SYNTHESIS AND REACTION WITH HEMIN OF FRAGMENT (62-93) OF THE POLYPEPTIDE CHAIN OF CYTOCHROME b_5

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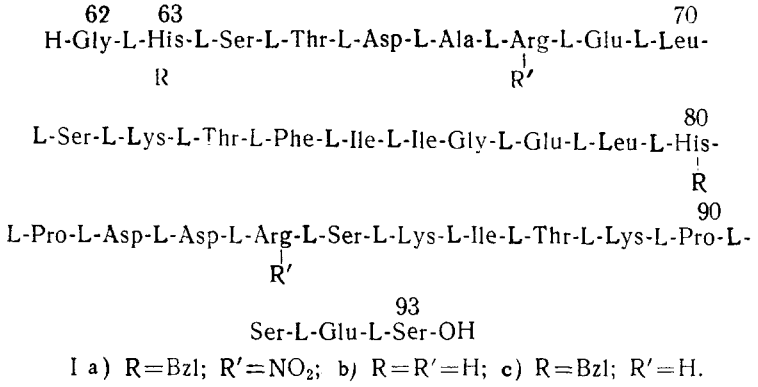
In the preceding paper [1] we have described the synthesis of the (74-93) eicosapeptide. Continuing the growth of the polypeptide chain on the peptidylpolymer we have obtained a compound containing 32 amino-acid residues. Since it includes two histidine residues (63 and 80) which, as has been assumed [2], can participate in coordination with heme in cytochrome b_5, it appeared of interest to perform the reaction of the ditriacontapeptide (I) with hemin.



I a) R=Bzl; R′=NO_2; b) R=R′=H; c) R=Bzl; R′=H.

In the synthesis of the ditriacontapeptide (I) we used 15-fold excesses of the protected amino acids. In all stages of the condensation, before the removal of the Boc protection the unchanged amino groups were acetylated.

During the synthesis of the polypeptide (I), the polymer underwent degradation and some of it washed through the pores of the glass filter of the reaction vessel, as a result of which the weight of the dried ditriacontapeptidylpolymer fell to 27%.

The polypeptide (I) was separated from the polymer support by means of hydrogen bromide in trifluoroacetic acid. The passage of hydrogen bromide for 30 min liberated 77% of the peptide, and its passage for another 30 min liberated 18%; when hydrogen bromide was passed for 90 min, the whole of the peptide fixed to the polymer was liberated, but some of the protective groups remained unremoved. An increase in the time of passage of hydrogen bromide to 100, 110, and 120 min led to an accumulation of peptide decomposition products.

The peptide (Ia) split off from the polymer with the partial retention of the benzyl-protected side chains of the amino acids was hydrogenated with hydrogen over palladium black. In addition to the splitting off of the benzyl groups, the reduction of the nitro group of the arginine and the splitting off of the benzyl protection of the imidazole rings of the histidine took place. The completely reduced ditriacontapeptide (Ib) was purified by ion-exchange chromatography on DEAE-Sephadex in an ammonium-acetate-buffer concentration and pH gradient. The N-terminal acid determined by means of dansyl chloride was glycine.

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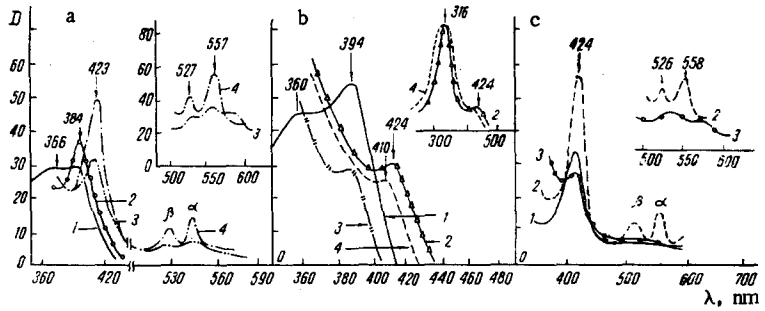

Fig. 1. Electronic spectra in 0.1 M tris acetate buffer, pH 7.6 (reducing agent sodium dithionite): a. 1) hemin; 2) heme; 3) histidine-hemochromogen (beginning of coordination); 4) histidine-hemochromogen (saturation); b. 1) heme-ditriacontapeptide (II, oxidized form); 2) heme-ditriacontapeptide (II, reduced form); 3) heme-di-N^{im}-Bzl-His-ditriacontapeptide (III, oxidized form); 4) heme-di-N^{im}-Bzl-His-ditriacontapeptide (III, reduced form); c. 1) cytochrome b_5 (oxidized form); 2) cytochrome b_5 (reduced form); 3) heme-ditriacontapeptide (II, reduced form).

When the peptide (I) was split off from the polymer support under the action of anhydrous liquid hydrogen fluoride in the presence of anisole, a more homogeneous product was obtained with a very small amount of impurities. Compound (Ic) was purified successively by gel filtration on Sephadex G-25 and ion-exchange chromatography on DEAE-Sephadex in an ammonium-acetate-buffer concentration and pH gradient. The polypeptide (Ic) isolated was homogeneous on paper chromatography and electrophoresis and gave a single peak on ion-exchange chromatography. The N-terminal amino acid determined with the aid of dansyl chloride was glycine. The reduction of the compound (Ic) obtained over palladium led to the free ditriacontapeptide (Ib).

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

The peptides (Ib and c) were investigated in the complex-forming reaction with hemin in 0.1 M tris acetate buffer (pH 7.6) under the conditions described by F. Strittmatter [3].

The formation of coordinated complexes of the peptides with hemin was judged from the nature of the absorption spectrum in the visible region expressed in the shift of the Soret band and in the appearance of absorption bands in the 520-580 nm region. The shape of the spectrum in the 520-580 nm region on the formation of a coordination bond of the peptide with the hemin resembles the spectrum of histidine-hemochromogen in the initial stage of complex-formation (Fig. 1a, curve 3), which must obviously correspond to the coordination of only one histidine with the heme. The electronic spectrum of the heme-ditriacontapeptide (II) formed differed from the spectrum of the heme-di-N^{im}-Bzl-His-ditriacontapeptide (III). This was shown in the difference in the nature of the Soret band in the oxidized form (see Fig. 1b, curves 1 and 3) and in the hypsochromic shift of the Soret band in the reduced form (curve 4) for (III) as compared with the heme-ditriacontapeptide (II) (curve 2).

The spectrum of the heme-ditriacontapeptide (II, reduced form) differed from that of the cytochrome b_5 (Fig. 1c) by the fact that no separation of the α and β bands was observed in it. A similar phenomenon has been reported by Strittmatter and Huntley [2] in an investigation of the complexes of hemin with the tryptic peptides 3-68 and 69-84 of cytochrome b_5.

One of the possible explanations of the difference in the electronic spectra of the heme-ditriacontapeptide (II) and cytochrome b_5 may be that in natural cytochrome b_5 the bond of the apoenzyme with the heme is not effected through the pair of histidine 63 and 80.* Another possible cause of the observed difference in the spectra may be a difference in the fine spatial structure of the peptide chain in the complex obtained and in cytochrome b_5.

*An x-ray structural analysis of cytochrome b_5 with a resolution of 2.8 Å has recently been published [4]. This showed that the bond of the apoenzyme with the heme takes place through the pair of histidine residues in positions 39 and 63.

EXPERIMENTAL

Chromatography and electrophoresis were performed on type B (fast) paper of the Leningrad mill.

The following solvent systems were used for chromatography: 1) 2-methylpropan-2-ol–88% formic acid–water (75:15:15); 2) butan-1-ol–butyl acetate–acetic acid–water (19:1:5:25); 3) butan-2-ol–formic acid–water (15:3:1); and 4) butan-2-one–acetic acid–water (10:30:25).

Hydrobromide of Glycylhistidylserylthreonylasparagylalanylarginylglutamylleucylseryllysylthreonylphenylalanylisoleucylisoleucylglycylglutamylleucylhistidylprolylasparagylasparagylarginylseryllysylisoleucylthreonyllysylprolylserylglutamylserine (I). The ditriacontapeptide (I) was obtained by the addition of protected amino acids to the eicosapeptidylpolymer [1] (1.41 g, peptide content 0.223 mmole) by the carbodiimide method (Boc-O-Bzl-Ser, Boc-O-Bzl-Thr, Boc-N-NO_2-Arg and Boc-N^{im}-Bzl-His) and by the p-nitrophenyl ester method (Boc-β-OBzl-Asp-ONP, Boc-γ-O-Bzl-Glu-ONP, Boc-N^{ε}-L-Lys-ONP, Boc-Leu-ONP, Boc-Ala-ONP and Boc-Gly-ONP) in the presence of 1,2,4-triazole. At each stage of the condensation, before the removal of the Boc protection, the amino groups that had not reacted were acetylated with acetic anhydride (0.5 ml) in the presence of triethylamine (0.3 ml). The amino acids and the carbodiimide were used in 15-fold excess and the 1,2,4-triazole in 18-fold excess. After the performance of twelve cycles of condensation, the peptidylpolymer was acetylated, washed with dimethylformamide, with anhydrous methanol, and with ether, and was dried in vacuum over caustic potash. The weight of dried peptidylpolymer was 1.39 g (theoretically calculated: 1.91 g; loss 0.5 g $\approx$ 27%).

Splitting Off of the Peptide (Ia) from the Polymer Support by the Action of Hydrogen Bromide. A current of dry hydrogen bromide was passed through a suspension of 500 mg of the peptidylpolymer in 5 ml of trifluoroacetic acid containing 0.5 ml of anisole for 90 min. After the treatment described for the eicosapeptide [11] and reprecipitation from methanol–ether, 0.193 mg of the peptide (Ia) with some of the protective benzyl groups not eliminated from the side chains of the amino acids, was obtained. A solution of 180 mg of the peptide (Ia) in 5 ml of water was deposited on a column of Sephadex G-25 (3.5 × 50 cm, V = 60 ml/h) equilibrated with water. After 110 ml, the main fraction was collected (90 ml), and its freeze-drying gave 162 mg of the peptide (Ia).

Hydrogenation of the Ditriacontapeptide (Ia) over Palladium Black. A solution of 70 mg of the partially purified peptide (Ia) in 20 ml of aqueous methanol (1:1) was treated with 0.12 g of Raney nickel, and the mixture was stirred for 15 min. Then the catalyst was separated off and was washed with 10 ml of aqueous methanol (1:1). The filtrate and washings were combined, acidified with acetic acid (3–5 drops), and hydrogenated in the presence of 0.12 g of palladium black for 48 h. The catalyst was separated off and was washed with 100 ml of 2% acetic acid. The filtrate and the washings were freeze-dried, the residue was dissolved in 1 ml of water, and the solution was deposited on a column of Sephadex G-25 (3.5 × 50 cm, V = 24 ml/h). The freeze-drying of the main fraction gave 27 mg of substance (Ib) with R_{f_1} 0.40 and R_{f_2} 0.31. Electrophoresis in 0.05 M acetate buffer (pH 2.9, i = 4.5 mA, 1200 V, 80 min) and in phosphate buffer in the presence of 8 M urea (pH 7.2, 1200 V, 2 h, i = 4.5 mA) showed a single ninhydrin-positive substance, which was also revealed by the Pauli and Sakaguchi reagents. The N-terminal amino acid, determined by means of dansyl chloride, was glycine [slight contaminations (< 1%) with serine, lysine, and glutamic acid].

Purification of the Ditriacontapeptide (Ib) on DEAE-Sephadex. A solution in 1 ml of 0.1 M ammonium acetate buffer (pH 7.6) of 64 mg of the peptide (Ib) that had been reduced and purified on Sephadex G-25 was deposited on a column (1.4 × 35 cm, V = 40 ml/h) of DEAE-Sephadex equilibrated with the same buffer. After 150 ml of eluent had been passed, 1 M ammonium acetate buffer, pH 7.6, was added to the mixing flask (V = 100 ml) filled with the initial buffer, and after 400 ml had been passed a similar buffer with pH 6.25 was added. After a total of 220 ml had been eluted, the main fraction was collected (50 ml). It was freeze-dried and desalted on Sephadex G-25. The freeze-dried peptide (Ib), 42 mg (20.2% calculated on the Boc-O-benzylserine attached to the polymer), was homogeneous on paper electrophoresis in 0.05 M acetate buffer (pH 2.9), in pyridine acetate buffer (pH 3.7), and in phosphate buffer (pH 7.2). $[\alpha]_D^{24}$ –29.4° (c 0.7; 50% MeOH), R_{f_2} 0.31 and R_{f_3} 0.40. Amino-acid analysis of an acid hydrolysate (6 N HCl, 110°C, 24 h, vacuum treatment): Lys 3.3 (3); His 1.55 (2); Arg 1.95 (2); Asp 3.8 (3); Thr 2.8 (3); Ser 5.0 (5); Glu 3.4 (3); Pro 2.1 (2); Gly 1.75 (2); Ala 0.88 (1); Ile 3.5 (3); Leu 1.9 (2); Phe 0.63 (1). The N-terminal acid determined by means of dansyl chloride was glycine.

The Splitting Off of the Ditriacontapeptide (Ic) from the Polymer Support by the Action of Hydrogen Fluoride. To 0.5 g of the ditriacontapeptidylpolymer was added 0.5 ml of freshly distilled anisole and

anhydrous liquid hydrogen fluoride. The mixture was stirred at 0°C for 45 min. The hydrogen fluoride was evaporated off in a current of nitrogen at 20°C, and the residue was treated with dry ethyl acetate (2× 5 ml). The colored product of the oxidation of the anisole passed into the ethyl acetate. The residue was dried in vacuum over caustic potash and was then washed succesively with methanol, aqueous methanol (1 : 1), water, and 5% acetic acid (2×10 ml in each case). The methanol was evaporated off in vacuum, and the aqueous washings were immediately freeze-dried. This gave 206 mg of substance (Ic), the paper chromatography and electrophoresis of which showed the presence in it of a single substance revealed by means of ninhydrin and the Sakaguchi reagent, contaminated with compounds having a lower chromatographic mobility.

Purification of the Ditriacontapeptide (Ic) on DEAE-Sephadex. A solution in 1 ml of 0.1 M ammonium acetate buffer (pH 7.6) of 28 mg of the peptide split off from the polymer with hydrogen fluoride was passed through a column of DEAE-Sephadex (in a similar manner to compound Ib). After 210 ml of eluate had issued, the main fraction was collected (25 ml), and it was desalted on Sephadex G-25 and freeze-dried. The yield of (Ic) was 21 mg (61.2% calculated on the Boc-O-benzylserine attached to the polymer).

The peptide was homogeneous on chromatography on paper (R_{f_1} 0.37, R_{f_2} 0.40) and in a thin layer of silica (R_{f_4} 0.84). Electrophoresis in 0.04 M acetate buffer (pH 2.9) and ammonium acetate buffer (pH 7.6) (1200 V, 80 min), showed a single ninhydrin-positive substance. The peptide (Ic) was revealed by the Sakaguchi reagent but not by the Pauli reagent. $[\alpha]_D^{21}-41.6°$ (c 0.6; 50% MeOH + 0.03 M. CH_3COOH). Amino-acid analysis of an acid hydrolyzate (6 N HCl, 110°C, 24 h, vacuum treatment): Lys 3.23 (3); Arg 1.57 (2); Asp 3.0 (3); Thr 3.16 (3); Ser 4.57 (5); Glu 2.73 (3); Pro 2.3 (2); Gly 2.0 (2); Ala 0.86 (1); Ile 2.89 (3); Leu 1.85 (2); Phe 0.87 (1). The N-terminal amino acid, determined by means of dansyl chloride, was glycine.

Reduction of the Peptide (Ic) over Palladium Black. The ditriacontapeptide (Ic) (178 mg) was hydrogenated in the presence of palladium black under the conditions described previously for 48 h. After purification on DEAE-Sephadex, 55 mg of the ditriacontapeptide (Ib) was obtained (31.2% on the Boc-O-benzylserine attached to the polymer). R_{f_2} 0.31 and R_{f_3} 0.40.

Reaction of the Peptides (Ib and c) with Hemin. The experiments were performed with a freshly prepared (not more than 3 h) solution of hemin made by dissolving 3.25 mg of hemin in 0.5 ml of 1 N caustic soda solution and diluting with 0.1 M tris acetate buffer, pH 7.6, to 150 ml.

The peptide was added in portions (2-3 mg each) to 2.5 ml of the solution of hemin in 0.1 M tris acetate buffer. The electronic spectra of the oxidized and reduced forms of the complexes were recorded after 2-3 min (see Fig. 1). Sodium dithionate was used as the reducing agent. With a 50-fold excess of the peptide (Ib), the complex precipitated.

SUMMARY

1. The synthesis of the ditriacontapeptide forming the (62-93) fragment of the polypeptide chain of cytochrome b_5 has been effected using the solid-phase method.

2. Complexes of the free ditriacontapeptide with hemin and the ditriacontapeptide with the histidine residues protected by benzyl groups have been obtained, and a comparison of their electronic spectra with the spectrum of cytochrome b_5 has been made.

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