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THE SYNTHESIS OF PROTECTED PEPTIDE FRAGMENTS OF A STAPHYLOCOCCAL NUCLEASE

BY CHRISTIAN B. ANFINSEN, DAVID ONTJES, MOTONORI OHNO, LILA CORLEY, AND ANN EASTLAKE

LABORATORY OF CHEMICAL BIOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, BETHESDA, MARYLAND

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We have recently determined the total amino acid sequence of an extracellular nuclease of *Staphylococcus aureus* consisting of a single chain of 149 residues devoid of half-cystine[1, 2] (see Fig. 1). This globular protein contains some regions of helical structure,[3] can undergo completely reversible unfolding in a variety of denaturing solvents,[4] and readily yields crystals suitable for X-ray analysis.[5] Because of these features, organic synthesis of the polypeptide chain of the nuclease would be of particular value in the direct study of catalytic and binding properties, and of factors determining tertiary structure.

The proposed plan for synthesis involves an assembly of the chain through successive couplings of protected peptide fragments to the free α-amino group of a protected C-terminal sequence. Each peptide fragment must be fully protected except for a free terminal carboxyl group. Short of total stepwise assembly of the nuclease chain in one operation, these considerations preclude the use of the solid phase method[6] of Merrifield, as usually employed, since in this method most blocking groups are lost upon detachment of the completed peptide from the supporting resin. At the present time, the uninterrupted solid phase synthesis of a chain of

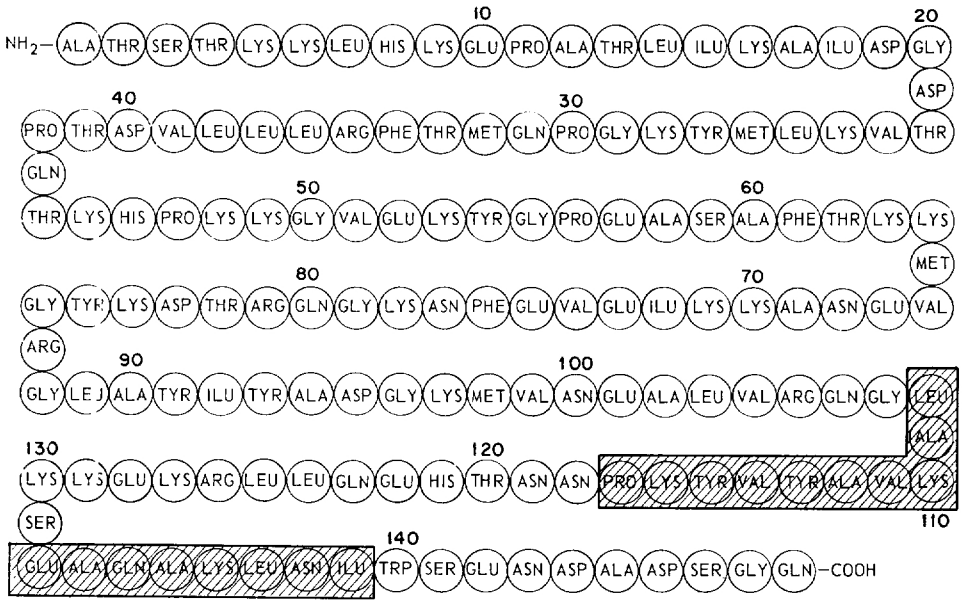

FIG. 1.—The amino acid sequence of staphylococcal nuclease.[2] The two protected peptides, whose syntheses are described in the present communication, are indicated by cross-hatching.

149 residues seems likely to yield an unacceptably heterogeneous product offering a formidable task of purification.

The present communication reports the synthesis of two peptides to illustrate the general approach we have used in the synthesis of a number of protected fragments of the nuclease sequence. The desired peptide, except for its NH_2-terminal residue, is made by solid phase synthesis.[6] The ϵ-amino groups of lysine residues have been blocked by the trifluoroacetyl (TFA) group. This protecting group is not removed by HBr in anhydrous trifluoroacetic acid or by anhydrous HF,[7] the reagents used to cleave the peptide from its resin support in the solid phase method.[6, 8] Carboxyl groups in the final peptide fragment, other than the α-carboxyl of the COOH-terminal residue, must also be blocked since branching of the polypeptide chain would otherwise occur at each β- or γ-carboxyl during the subsequent coupling of protected fragments of the growing COOH-terminal portion of the chain. Peptides to be synthesized may therefore be selected so that they terminate in t-Boc-ω-benzyl-glutamic or aspartic acids. A similar synthetic strategy has been employed by Marshall and Merrifield in the synthesis of two derivatives of angiotensin.[6]

Following cleavage from the resin the peptide is coupled to the N-hydroxysuccinimide (OSu)[13] ester of the appropriate t-butyloxycarbonyl (t-Boc) amino acid to give a blocked peptide product. For peptides terminating in a dicarboxylic amino acid, t-Boc-β-benzyl-aspartic acid-OSu and t-Boc-γ-benzyl-glutamic acid-OSu have been used.

$$\text{Boc amino acyl-resin} \xrightarrow[\text{solid phase coupling)}]{\text{(repeated}} \text{Boc-peptidyl resin}$$

$$\xrightarrow[\text{TFA}]{\text{HBr}} \text{Peptide hydrobromide} \xrightarrow[\text{(soluble coupling)}]{\text{Boc-amino acid-OSu + Base}} \text{Boc-amino acyl-peptide}$$

Maerials and Methods.– Monomers; t-Boc-L-amino acids were obtained from Cyclo Chemical Corp. for Fox Chemical Co. T-Boc-amino acid-N-hydroxysuccinimide (OSu) and p-nitrophenyl (ONP) esters, as well as chloromethylated-copolystyrene divinylbenzene resin, were obtained from Cyclo. All monomers were examined by thin-layer chromatography, and in most cases by measurements of optical rotation and melting point. The N-OH-succinimide esters were often contaminated with minor amounts of the parent t-Boc-amino acids. Since a threefold excess of the N-hydroxysuccinimide esters was used in coupling reactions, recrystallization of these compounds was omitted unless contamination became severe. T-Boc-asparagine-ONP and t-Boc-glutamine-ONP were recrystallized from ethanol to homogeneity on thin-layer chromatography (Silica gel H, Brinkmann Inst. Co.) in solvent systems I, II, and III (BuOH:AcOH:pyridine: H_2O, 4:2:1:1; $CHCl_3$:AcOH, 95:5; MeOH:EtAC, 1:2). The $[\alpha]_D^{25}$ of t-Boc-asparagine-ONP, after 2 recrystallizations was $-39.2°$ (c = 1, DMF), a value slightly lower than that reported by Schröder and Klieger.[9]

T-Boc-ϵ-TFA-lysine was synthesized as follows: 10mM (2.42 gm) ϵ-TFA-L lysine[10] ($[\alpha]_{580}^{25°}$ mμ; C, 3 in dichloroacetic acid; $+21.7°$), 14 mM BOC-N_3 (2.0 gm), 35 mM $NaHCO_3$ (3.1 gm), 30 ml water, and 20 ml dioxane were added together and stirred vigorously at 40° for 2.5 days. The reaction mixture was evaporated *in vacuo* to remove dioxane and then extracted with ether to remove unchanged BOC-N_3 and residual dioxane. The aqueous solution was acidified with a slight excess of citric acid and extracted 2 times with 40-ml portions of ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and evaporated nearly to dryness *in vacuo*. The oily residue was crystallized by adding petroleum ether. Yield, 1.70 gm (5.2 mM) 52%, melting point 101–103°. Recrystallization from CH_2Cl_2 yielded material which melted sharply at 103°. Calc. for $C_{13}H_{21}O_5N_2F_3$: C, 45.6; H, 6.1; N, 8.2; F, 16.7. Found: C, 45.4; H, 6.3; N, 8.0; F, 16.5.

Solvents: Dimethylformamide (DMF), Spectro Grade (Eastman), was purified by stirring

with t-Boc-glycine-OSu, 1 mM/100 ml, for 3 hr at room temperature, followed by distillation from CaO at 15 mm, 40°C. Only the middle fraction was retained.

Dioxane was purified by filtration through activated alumina to remove peroxides. Dioxane-HCl solution, for removal of t-Boc groups, was prepared by saturation of dioxane with HCl gas, giving an HCl concentration of approximately 4 *N*.

T-Boc-isoleucine resin and t-Boc-proline resin were prepared by mixing 10 gm of chloromethylated resin (substitution 1.20 mEq/gm), 9.8 mM t-Boc-amino acid, 9.8 mM triethylamine, and 30 ml of ethanol. The mixture was refluxed at 70°C for 55 hr and the resin then rinsed several times with ethanol and chloroform or methylene chloride.

Dried resin samples, hydrolyzed in evacuated, sealed tubes, containing 50% dioxane/50% concentrated HCl, for 20 hr at 110°C gave amino acid values of 0.36 mM/gm for t-Boc-Ile resin and 0.30 mM/gm for t-Boc-Pro resin.

Solid phase coupling procedures were based on published studies of solid phase synthesis[6] and on the generous advice furnished by Drs. R. B. Merrifield and A. Marglin of the Rockefeller Institute. All operations were carried out at room temperature in the rocking apparatus described by Merrifield, using solvent volumes of about 10 ml per gm of resin.

The t-Boc-peptidyl resin was rinsed 3 times with dioxane, shaken for 30 min with dioxane-HCl to remove the t-Boc group, and rinsed 3 times with fresh dioxane. The resin was then rinsed 3 times with $CHCl_3$, shaken for 10 min with 10% triethylamine in $CHCl_3$, and rinsed 3 times each with $CHCl_3$ and CH_2Cl_2. The desired t-Boc-amino acid was added in 3-fold molar excess in CH_2Cl_2 and, after 10 min of shaking, a 3-fold excess of dicyclohexylcarbodiimide (Aldrich) in CH_2Cl_2 was added. After 2 hr of shaking, the resin was rinsed 3 times with CH_2Cl_2 and was ready for the next cycle.

T-Boc-asparagine and glutamine were added as the p-nitrophenyl esters in 4-fold excess in DMF and were shaken 12 hr. Appropriate DMF rinses were added to the cycle, and carbodiimide reagent was omitted.

For cleavage of the completed peptide, the dried t-Boc-peptidyl resin was suspended in anhydrous trifluoroacetic acid. After passage through a trap containing 20% resorcinol in trifluoroacetic acid, HBr gas was bubbled through the resin suspension for 90 min at room temperature at a rate just sufficient to keep the particles well dispersed. The trifluoroacetic acid was filtered off, and the resin rinsed twice with trifluoroacetic acid and twice with 50% trifluoroacetic acid/CH_2Cl_2. The combined filtrates were flash-evaporated. The resulting oil was generally re-evaporated after resuspension in CH_2Cl_2, triturated 3 times with ether, and filtered to yield a white, odorless powder, the peptide-HBr salt.

Results.—Synthesis of protected peptide comprising residues 108–117: 4.0 grams of t-Boc-proline-resin (containing 1.2 mM proline) were submitted to the cycle of operations described in *Methods*, eight residues being added before cleavage of the peptide from the solid phase support with HBr-trifluoroacetic acid. The crude nonapeptide hydrobromide was combined with 1.0 mM $NaHCO_3$ and 1.0 mM t-Boc-leucine-OSu in 75 ml ethanol-H_2O, 2:1, and shaken for 16 hours at 5°. The bulk of the ethanol was removed *in vacuo* and the suspension was centrifuged and the decapeptide product washed three times with water. Results of amino acid analysis of an aliquot of this material, after drying *in vacuo*, indicated slight contamination with t-Boc-leucine-OSu. The yield of crude, protected decapeptide was 1.1 grams.

A portion of the crude product (0.6 grams) was dissolved in the contents (60 ml) of the first three tubes of a 200-tube countercurrent apparatus. The solvent system employed for the partition was $CHCl_3$:MeOH:0.03 *M* sodium citrate buffer, pH 5.5; 3.0:2.5:1.0. The pattern shown in Figure 2 was obtained after 200 transfers. The contents of tubes containing the major and minor peaks were pooled and acidified to pH 3.0 with citric acid. At this pH the protected peptide was concentrated in the organic phase. The organic solvents were removed *in vacuo*, the semi-

dry residue was washed with water, and the final product was lyophilized from water suspension. The yield of the major component was 0.38 grams.

A sample of the purified, protected peptide was treated with anhydrous trifluoroacetic acid for 30 minutes at room temperature to remove the t-Boc group. After removal of solvent *in vacuo,* exposure to 1 *M* piperidine for 2 hours at 0°[11] effected removal of the TFA groups. The resulting solution was taken to dryness and the residue washed with ether to remove residual piperidine. An aliquot was digested with leucine aminopeptidase[2] and an identical aliquot hydrolyzed with constant boiling HCl for 22 hours in an evacuated, sealed tube at 110°. The results of amino acid analyses of the two hydrolysates are summarized in Table 1. The data show that no racemization had occurred during the solid phase synthesis or during addition of the NH_2-terminal leucine residue. The COOH-terminal Lys-Pro bond was, of course, not digested during the leucine amino peptidase treatment.

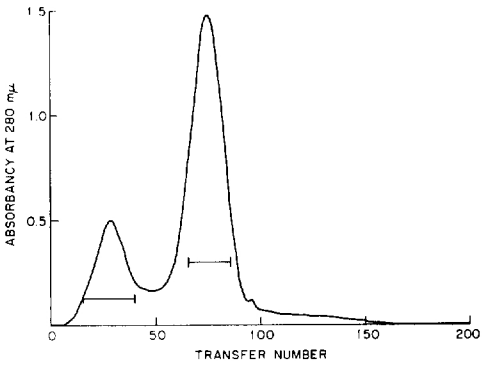

FIG. 2.—Countercurrent distribution pattern, after 200 transfers, of the protected peptide comprising residues 108–117 of the sequence of staphylococcal nuclease. Details are given in the text.

Synthesis of protected peptide comprising residues 132–139: The partially blocked peptide Ala-Gln-Ala-(ε-TFA)-Lys-Leu-Asn-Ilu (residues 133–139) was synthesized by submitting 5 gm of t-Boc-Ilu resin to the solid phase cycle as described, to yield 1.6 gm of crude product. Amino acid analysis of this product is shown in Table 2.

Approximately 1.3 mM (1.2 gm) of this heptapeptide-HBr salt was dissolved in 20 ml of DMF, and 2.6 mM of redistilled N-methyl-morpholine was added, causing the clear solution to become opaque and more viscous. After stirring for ½ hour at room temperature, 2.6 mM of recrystallized t-Boc-γ benzyl-Glu-OSu in 10 ml DMF was added and the mixture stirred for 16 hours. At the end of this time the solution had become clear and nonviscous. The DMF was then evaporated *in vacuo* at 30°C. The oily residue was triturated 3 times with ether, filtered, washed with ether, and redissolved in methanol. After evaporation to an oil, the peptide was precipitated by addition of excess ether, filtered, and dried, yielding 0.8 gm of white powder (approximately 0.7 mM).

Thin-layer chromatography of the protected octapeptide showed homogeneity in

TABLE 1

ANALYSIS OF PEPTIDE, RESIDUES 108–117

	Amino Acid Analyses (Molar ratios)									
	t-Boc-Leu-	Ala	TFA Lys	Val	Ala	Tyr	Val	Tyr	TFA Lys	Pro-OH
Acid hydrolysis of crude nonapeptide		1.12	1.04	0.98	1.12	0.94	0.98	0.94	1.04	*1.00*
Acid hydrolysis*	1.07	1.05	*1.00*	0.98	1.05	0.92	0.98	0.92	1.00	1.13
LAP after TFA, piperidine*	1.07	0.96	1.06	1.06	0.96	*1.00*	1.06	1.00	—	—

* Analyses of major countercurrent component. This component gave the following results on elemental analysis: *calc.* C, 54.99; H, 6.59; N, 11.65; F, 7.91; *found* C, 54.20; H, 6.23; N, 11.16; F, 7.81. The minor component shown in Fig. 2 gave a very low value for fluorine and was not studied further.

TABLE 2

ANALYSIS OF PEPTIDE, RESIDUES 132–139

	Amino Acid Analyses (Molar ratios)							
	OBZ t-Boc-Glu	Ala	Gln	Ala	ε-TFA Lys	Leu	Asn	Ilu - OH
Acid hydrolysis Residue 133–139 peptide	—	0.98	0.95*	0.98	0.87	0.96	1.00*	*1.00*
Acid hydrolysis Residue 132–139 peptide	1.00*	1.04	1.00*	1.04	0.96	0.99	1.04*	*1.00*
Aminopeptidase digestion after HF and piperidine deprotection	0.83	0.89	1.00†	0.89	0.94	1.05	1.00†	*1.00*

Elemental Analysis: *calc.* C, 53.27; H, 6.88; N, 13.15; F, 4.86; *found* C, 53.36; H, 7.01; N, 13.09; F, 4.79.
* As the dicarboxylic amino acids.
† Digestion was performed by incubating 10 mg of peptide and 0.05 mg aminopeptidase-*M* (Henley and Co., N. Y., N. Y.) in 0.1 *M* phosphate buffer at pH 7–8 for 16 hr. Incubation of equimolar amounts of the substituent free amino acids under identical conditions showed a loss of 32% of glutamine and asparagine as determined by amino acid analysis using color constants as follows: glutamine 16.5, asparagine 16.3, leucine 23.0. Glutamine and asparagine values for the peptide have been adjusted accordingly, and are therefore likely to be less accurate than values for other amino acids.

TABLE 3

STABILITY OF NUCLEASE

Nuclease derivative	Reagent	Purpose	Specific activity
Native nuclease	Anhydrous HF[7, 8, 15] 0°, 60 min	Removal of benzyl esters and ethers, ε-CBZ, -NO_2, and t-Boc	80%
Fully trifluoroacetylated nuclease[11]	1 *M* Piperidine 0°, 1–2 hr	Removal of α- and ε-TFA	75–100%
Native nuclease	Anhydrous CF_3COOH, room temp., 2 hr	Removal of t-Boc during successive coupling of protected peptides	100%

Following each of the treatments listed above, the reagent was removed *in vacuo* and a solution of the residual protein in water was lyophilized. Specific activities were determined by spectrophotometric assays[14] and estimations of protein concentration, the latter by measurements of absorbancy at 280 mμ.

system I (R_f = 0.85) and in 80% aqueous pyridine (R_f = 0.65). A 20-mg sample of the protected peptide was treated with 2 ml of anhydrous HF and 0.1 ml of anisole (Eastman) for 1 hr at 0°C, to remove the benzyl ester and t-Boc blocking groups. HF was then removed with a stream of nitrogen gas at room temperature and the peptide rinsed with ether and dried *in vacuo*. The ε-TFA blocking group was subsequently removed by dissolving the peptide in 1 ml of 1 *M* aqueous piperidine for 1 hr at room temperature. Following evaporation to dryness *in vacuo* the fully deprotected peptide was rinsed with ether. Descending paper chromatography (Whatman 1) in solvent system I and in 80% aqueous pyridine showed the deprotected product to be homogeneous, with R_f values of 0.35 and 0.40, respectively. Results of amino acid analysis of an acid hydrolysate of the protected peptide, and of an aminopeptidase-M digest of the deprotected peptide are shown in Table 2.

Discussion.—In using the combination of solid phase and soluble coupling methods described above, we have found that nearly all protected peptide products require purification by countercurrent distribution before they become adequate intermediates for further synthesis. For example, in the solid phase syntheses of heptalysine and tetralysine, the desired products constituted 82% and 93% of the total yields, respectively, while shorter lysine peptides comprised the remainder.[12] Therefore, the average coupling efficiency for each step may be calculated to be

approximately 98%. Soluble coupling of the NH_2-terminal amino acid has generally been less efficient.

In the case of the two peptides reported here, and with others similarly examined, close agreement has been observed between amino acid analyses performed after acid hydrolysis and enzymic digestion. While trace quantities of D-amino acids cannot be ruled out, it appears that neither the carbodiimide nor the N-hydroxysuccinimide ester coupling procedures cause significant racemization. This agrees with the experiences of others.[6, 13]

The successful organic synthesis of the entire polypeptide chain or a protein must eventually depend upon the stability properties of the native protein itself, since the final deprotection of functional groups requires exposure to several potentially destructive reagents. In our studies on staphylococcal nuclease, blocking groups have been chosen so that the conditions required for their removal do not irreversibly damage the enzyme. Table 3 summarizes experiments demonstrating the relative stability of nuclease to the reagents required.

We wish to thank Dr. A. B. Robinson for his help in the initial experiments on the stability of nuclease to HF.

[1] Taniuchi, H., and C. B. Anfinsen, *J. Biol. Chem.*, **241**, 4366 (1966).
[2] Taniuchi, H., C. B. Anfinsen, and A. Sodja, *J. Biol. Chem.*, in press.
[3] Heins, J. N., J. R. Suriano, H. Taniuchi, and C. B. Anfinsen, *J. Biol. Chem.*, **242**, 1016 (1967).
[4] Cuatrecasas, P., S. Fuchs, and C. B. Anfinsen, unpublished data.
[5] Cotton, F. A., E. E. Hazen, and D. C. Richardson, *J. Biol. Chem.*, **241**, 4389 (1966).
[6] Merrifield, R. B., *Science*, **150**, 178 (1965); Marshall, G. R., and R. B. Merrifield, *Biochemistry*, **4**, 2394 (1965).
[7] Sakabibara, S., and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
[8] Lenard, J., and A. B. Robinson, *J. Am. Chem. Soc.*, **89**:1, 180 (1967).
[9] Schröder, E., and E. Klieger, *Am. Chem. Liebigs*, **673**, 208 (1964).
[10] Schallenberg, E. E., and M. Calvin, *J. Am. Chem. Soc.*, **77**, 2779 (1955).
[11] Goldberger, R. F., and C. B. Anfinsen, *Biochem.*, **1**, 401 (1962).
[12] Anfinsen, C. B., and H. A. Sober (unpublished results).
[13] Cuatrecasas, P., S. Fuchs, and C. B. Anfinsen, *J. Biol. Chem.*, **242**, 1541 (1967).
[14] Anderson, G. W., J. E. Zimmerman, and F. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963).