Communications to the Editor

The Total Synthesis of an Enzyme with Ribonuclease A Activity[1]

Sir:

We wish to report our preliminary experiments on the chemical synthesis of a protein with high and specific ribonuclease activity. A protected linear polypeptide of 124 amino acid residues with the sequence of bovine pancreatic ribonuclease A (RNase A)[2] was synthesized by the solid-phase method.[3] After the peptide was removed from the resin support it was purified, and the disulfide bonds were formed by air oxidation to produce the synthetic enzyme. The product was active toward ribonucleic acid and 2′,3′-cyclic cytidine phosphate and was completely inactive toward deoxyribonucleic acid, 2′,3′-cyclic guanosine phosphate, and certain purine-containing dinucleotides, demonstrating its selective requirement for typical RNase A substrates. The conclusion that a protein with the composition and structure of ribonuclease A has been synthesized is further supported by the close agreement between the synthetic protein and natural RNase A with respect to the Michaelis constant, amino acid analyses, electrophoretic and chromatographic behavior, and peptide maps of tryptic digests.

The synthesis was carried out in a stepwise manner starting from the C terminus of RNase A with 0.42 mmol of *t*-butyloxycarbonyl-L-valine esterified to 2 g of 1% cross-linked polystyrene resin support. The general procedures[3-6] of the automated solid-phase method were followed. The following N^{α}-*t*-butyloxycarbonyl (Boc)[7] derivatives were used: Asp(β-OBzl), Glu(γ-OBzl), Cys(Bzl), Ser(Bzl), Thr(Bzl), Tyr(Bzl), Lys(Z), Arg(NO_2), Met(O). Histidine was unprotected. Coupling was usually mediated by N,N′-dicyclohexylcarbodiimide (DCCI) (threefold excess, 5 hr). Boc groups were removed with 50% (v/v) trifluoroacetic acid (TFA) in methylene chloride. Amino acid analysis of the fully protected 124-residue peptide–resin indicated a yield of 17% based on the amount of valine originally esterified to the resin, which means that an average of about 1.4% of the peptide chain was lost from the resin at each cycle of the synthesis.

Cleavage of the polypeptide from its solid support together with removal of all protecting groups was achieved in a single step by treatment with anhydrous HF[8] in the presence of anisole and TFA for 90 min at 0–15°. The yield of this cleavage step was 41%. The product was converted to the S-sulfonate [RNase(SSO_3^-)$_8$] with Na_2SO_3 and $Na_2S_4O_6$ in 8 *M* urea at pH 7.5.[9] Paper electrophoresis at pH 2.25 showed a major Pauly-positive spot, which moved toward the cathode with the same mobility (R_{His} 0.28) as that of the S-sulfonate of natural RNase, and two minor components. The synthetic RNase(SSO_3^-)$_8$ was purified first on Dowex 1-X2 in 2.4 *M* formic acid–4 *M* urea and then by gel filtration on Sephadex G-50 in 5 *M* urea–0.1 *M* NH_4OAc, pH 5.2. The faster moving Sephadex fraction eluted at the same volume as natural RNase(SSO_3^-)$_8$ and was homogeneous in the paper electrophoresis system; yield of purified RNase(SSO_3^-)$_8$, 216 mg; amino acid analyses are given in Table I.

Table I. Amino Acid Analyses[a]

Amino acid	Natural RNase Lit.[2]	Natural RNase Found	Synthetic RNase-(SSO_3^-)$_8$	Synthetic RNase	Natural RNase HF–TFA treated
Lys	10	10.0	10.3	9.1	9.2
His	4	3.8	3.3	3.3	3.5
Arg	4	4.1	4.6	4.2	3.9
Asp	15	15.7	15.9	16.0	16.2
Thr	10	9.8	9.4	10.5	10.4
Ser	15	14.0	15.3	15.5	15.5
Glu	12	12.1	12.4	12.1	12.8
Pro	4	3.8	3.9	3.9	3.6
Gly	3	3.1	3.1	3.3	3.4
Ala	12	12.0	12.0	12.0	12.0
Cys	8	7.8	6.7	7.2	7.4
Val	9	8.6	9.4	8.9	8.8
Met	4	4.1	3.9	3.8	3.4
Ile	3	2.3	2.1	2.1[b]	2.2
Leu	2	2.2	2.9	2.6	2.3
Tyr	6	5.6	5.1	5.2	5.9
Phe	3	3.3	3.2	3.5	3.2

[a] Samples were hydrolyzed in 6 *N* HCl in sealed, evacuated tubes, 24 hr, 110°, and analyzed on a Spinco 120B amino acid analyzer. Cystine was determined on performic acid oxidized samples. Values are expressed as moles per mole of RNase with alanine set at 12.0. All values are uncorrected. [b] A 48-hr hydrolysis time increased the value of isoleucine to 2.87.

The RNase(SSO_3^-)$_8$ was reduced to RNase(SH)$_8$ with mercaptoethanol in 8 *M* urea, pH 8.5, 20 hr. The mixture was passed through Sephadex G-25 in 0.1 *M* HOAc to remove urea and excess mercaptoethanol. The protein-containing fraction was diluted to 0.02 mg/ml in 0.1 *M* Tris, pH 8.3, and oxidized in air at 25° for 20 hr to form the four disulfide bridges of RNase.[10]

(1) Supported by Grant A-1260 from the U. S. Public Health Service.

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The crude product was acidified, lyophilized, and desalted on Sephadex G-25 in 0.1 *M* acetic acid. It was then fractionated on IRC-50 in 0.2 *M* sodium phosphate buffer, pH 6.47.[11] Five fractions were detected at 280 mμ. The largest one, fraction I, eluted at the same volume as reduced and reoxidized natural RNase. It was desalted on Sephadex G-25 in 0.1 *M* acetic acid and was lyophilized (yield, 85 mg). This synthetic ribonuclease was indistinguishable from the native enzyme by paper electrophoresis at pH 2.5 (R_{His} 0.58). Peptide maps from tryptic digests[12] of performic acid oxidized samples showed the 14 expected[12] ninhydrin-positive spots in the same relative positions as the natural ribonuclease control. There was one small additional spot in the synthetic preparation near the position of free lysine. Amino acid analyses of acid hydrolysates compared well with those of natural RNase which had been treated with HF and TFA (Table I). Enzymic digestion (papain followed by aminopeptidase M)[13] was complete. It also showed that 79% of the methionine sulfoxide residues had been converted to methionine during the mercaptoethanol reduction of the RNase$(SSO_3^-)_8$. The remainder was recovered as methionine sulfone.

The synthetic enzyme (fraction I) showed a specific activity of 13% by two methods[14,15] with yeast RNA as substrate, and 24% with 2′,3′-cyclic cytidine phosphate[16] as substrate when compared with pure natural bovine pancreatic ribonuclease A. Fraction II contained some activity, but the other fractions were inactive. The synthetic enzyme was completely inert toward DNA, 2′,3′-cyclic guanosine phosphate, or 5′-(3′-guanylyl)cytidylic acid (GpCp)[17] under conditions where DNase[18] or RNase T_1[19] were active and also was without effect on 5′-(3′-adenylyl)adenylic acid (ApAp), demonstrating the high substrate specificity to be expected of RNase A. A further indication that the synthetic material contained the same active enzyme species as natural RNase was obtained from the Michaelis constant. Initial velocities were measured spectrophotometrically[14] and the K_m values, calculated from Eadie plots, were found to be 2.4 mg/ml for natural RNase and 2.5 mg/ml for the synthetic product. These results provide direct evidence for the hypothesis[10a,20] that the linear amino acid sequence of a protein contains all the information necessary to direct the formation of an active enzyme.

Although the physical and chemical methods so far applied to the synthetic protein indicate a good degree of homogeneity, the presence of very closely related molecules cannot be excluded, and the failure to obtain a fully active enzyme means that our product is not yet pure. Assembly of the 124 amino acid residues into the protected, resin-bound straight-chain precursor of RNase required 369 chemical reactions and 11,931 steps of the automated peptide synthesis machine without any intermediate isolation steps. Deficiencies in these peptide-forming reactions, in the work-up conditions, and in the final oxidation and refolding of the protein probably all contributed to the decreased activity, but an assessment of the relative effects of each step much await the completion of further work.

These experiments demonstrate for the first time that a protein molecule with true enzymic activity toward its natural substrate can be totally synthesized from the component amino acids.

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Received November 25, 1968

Studies on the Total Synthesis of an Enzyme. I. Objective and Strategy

Sir:

The remarkable advances in polypeptide synthesis during the past 15 years have made the synthesis of an enzyme a feasible objective. From a number of considerations the synthesis of RNase S′ appeared to be an attractive goal.

The studies of Richards[1] and his collaborators have shown that RNase A may be cleaved at a single bond without impairment of enzymatic activity to produce RNase S, which may be separated into a tetrahectapeptide (S-protein) and an eicosapeptide (S-peptide). Recombination of these two enzymatically inactive fragments in equimolar ratio restored full enzymatic activity (RNase S′).

Because the work of Smyth, Stein, and Moore[2] had established the complete primary structure of RNase A, the amino acid sequence of S-protein is thus known. Furthermore, since S-peptide had been synthesized by Hofmann, Smithers, and Finn,[3] S-protein, a tetrahectapeptide, appeared to be the smallest protein whose synthesis would constitute a total synthesis of an enzyme. Finally the observation by Haber and Anfinsen[4] that the oxidation of reduced S-protein (eight cysteine residues) regenerates enzymatic activity when the resulting protein is assayed in the presence of S-peptide allowed these workers to infer that "information determining secondary and tertiary structure of RNase" is contained in the amino acid sequence of S-protein.

The molecular weight of S-protein and especially the

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