SYNTHESIS OF AN EICOSAPEPTIDE CORRESPONDING TO THE SEQUENCE 74-93 OF THE POLYPEPTIDE CHAIN OF CYTOCHROME b_5

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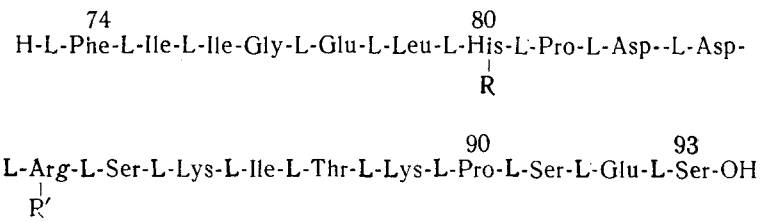
Continuing investigations [1-3] on the synthesis of histidine-containing fragments of cytochrome b_5 isolated from the microsomes of the calf thymus [4], we have synthesized the eicosapeptide (I) corresponding to the sequence 74-93 of the polypeptide chain of cytochrome b_5. It includes the histidine residue 80.



I a) R=Bzl, R′=NO_2; b) R=R′=H.

Merrifield's solid-phase method [5] with the sequence of growth of the chain from the C-end, predominantly through the p-nitrophenyl esters in the presence of 1,2,4-triazole as catalyst [6] was used for the synthesis.

In order to decrease the number of "false" peptides, considerable excesses of the amino acids and reagents were used (Table 1), and some of the amino acids were introduced into the growing peptide chain in the form of blocks – the dipeptides Boc-Ile-Gly-OH (III) and Boc-Phe-Ile-OH (IV) – by the carbodiimide method. So that the peptide-forming reaction should take place more completely, the time of condensation was increased (to 16-24 h in the p-nitrophenyl ester and the carbodiimide methods) and the unchanged amino groups were acetylated with acetic anhydride in the presence of triethylamine [5].

To eliminate the Boc protection more completely, the peptidylpolymer was treated twice with a 1 N solution of hydrogen chloride in acetic acid. The reagents were eliminated from the pores of the polymer by the action of methanol, ether, and petroleum ether on the peptidylpolymer after each stage.

The purity of the peptide chain growing on the polymer was determined after the ninth, tenth, twelfth, fourteenth, sixteenth, and eighteenth stages of the condensation by the paper electrophoresis of the products separated from the pores of the peptidylpolymer by its treatment with a 40% solution of hydrogen bromide in acetic acid for 90 min. In each case, the ether-precipitated peptide hydrobromides were subjected to electrophoresis in various buffers at various pH values. The results of electrophoresis showed that the peptides split off from the polymer contained the amino acid added last as an impurity in the main substance. This is apparently due to the ionic binding of the free amino acids by the triethylammonium groups formed in the polymer as a result of the reaction of the triethylamine with the chloromethyl groups. After the fifth stage of condensation, the amount of N-terminal amino acid was determined by the dansylation of

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TABLE 1. Molar Excesses of the Reagents in the Synthesis of the Eicosapeptide

Amino acids and peptides	Sequence	Molar excesses of		
		amino acids or peptides	carbodiimide	1,2,4-triazole
	92–93	5	—	6
	91–92	7	7	—
	83–90	10	10	12
	78–82	15	15	18
Boc-Ile-Gly-OH (II)	76–77	15	15	—
Boc-Phe-Ile-OH (III)	74–75	15	15	—

a sample of the peptidylpolymer [7]. In addition to dansylated lysine, less than 1% of dansylated serine was found.

The use of the dipeptide blocks Boc-Ile-Gly-OH (II) and Boc-Phe-Ile-OH (III) and the acetylation of the free amino groups in the last two stages of the synthesis of the eicosapeptide (I) considerably facilitated its purification after its splitting off from the polymer under the action of hydrogen bromide in trifluoroacetic acid. According to paper chromatography, the peptide split off from the polymer contained mainly a substance with R_{f1} 0.64 contaminated with a small amount of a substance with R_{f1} 0.5. After two purifications on Sephadex G-25 the freeze-dried peptide on paper chromatography (R_{f1} 0.64) and paper electrophoresis in acetate buffer, pH 2.9, showed one ninhydrin-positive spot.

The N-terminal amino acid determined by dansylation was phenylalanine; small amounts of isoleucine and serine (less than 1%) were found.

The bulk of the partially protected peptide (Ia, R = Bzl, R' = NO_2) that was isolated was reduced. The reduction of the peptide (Ia) with sodium in liquid ammonia led to the cleavage of peptide bonds. The results of a preliminary analysis of the decomposition product formed permitted the assumption that it was possibly the cleavage of the $\overset{90}{\text{Pro}}$–$\overset{91}{\text{Ser}}$ bond that had taken place, with the splitting off of the tripeptide H-$\overset{91}{\text{Ser}}$-$\overset{92}{\text{Glu}}$-$\overset{93}{\text{Ser}}$-OH.

Catalytic hydrogenation in the presence of 5% palladium adsorbed on carbon led to the irreversible adsorption of the bulk of the free peptide on the catalyst. Because of this, the peptide (Ia) was hydrogenated in the presence of palladium black in aqueous (1 : 1) methanol in acetic acid. The free eicosapeptide after reduction (Ib) was purified by ion-exchange chromatography on DEAE-Sephadex in a concentration and pH gradient of an ammonium acetate buffer.

The homogeneity of the peptide (Ib) obtained was confirmed by the results of paper electrophoresis in various buffers at various pH values, and by thin-layer, paper, and ion-exchange chromatography, and its amino acid composition was confirmed by quantitative amino acid analysis.

EXPERIMENTAL

The purity of the peptides obtained was checked by electrophoresis and paper chromatography on type B (fast) paper of the Leningrad mill.

Electrophoresis was performed in a horizontal instrument at 1200 V in 0.04 M acetate buffer (pH 2.9), 0.05 M ammonium acetate buffer (pH 7.6), 0.05 M borate buffer (pH 6.7), and 0.1 M pyridine acetate buffer (pH 3.7). The following solvent systems were used for chromatography: 1) butan-2-ol–acetic acid–water (12 : 3 : 5); 2) butan-2-ol–10% ammonia (3 : 2), 3) 2-methylpropan-2-ol–88% formic acid–water (70 : 15 : 15); 4) butan-1-ol–butyl acetate–acetic acid–water (19 : 1 : 5 : 25); and 5) butan-2-one–acetic acid–water (10 : 30 : 25).

All the amino acids used in the synthesis belonged to the L series.

Hydrobromide of Phenylalanylisoleucylisoleucylglycylglutamylleucylhistidylprolylasparagylarginylseryllysylisoleucylthreonyllysylprolylserylglutamylserine (I). The peptide was synthesized on a chloromethylated copolymer of styrene with 2% of divinylbenzene (7% of chlorine). N^{α}-Boc-O-benzylserine was added by boiling 1.1 g of the polymer with 0.59 g of the protected amino acid and 0.27 ml of triethylamine in 20 ml of anhydrous ethanol for 24 h. The amount of Boc-O-benzylserine added to the polymer, determined colorimetrically [8] after a sample had been subjected to acid hydrolysis in 6 N hydrochloric acid at 110°C for 24 h was 0.315 mmole/g of polymer. The synthesis was continued with 1.09 g of the Boc-aminoacylpolymer. N,N'-Dicyclohexylcarbodiimide was used as the condensing agent for Boc-O-benzylserine, Boc-isoleucine, Boc-O-benzoylthreonine, Boc-N^{ω}-nitroarginine, and Boc-N^{im}-benzylhistidine. The remaining amino acids were introduced into the peptide chain by the p-nitrophenyl ester method in the presence of 1,2,4-triazole. For each amino acid the cycle included the splitting off of the Boc protection by

the action of a 1 N solution of hydrogen chloride in acetic acid (2×20 min, 40 ml), neutralization of the hydrochloride with 10% triethylamine solution in chloroform (20 ml, 20 min), and condensation with the subsequent amino acid (the excesses of the reagents are given in Table 1).

The amino groups that had not reacted were acetylated before the elimination of the Boc protection in the sixth, twelfth, and seventeenth cycles of the synthesis. The last four amino acids were added in the form of the dipeptide blocks (II) and (III) by the carbodiimide method.

After the performance of 17 cycles of condensation, a suspension of the peptidylpolymer in dimethylformamide was treated with acetic anhydride (1.5 ml) in the presence of triethylamine (1.5 ml) for 40 min and was then washed with dimethylformamide and with anhydrous ethanol and was dried in vacuum over caustic potash. The weight of the dried peptidylpolymer was 2.35 g (increase in weight 1.35 g).

Splitting Off of the Eicosapeptide (Ia) from the Polymer Support. A current of dry hydrogen bromide freed from bromine was passed through a suspension of the peptidylpolymer (0.76 g) in 10 ml of trifluoroacetic acid at 20°C for 60 min and at 0°C for 30 min. The polymer was separated off and washed with trifluoroacetic acid (3×10 ml). Then the solvent was driven off in vacuum at 25°C. The residue was hardened in anhydrous ether, separated off, and reprecipitated from methanol (3 ml) with anhydrous ether (50 ml). The weight of substance isolated was 0.312 g. Paper chromatography of the product split off showed one ninhydrin-positive substance with R_{f1} 0.64 and a weak spot at 0.49. The substance (0.312 g) was dissolved in 5 ml of water and was passed in three stages through a column of Sephadex G-25 (1.4×48 cm, V = 24 ml/h). The freeze-drying of the main fractions (3×40 ml) gave 265 mg of an amorphous product (R_{f_1} 0.64) slightly contaminated with the substance having R_{f1} 0.49. The peptide (20 mg) was rechromatographed on Sephadex G-25. Freeze-drying of a narrow fraction (1.5 ml, peak I) gave 3 mg of the eicosapeptide (Ia), homogeneous on paper chromatography and on electrophoresis in acetate buffer (pH 2.9). The N-terminal amino acid determined with the aid of dansyl chloride was phenylalanine, contaminated with small amounts of isoleucine and serine.

Hydrogenation of the Eicosapeptide over Palladium Black. A solution in 10 ml of 50% methanol of 40 mg of the eicosapeptide (Ia) that had been twice purified on Sephadex G-25 was acidified with acetic acid (3-5 drops) and 100 mg of palladium black was added. Hydrogenation with hydrogen was carried out for 48 h. The course of the hydrogenation reaction was monitored by paper chromatography and by the Pauli reagent (free His) and the Sakaguchi reagent (free Arg). The catalyst was separated off and washed with 100 ml of 1% acetic acid. The filtrate and the washings were freeze-dried and dissolved in 1 ml of water, and the solution was passed through a column of Sephadex G-25 (2.5×45 cm, V=60 ml/h) equilibrated with water. After 120 ml, the main fraction (20 ml) was collected, and the freeze drying of this gave 23 mg of the peptide (51.7% calculated on the Boc-O-benzylserine fixed to the polymer).

Chromatography of the Eicosapeptide on DEAE-Sephadex. A solution of 5 mg of the peptide (Ib) in 1 ml of water was deposited on a column of DEAE-Sephadex (1.2×12 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 7.6). After the passage of 100 ml, the addition of 1 M ammonium acetate buffer with pH 7.6 to the mixing flask (V = 200 ml) filled with the initial buffer was begun, and after the passage of 250 ml the addition of 1 M ammonium acetate buffer with pH 6.25 was begun. Elution in the concentration and pH gradient gave only peak I of a substance with R_{f1} 0.30, R_{f2} 0.18, R_{f3} 0.35, and R_{f4} 0.24 (paper chromatography) and R_{f5} 0.65 (chromatography on a plate with a thin layer of silica). Electrophoresis in 0.04 M acetate buffer (pH 2.9, i = 7.5 mA, 90 min), in 0.05 M ammonium acetate buffer (pH 7.6, i = 4.5 mA, 90 min), and in 0.05 M borate buffer (pH 6.7, i = 4.5 mA, 90 min) showed a single ninhydrin-positive substance, which was also stained by the Pauli and Sakaguchi reagents. $[\alpha]_D^{21}$ + 9.58° (c 1.8; H_2O). Amino acid analysis of the free eicosapeptide: Lys 2.02 (2); His 1.05 (1); Arg 1.01 (1); Asp 1.96 (2); Thr 1.21 (1); Glu 2.13 (2); Ser 2.86 (3); Pro 2.01(2); 1.01 (1); Leu 1.0 (1); Ile 3.26 (3); Phe 0.98 (1). The N-terminal amino acid was Phe (determined by means of dansyl chloride).

SUMMARY

The synthesis of an eicosapeptide corresponding to the sequence 74-93 of the polypeptide chain of cytochrome b_5 isolated from the microsomes of calf thymus has been performed by the solid-phase method.

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