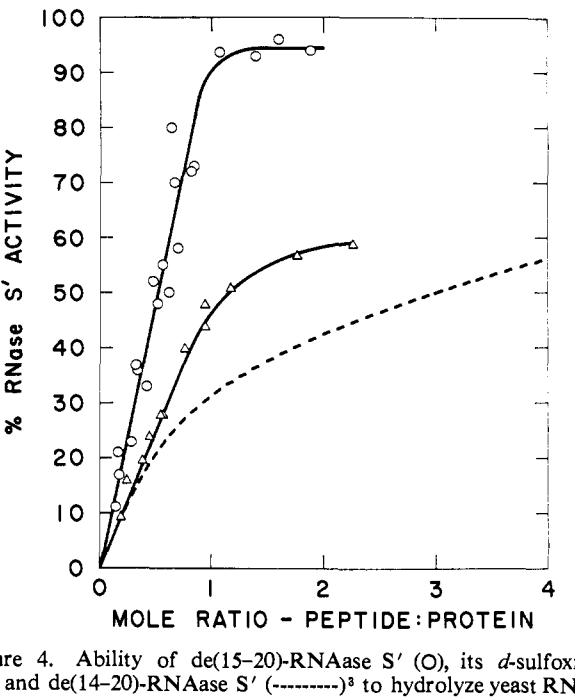

Figure 4. Ability of de(15–20)-RNAase S′ (O), its *d*-sulfoxide (△), and de(14–20)-RNAase S′ (---------)[3] to hydrolyze yeast RNA.

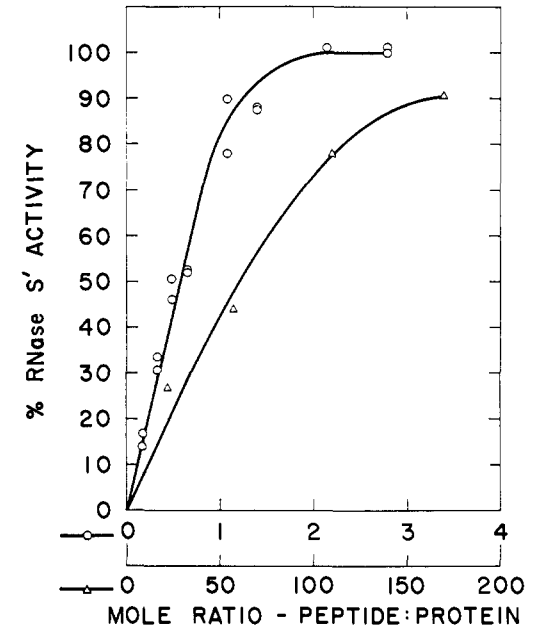

Figure 5. Ability of de(15–20)-RNAase S′ (O) and its *d*-sulfoxide (△) to hydrolyze cytidine 2′,3′-phosphate.

analyses of AP-M digests also served to determine the methionine sulfoxide content of the peptides.

We have presented evidence[3] to support the hypothesis that certain amino acid residues in the S-peptide chain act as "binding sites," *i.e.*, sites which align the peptide properly on the S-protein molecule. In order to explore contributions made by individual amino acid residues located in the N-terminal portion of S-peptide (I) to the activation of S-protein we have previously investigated a series of partially synthetic RNAases S′, namely the de(14–20), de(1)(14–20), de(1–2)-(14–20), and de(1–3)(14–20) enzymes.[3] The results pointed to Glu-2 as an important "binding site."

In this communication we identify a "binding site" in the C-terminal region of S-peptide. Combination of S-protein with the pentadecapeptide IV affords an enzyme de(16–20)-RNAase S′; the 50% activation ratio of this peptide is identical with that of S-peptide both as concerns RNA and cytidine 2′,3′-cyclic phosphate (Figures 2 and 3). Indeed within the limits of the errors of assay de(16–20)-RNAase S′ exhibits activation characteristics identical with those of RNAase S′.[6] De(15–20)-RNAase S′ which lacks six amino acid residues of S-peptide including serine 15 shows identical behavior (Figures 4 and 5). The significant

increase in S-protein activating ability which is brought about by addition of the aspartic acid moiety to the C terminus of peptide II (Figure 4) implicates this aspartic acid as another important "binding site." Clearly it is aspartic acid and not aspartylserine, as has been suggested,[23] which is responsible for the enhancement of activity.

Vithayathil and Richards[24] esterified S-peptide with methanolic hydrogen chloride and found that the ensuing tetramethyl ester retained the ability to activate S-protein. At a molar peptide ester–protein ratio of 4:1 only 28% of the activity of RNAase S′ was achieved. A competitive inhibition experiment gave the authors "a clear indication of stronger binding between S-protein and S-peptide than between S-protein and tetramethyl S-peptide." However, based on their inability to separate S-protein from tetramethyl S-peptide on paper electrophoresis at pH 6.0 and other evidence, they concluded that "direct charge–charge interaction plays only a minor role in the binding of the peptide and protein components." Evidence presented in this and a previous communication[3] suggests that at least two of the four carboxyl groups of S-peptide are significantly involved in binding.

As has been observed previously, replacement of methionine by its *d*-sulfoxide in peptides I and II[3] or oxidation of natural S-peptide to the *dl*-sulfoxide[3] or the sulfone[25] markedly lowers S-protein activating potency. The same behavior is characteristic of the *d*-sulfoxides V and VI of peptides III and IV (Figures 2 and 4). It has been reported that the *dl*-sulfoxide[3] and sulfone[25] of natural S-peptide bring about practically full activation of S-protein at a molar ratio of 3:1. However, more extensive investigations have demonstrated that all these oxidized forms exhibit activation characteristics identical with those of peptides V and VI (Figures 2 and 4). With RNA, the 50% activation ratios are between 1 and 2.

More striking is the difference between the oxidized and reduced forms of de(15–20)-RNAase S′ with cytidine 2′,3′-phosphate as substrate. At a peptide to protein ratio of 2:1 where the reduced form exerts maximal activity the sulfoxide brings about negligible activation (Figure 5). De(16–20)-RNAase S′ exhibits a similar behavior.

It appears to be more than a coincidence that oxidation of the methionine in the melanocyte-expanding hormones, the corticotropins, and the parathyroid hormones likewise is accompanied by a marked decrease in biological activity.[26] Although the reason for the behavior of these methionine-containing peptides cannot be defined at this time, a similar fundamental principle appears to be involved.

Riehm and Scheraga[27] found that five of the ten free carboxyl groups of RNAase A failed to react with N-cyclohexyl-N′-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate at pH 4.5. The carboxyls of glutamic acid 2 and aspartic acid 14 are among the nonreactive groups. Our findings in this and a preceding study[3] have implicated these same carboxyls as important "binding sites" in S-peptide. The results of both approaches suggest that these polar groups are not located on the surface of the enzyme but rather that they are directed toward the interior of the molecule.

Acknowledgment. The authors wish to express their appreciation to Mrs. Elaine Gleeson and Mr. Albert R. Frazier for skillful technical assistance.

(23) (a) J. T. Potts, Jr., D. M. Young, and C. B. Anfinsen, *J. Biol. Chem.* **238**, 2593 (1963); (b) J. T. Potts, Jr., D. M. Young, C. B. Anfinsen, and A. Sandoval, *ibid.*, **239**, 3781 (1964).

(24) P. J. Vithayathil and F. M. Richards, *ibid.*, **236**, 1380 (1961).

(25) P. J. Vithayathil and F. M. Richards, *ibid.*, **235**, 2343 (1960).

(26) For a review see K. Hofmann, *Ann. Rev. Biochem.*, **31**, 213 (1962).

(27) J. P. Riehm and H. A. Scheraga, *Biochemistry*, **5**, 99 (1966).