SOLID-PHASE SYNTHESIS OF A NONAPEPTIDE CORRESPONDING TO SEQUENCE 26-34 OF CYTOCHROME B_5

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Cytochrome B_5, isolated from the microsomes of mammalian liver [1], is a hemoprotein participating in electron transfer in the respiratory chain of cells. The primary structure of the apoprotein and the physicochemical properties of this enzyme have been established by the work of Strittmatter and Osols [2]. Cytochrome B_5 consists of a complex of ferriprotoporphyrin (IX) with a protein consisting of 85 amino acid residues. Two of the four histidine residues are probably involved in the coordination bond of the iron of the heme with the protein.

This paper forms part of studies on the synthesis of histidine-containing peptides of cytochrome B_5. It gives a study of the nature of the bond of the apoprotein with the prosthetic group. We have obtained the nonapeptide

$$\begin{array}{l} \qquad\qquad\quad Tos^{\varepsilon} \\ \qquad\qquad\quad | \\ HBr\cdot L\text{—}Lys\text{—}L\text{—}Phe\text{—}L\text{—}Leu\text{—}L\text{—}Glu\text{—}L\text{—}Glu\text{—}L\text{—}His\cdot L\text{—} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad Bzl^{im} \\ \qquad\qquad\qquad\qquad\quad \text{—}Pro\text{—}Gly\text{—}Gly \quad (I), \end{array}$$

corresponding to sequence 26-34 of cytochrome B_5 by Merrifield's solid-phase method [3]. All the amino acids except N^{α}-tert-butoxycarbonyl-N^{ε}-tosyl-L-lysine, which was brought into the reaction by the p-nitrophenyl ester method, were synthesized by the carbodiimide method. To facilitate subsequent purification, after each stage of condensation the free amino groups were acetylated [4]. The peptide was separated from the resin by the action of hydrogen bromide on a suspension of the polymer in trifluoroacetic acid for 90 min. A product was isolated in which paper chromatography in a butan-1-ol—acetic acid—water (4 : 1 : 5) system (system 1) showed the presence of three ninhydrin-positive spots. The mixture of substances was separated on Sephadex G-10 in 0.1 M acetic acid. The N-acetyl derivatives of the peptides (revealed with iodine) were eluted first, and then substances with R_{f_I} 0.30, 0.20, and 0.80. An amino acid analysis of the last substance showed that it was the nonapeptide I. The compound with R_{f_I} 0.30 was found to contain the same amino acids as the nonapeptide, with the exception of proline and glycine. Thus, when the nonapeptide is split off from the polymer support, cleavage of the peptide bond between proline and the amino acids adjacent to it takes place. This is in agreement with literature information [5] on the instability of prolyl-containing peptides. A similar cleavage of the polypeptide was also observed during its purification.

To study the influence of the method of separation from the polymer on the stability of the propylglycine bond, we synthesized the tripeptide L-Pro-Gly-Gly (II), corresponding to sequence 32-34 of cytochrome B_5, by the solid-phase method. The chain was extended by the carbodiimide method, or by the N-hydroxysuccinimide or p-nitrophenyl ester methods using 1,2,4-triazole as a catalyst [6]. The completeness of the condensation in the last two methods was determined by potentiometric titration [7]. At the glycylglycyl-polymer stage, the yield was 98%, and at the L-prolylglycylglycyl-polymer stage it was 96.5%. The peptide was separated from the resin by acidic (HBr/CF_3COOH or HBr/CH_3COOH) or alkaline (methanolic solution of caustic soda) agents.

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Paper chromatography showed that in each case the products of the separation of the peptide from the polymer contained, in addition to the tripeptide (II), the dipeptides L-Pro-Gly (III) and Gly-Gly (IV) [8]. The tripeptide II and the dipeptides III and IV used as markers in paper chromatography were obtained by independent synthesis from the tripeptide N^{α}-BOC-L-Pro-Gly-Gly-OBzl (V) and the dipeptides N^{α}-BOC-L-Pro-Gly-OBzl (VI) and N^{α}-BOC-Gly-Gly-OBzl (VII) after saponification and elimination of the tert-butoxycarbonyl protection.

EXPERIMENTAL

The compounds were chromatographed on type "B" (fast) Leningrad paper in a butan-1-ol–acetic acid–water (4 : 1 : 5) system (system 1) and in a thin layer of silica in benzene–ethyl acetate (1 : 1) and ethyl acetate–methanol (9 : 1) systems (system 2 and 3, respectively).

Solid-Phase Method of Synthesis. Hydrobromide of N^{ε}-Tosyl-L-lysyl-L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-N-benzyl-L-histidyl-L-prolylglycylglycine (I). The solid-phase synthesis was carried out on a chloromethylated copolymer of styrene with 2% divinylbenzene (7.9% Cl). tert-Butoxycarbonylglycine was added by boiling 2.8 g of the polymer with 0.97 g of tert-butoxycarbonylglycine and 0.76 ml of triethylamine in 40 ml of absolute ethanol for 24 h. The amount of amino acid bound to the polymer, determined colorimetrically [9] after acid hydrolysis of a sample of the polymer at 110°C in 6 N HCl for 24 h, was 0.29 mmole/g of polymer. N,N'-Dicyclohexylcarbodiimide was used as the condensing agent for all the amino acids.

N^{α}-tert-Butoxycarbonyl-N^{ε}-tosyl-L-lysine was added to the peptide by condensation using the p-nitrophenyl ester method.

For each amino acid the cycle was as follows:

A) Splitting off the tert-butoxycarbonyl group by the action of 1 N HCl in acetic acid (25 ml, 30 min);

B) Neutralizing the hydrochloride with 10% triethylamine in chloroform (25 ml, 15 min);

C) Condensation with the next amino acid (4 equiv) in the presence of N,N'-dicyclohexylcarbodiimide (4 equiv) in methylene chloride for 8 h, or for 24 h in DMF for the activated ester of protected lysine;

D) Acetylation of the free amino groups with a mixture of acetic anhydride (0.7 ml) and triethylamine (0.7 ml) in DMF (25 ml).

After nine cycles the polymer was washed with DMF and methanol and dried in vacuo over KOH. The weight of the polymer with the peptide added to it was 4.15 g (weight increase of 1.3 g). The peptide was split off from the resin by the passage of a current of HBr through a suspension of the polymer in trifluoroacetic acid (90 min). The suspension was filtered, the residue was washed with trifluoroacetic acid (3 × 10 ml), and the solvent was driven off in vacuo at 25°C. The residue was crystallized from ether. Yield 1.05 g. Paper chromatography in system 1 showed the presence of three ninhydrin-positive spots with R_{f_I} 0.80, 0.30, and 0.20. A 50-mg quantity of the mixture in solution in 0.1 M acetic acid was deposited on a column of Sephadex G-10 (20 × 150 mm). The rate of elution was 10 ml/h (0.1 M acetic acid). The first fraction (17 ml), negative to ninhydrin and readily revealed with iodine, contained acetyl peptides. The second fraction (8 ml) consisted of a mixture of substances with R_{f_I} 0.20 and 0,30, and the third fraction (10 ml) contained the nonapeptide I with R_{f_I} 0.80. It was freeze-dried, giving 20 mg of the nonapeptide I in the form of an amorphous substance. Yield 38.3% calculated on the BOC-Gly-polymer. An amino acid analysis of an acid hydrolyzate gave the following ratio of the amino acids: Lys 0.88, Phe 1.0, Leu 1.0, Glu 2.03, His 1.16, Pro 1.21, and Gly 2.11. Electrophoresis in a phosphate buffer (pH 6.2; 600 V, 30 min) showed one ninhydrin-positive spot.

Hydrobromide of L-Prolylglycylglycine (II). The fixing of tert-butoxycarbonylglycine to the polymer was effected under the conditions described above during 48 h. The amount of tert-butoxycarbonylglycine was 0.65 mmole/g of polymer. The chain was extended by the carbodiimide method (4 equiv of amino acid and 4 equiv of N,N'-dicyclohexylcarbodiimide) in methylene chloride, by the p-nitrophenyl method (4 equiv of amino acid and 5 equiv of 1,2,4-triazole), or by the N-hydroxysuccinimide ester method (4 equiv of amino acid and 5 equiv of 1,2,4-triazole). After each stage acetylation was carried out as described for the pre-

ceding synthesis. The peptide was separated from the resin by the passage of dry HBr through a suspension of the resin in trifluoroacetic acid for 90 min, by the action of 36% HBr in acetic acid for 60 min, or by treatment with 1% caustic soda in methanol for 20 or 60 min. The products obtained were chromatographed on type B Leningrad paper in a butan-1-ol–acetic acid–pyridine–water (40 : 6 : 20 : 24) system. In each case, the dipeptides III and IV were formed as well as the tripeptide II, but the tripeptide II predominated.

Benzyl Ester of tert-Butoxycarbonylglycylglycine (VII). To 1.50 g of the p-toluenesulfonate of the benzyl ester of glycine in 20 ml of dioxane was added 0.65 ml of triethylamine, the mixture was stirred at 20°C for 15 min, then 1.23 g of the N-hydroxysuccinimide ester of tert-butoxycarbonylglycine was added. After 4 h the reaction mixture was evaporated, and the residue was dissolved in 50 ml of chloroform, washed with 3% sodium bicarbonate solution (3 × 10 ml), with water, with 5% citric acid solution, and with water again, and then dried with $MgSO_4$ and evaporated, and the residue was crystallized from aqueous methanol (1 : 1). Yield 1.32 g (94%). mp 86.3-87°C, $R_{f_{II}}$ 0.53. Found %: C 59.68; H 7.06; N 8.66. $C_{16}H_{22}N_2O_5$. Calculated %: C 59.61; H 6.88; N 8.69.

Benzyl Ester of tert-Butoxycarbonyl-L-prolylglycine (VI). The compound was obtained in the form of an oil by the method described above from 0.43 g of the p-toluenesulfonate of the benzyl ester of glycine and 0.20 g of the N-hydroxysuccinimide ester of tert-butoxycarbonyl-L-proline. Yield 0.22 g (95.1%). $R_{f_{III}}$ 0.79, $[\alpha]_D^{22}$ −45.5° (c 3 DMF). Found %: C 62.83; H 6.95; N 7.76. $C_{19}H_{26}N_2O_5$. Calculated %: C 62.96; H 7.23; N 7.71.

tert-Butoxycarbonylglycylglycine (VIII). A solution of 1.0 g of the dipeptide VII in 10 ml of aqueous methanol (1 : 1) was treated with 3.2 ml of 1 N caustic soda, and the mixture was stirred at 20°C for 1 h and then evaporated. The residue was dissolved in 5 ml of water and, with cooling, the solution was acidified with 1 N H_2SO_4 to pH 4.5. The substance was extracted with ethyl acetate, and the extract was washed with 5 ml of ice water, dried with $MgSO_4$, and evaporated. The residue was crystallized from petroleum ether and recrystallized from ethyl acetate. Yield 0.69 g (96%). Mp 131-132°C, $R_{f_{III}}$ 0.52. Found %: C 46.40; H 6.84; N 11.88. $C_9H_{16}N_2O_5$. Calculated %: C 46.55; H 6.95; N 12.04.

Benzyl Ester of tert-Butoxycarbonyl-L-prolylglycylglycine (V). A 2-ml quantity of 1 N HCl in glacial acetic acid was added to 0.28 g of the dipeptide VII, and then, after 30 min, 50 ml of ether was added. The precipitate was separated off and dried over KOH. Yield 0.20 g (95%). Mp 159.5-160°C (according to the literature, mp 160°C [10]). To 0.20 g of the dipeptide hydrochloride so obtained in 10 ml of THF 0.3 ml of triethylamine was added, and the mixture was stirred for 15 min. Then 0.20 g of the N-hydroxysuccinimide ester of tert-butoxycarbonyl-L-proline was added and the mixture was left for 12 h. After the working up process described for VII, an oil was obtained which solidified after prolonged drying. Yield 0.23 g (85.3%). $R_{f_{III}}$ 0.67, $[\alpha]_D^{22}$ −20.5° (c 4.5 DMF). Found %: C 59.90; H 7.02; N 10.09. $C_{21}H_{29}N_3O_6$. Calculated %: C 60.15; H 6.96; N 10.09.

CONCLUSIONS

1. The hydrobromide of N^{ε}-tosyl-L-lysyl-L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-N^{im}-benzyl-L-histidyl-L-prolylglycylglycine, corresponding to sequence 26-34 of cytochrome B_5, has been synthesized by the solid-phase method.

2. The lability of the bond between proline and the amino acids adjacent to it under the conditions of the splitting off of the peptide from the resin has been shown.

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