An improved synthesis of crystalline mammalian glucagon

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Mammalian glucagon was synthesized by the stepwise solid-phase method using several improvements developed in recent years. Peptide was assembled on a 4-(oxymethyl)phenylacetamidomethyl-copoly(styrene-divinyl benzene) resin support with N\(^2\)-t-butoxycarbonyl and benzyl-based side-chain protection for most of the trifunctional amino acids. Crude synthetic glucagon was obtained in 75% yield by deprotection and cleavage from the resin with a new modified HF procedure. Pure material was isolated in 48% overall yield by a one-step purification on preparative C\(_{18}\) reverse-phase chromatography. It was crystallized from aqueous solution at pH 9.2. The synthetic glucagon activated adenylate cyclase in rat liver membranes in the same manner as natural glucagon, with both achieving half-maximum activation at a concentration of 7 nM.

Glucagon, a 29-residue peptide hormone that regulates glycogenolysis and gluconeogenesis, has in recent years been the subject of numerous studies to determine the relation of the structure of the molecule to its function [1 — 5]. Our efforts have been directed toward developing a rapid and efficient stepwise solid-phase synthesis for the preparation of glucagon analogues for structure—function studies. We reported [6] the first stepwise solid-phase synthesis of glucagon that produced crystalline material in reasonable yield. In that synthesis, an alkoxycarbonyl alcohol resin was used in combination with acidlabile N\(^2\)-biphenylisopropoxycarbonyl amino acid derivatives and tert-butyl-based side-chain protection. These amino acid derivatives are quite unstable during synthetic manipulations and, in general, are not readily available. Because rapid syntheses of glucagon analogues will be facilitated by commercially available reagents that can be used without any purification, we decided to synthesize glucagon by a different synthetic scheme. Most of the problems previously encountered in the synthesis of glucagon were circumvented by the new approach which was based on N\(^2\)-t-butoxycarbonyl and benzyl-based side-chain protection [7] and relied on use of the recently developed 4-(oxymethyl)phenylacetamidomethyl-copoly(styrene-divinyl benzene) resin support (-OCH\(_2\)-Pam-resin) with improved acidolytic stability [8], the cyclohexyl-protecting group for the \(\beta\)-carboxyl of aspartic acid [9] that minimizes aspartimide formation, and new modified HF conditions for deprotection and cleavage of peptide from the resin [10]. In addition to benzyl-based protecting groups for the side chains of the nine hydroxyl-containing residues and one lysyl residue, the guanidine groups of the two arginine residues and the imidazole ring of the N-terminal histidine were protected by the p-toluenesulfonyl group. The indole ring of tryptophan was protected with the formyl group against alkylation and oxidation, whereas the thioether group of methionine was left unprotected. The protection scheme is shown in Fig. 1. Amino acid derivatives were coupled by activation as anhydrides or active esters and the reactions were monitored by a quantitative ninhydrin test. The synthetic strategy allowed removal of all protecting groups and cleavage of the peptide from the resin to be accomplished in a single step. Homogeneous glucagon was obtained by reverse-phase chromatography in high overall yield. The product was crystallized and was fully active in the adenylate cyclase assay.

**EXPERIMENTAL PROCEDURES**

**Materials**

Commercial reagents were as follows: t-butoxycarboxylin (Boc) amino acid derivatives and Aoc-Arg(Tos) (Peninsula) Boc-Asp-OBzI (Vega), copoly(styrene-1%-divinylbenzene) resin beads (Bio-Rad), 4-(bromomethyl)phenylacetic acid phenacyl ester (RSA Corp, Ardsley, NY, USA), trifluoroacetic acid (Ha-locarbon Products), dicyclohexylcarbodiimide (Pierce), 1-hydroxybenzotriazole (Aldrich) recrystallized from 70% ethanol, dichloromethane (Fisher), dimethyl sulfoxide, and ethanedithiol (Fluka), 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole and toluenesulfonic acid (Pierce). \([^{14}\text{C}]\) glycine (New England Nuclear, Boston, MA, USA). Preparative reverse-phase C\(_{18}\) resin (50 — 100 \(\mu\)m) was from Waters Associates. Natural glucagon was purchased from Sigma.

**Methods**

The purity of the commercial amino acid derivatives was confirmed by melting points and thin-layer chromatography. Boc-[\(^{14}\text{H}\)Leu (specific activity 3.1 \(\times\) 10\(^{9}\) dpm/mmol) and Boc-[\(^{14}\text{C}\)Gly (specific activity 1.1 \(\times\) 10\(^{9}\) dpm/mmol) were synthesized with the help of t-butoxycarboxylin anhydride (Fluka). Boc-Asp(OcHex) was prepared from Boc-Asp-OBzI according to Tam et al. [9].
Hydrolysates of peptide resins were obtained in 12 M HCl/phenol/AcOH (2:1:1) at 110°C, 24 h, as described by Gutte and Merrifield [11]. Hydrolysates of natural and synthetic glucagon were obtained in 3 M touluenesulfonic acid [12] and in 4 M methanesulfonic acid containing 0.2% of 3-(2-aminophenyl)indole for determination of tryptophan [13].

The synthesis was performed with a manual shaker in a reaction vessel described by Merrifield et al. [14]. Cleavage with HF was in a Diaflo HF line from Toho Co. (Osaka, Japan). Analytical HPLC was on the thermostated reverse-phase μBondapak C18 column (4 mm × 30 cm, Waters Associates). The chromatograms were monitored by a Schoeffel variable-wavelength ultraviolet photometer at 225 nm and 280 nm. Peptide samples were counted on a Beckman liquid scintillation counter, with 30% efficiency for tritium and 64% efficiency for 14C.

Adenylate cyclase assays were performed according to the method of Solomon et al. [15] with the addition of GTP in the assay system.

RESULTS

Assembly of the amino acid sequence of glucagon

Boc-Thr(Bzl)-OCH3-Pam-resin was synthesized as previously described [14]. Substitution of C-terminal Boc-Thr(Bzl) on the resin was determined to be 0.175 mmol/g by quantitative ninhydrin reaction [16] and picric acid titration [17] on small deprotected samples. The two results were in good agreement. For the synthesis 1.0 g was used.

One synthetic cycle consisted of the following steps: (1) CH2Cl2 wash and pre-swell (3 × 35 ml for 1 min); (2) deprotection with 50% F3CCOOH/CH2Cl2 (1 × 35 ml 1-min prewash + 1 × 35 ml for 20 min); (3) CH2Cl2 wash (6 × 35 ml for 1 min); (4) neutralization with 5% iPr2EtN/CH2Cl2 (3 × 35 ml for 1 min); (5) CH2Cl2 wash (6 × 35 ml for 1 min); (6) preformed symmetrical anhydride coupling in CH2Cl2 (4 equivalents, 0.1 M) for 60 min; (7) CH2Cl2 wash (6 × 35 ml for 1 min); (8) neutralization with 5% iPr2EtN/CH2Cl2 (3 × 35 ml for 1 min); (9) CH2Cl2 wash (6 × 35 ml for 1 min); (10) repeat steps (6) and (7). For monitoring of the extent of coupling reaction, after the repeat of step (7), the entire peptide-resin was neutralized with 5% iPr2EtN/CH2Cl2 (1 × 35 ml for 1 min), followed by CH2Cl2 washes (6 × 35 ml for 1 min) and removal of an aliquot (3–6 mg) of peptide resin. Aliquots (3–6 mg) were also removed after the first coupling and neutralization (after step 9).

The symmetrical anhydrides of amino acids were prepared at 0°C for 20 min with 8 equivalents of t-butoxycarbonyl amino acid derivative and 4 equivalents of dicyclohexylcarbodiimide in CH2Cl2. Dicyclohexyl urea was removed by filtration before addition of the anhydride to the peptide-resin. Exceptions to the above coupling protocol were: (a) Boc-[3H]Leu and Boc-[14]Gly in 1.6 equivalents and 1.2 equivalents respectively, were coupled by dicyclohexylcarbodiimide activation; (b) Boc-Asn and Boc-Gln were coupled with the HOBt/dicyclohexylcarbodiimide method [18] as described by Mojsov et al. [19] with 4 equivalents of reagents, and (c) Aoc-Arg (Tos) at positions 18 and 17 was coupled both times with dicyclohexylcarbodiimide (Merrifield, unpublished results) in fourfold excess. Special precautions were taken in the deprotection of and subsequent couplings to the three glutamine residues [20].

After incorporation of the methionine residue at position 27, ethanedithiol (0.03% v/v) was added to the 50% F3CCOOH/CH2Cl2 as an alkyl scavenger. The N-formyl group was found to be stable to this dilute solution of ethanedithiol in acid.

For most residues, the monitoring data indicated that the second coupling was necessary for quantitative incorporation. In some cases, such as the glutamine residue at position 24, aspartic acid at 21, glutamine at 20, arginine at 17, and lysine at 12, the ninhydrin test indicated that >1% of uncoupled amino groups remained after the second coupling. A third coupling was then performed, also for 60 min, with HOBt/dicyclohexylcarbodiimide for the glutamine residues, dicyclohexylcarbodiimide alone for arginine, and preformed symmetrical anhydride in dimethylformamide [21] for aspartic acid and lysine. The ninhydrin values were then satisfactory.

After deprotection of the glutamine residue at position 24 and phenylalanine at 22, the trifluoroacetic acid/CH2Cl2 (1:1) solution and subsequent CH2Cl2 washes were collected, the volume of solution was reduced (10 ml) and an aliquot (25 μl) was counted. From radioactivity recovered in solution, it was calculated that there was about a 0.13% loss of chains per deprotection step.

Amino acid analysis of the hydrolysate after residues 26 and 4 showed that the amount of growing peptide chains remained the same at the beginning (residue 26) and toward the end (residue 4) of the synthesis, indicating that no detectable chain termination occurred during the assembly of the peptide.

Isolation and purification of synthetic glucagon

Before final deprotection and cleavage of the peptide-resin with HF, an aliquot (0.39 g, 0.07 mmol) was treated with 50% F3CCOOH/CH2Cl2 (10 ml) containing ethanedithiol (0.03% v/v) for 30 min to remove the N-terminal t-butoxycarbonyl group, followed by CH2Cl2 washes (6 × 10 ml for 1 min), neutralization with 5% iPr2EtN/CH2Cl2 (3 × 10 ml for 1 min) and CH2Cl2 washes (6 × 10 ml for 1 min).

Preparation of fully deprotected glucagon by deprotection and cleavage of protected glucagon-OCH3-Pam-resin with new HF procedure. Protected glucagon-OCH3-Pam-resin (0.17 g; 0.03 mmol) was placed in the Teflon vessel of the HF apparatus, with addition of the following reagents: melted p-cresol (0.25 ml), p-thiocresol (0.25 ml), dimethylsulfide (3.25 ml) [10]. For HF treatment of the protected glucagon-OCH3-Pam-resin, methionine was added (0.27 g, 1.7 mmol, 50-fold molar excess over peptide). The vessel was cooled to −78°C; HF was condensed up to the 5-ml mark and the mixture was kept for 2 h at −78°C. The solution was then evaporated to remove HF and dimethylsulfide, and HF was condensed again to the
An aliquot of protected glucagon-OCH$_3$Pam-resin with HF/p-cresol (9:1) was placed in the Teflon vessel of the HF procedure; column: CM-cellulose (CM-52, 1.6 x 28 cm). Conditions: linear gradient of 0.01 M ammonium acetate in 6 M urea, pH 4.5 (250 ml), and 0.1 M ammonium acetate in 6 M urea, pH 5.4 (250 ml); flow rate: 18 ml/h.

The sample (1.6 µmol) directly after deprotection and cleavage by modified HF procedure was eluted with a linear gradient of 0.01 M ammonium acetate in 6 M urea, pH 4.5 (250 ml), and 0.1 M ammonium acetate in 6 M urea, pH 5.4 (250 ml); flow rate: 18 ml/h.

A shallow linear gradient of 30% solution B in A (200 ml) to 50% B in A (200 ml) was applied to obtain a greater separation of the two by-products from the major desired peak. The flow rate was 54 ml/h; the eluant was monitored by radioactivity. The early by-product eluted at a volume of 180 ml (45% B in A), and synthetic glucagon at 234 ml (47% B in A). To obtain the by-product eluting after the synthetic glucagon peak at the end of the gradient, the column was washed with 50% B in A (100 ml), and the material was eluted at 30-ml volume. Pooled fractions for each of the three peaks were neutralized with 1 M NaOH, acetonitrile was removed by evaporation under vacuum and the material was recovered by lyophilization. The peptides were desalted on Sephadex G-10 (2.5 x 23 cm) using 10% acetic acid as the eluant. The material from the second peak of the reverse-phase column, which corresponded to synthetic glucagon, was isolated after desalting in 5 mg yield (48% overall yield). Chromatography of an aliquot (20 µg) of purified synthetic glucagon on the analytical HPLC reverse-phase C$_{18}$ column showed only a sharp single peak eluting at 21.5 min.

Retention Time (min)

Absorbance

Effluent Volume (ml)

Radioactivity (cpm x 10$^5$)

Conductivity (mS)

Fig. 2. Ion-exchange chromatography of crude synthetic glucagon. The sample (1.6 µmol) directly after deprotection and cleavage by modified HF procedure; column: CM-cellulose (CM-52, 1.6 x 28 cm). Conditions: linear gradient of 0.01 M ammonium acetate in 6 M urea, pH 4.5 (250 ml), and 0.1 M ammonium acetate in 6 M urea, pH 5.4 (250 ml); flow rate: 18 ml/h.

Fig. 3. HPLC on analytical reverse-phase µBondapak C$_{18}$ column (4 mm x 30 cm) of 20 µg of synthetic glucagon purified by CM-cellulose ion-exchange chromatography. Solvent systems were as follows: buffer A, 90% phosphoric acid (0.1%) and 10% CH$_3$CN; buffer B, 50% phosphoric acid (0.1%) and 50% CH$_3$CN. The sample was eluted with a linear gradient of 30% B to 80% B obtained in 45 min at flow rate of 2 ml/min. Elution profile was monitored at 225 nm.
apparatus, and p-cresol (0.5 ml) and methionine (0.1 g, 0.67 mmol) were added. After cooling to −78°C, HF (4.5 ml) was distilled into the vessel, and the reaction proceeded with stirring for 1 h at −5°C. Work-up of the sample was the same as that described earlier for the sample treated with the modified HF. Absorption spectra of this sample (0.3 mg in 1 ml 10% acetic acid) had maxima at 251 nm, 286 nm, and 304 nm, indicating the stability of N'-formyl group to the HF/p-cresol (9:1) reagent.

An aliquot (20 µl) of the crude synthetic N'-formyl glucagon was eluted from the analytical HPLC reverse-phase C$_{18}$ column under the same conditions as those described in Fig. 3. [Trp(For)$_{25}$]glucagon eluted at 21 min, confirming previous observation that peptides containing Trp and Trp(For) are not well separated in this system. An additional peak, eluting at 16 min and representing 46% of the material applied was also present. Under the same chromatographic conditions, a sample of authentic synthetic [methionine sulfoxide$_{25}$]glucagon eluted with a retention time of 16 min.

Treatment of this crude material with the solvent mixture of 25% HF, 65% (CH$_3$)$_2$S, 10% p-cresol that contained 50-fold molar excess of methionine quantitatively converted it to the desired synthetic glucagon. Since no sulfoxide was obtained during the work-up following the new HF procedure, we concluded that oxidation occurred during the synthesis of the peptide chain rather than during the work-up.

**Characterization of synthetic glucagon**

**Amino acid analysis.** Samples of purified synthetic, as well as natural, glucagon were hydrolyzed with 3 M toluenesulfonic acid for 24 h at 130°C and in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole also for 24 h at 110°C. The ratio of amino acids in the synthetic material was: Lys 0.99 (1.00), His 0.98 (1.00), Arg 2.10 (2.00), Trp 0.92 (1.00), Asp + Asn 3.94 (4.00), Thr 2.86 (3.00), Ser 3.90 (4.00), Glu(Gln) 2.96 (3.00), Gly 1.02 (1.00), Ala 1.00 (1.00), Val 0.98 (1.00), Met 0.94 (1.00), Leu 1.97 (2.00), Tyr 1.90 (2.00), Phe 1.96 (2.00). The values for serine and threonine are not corrected.

**Chromatography of synthetic and natural glucagon.** Chromatographic analysis of synthetic and natural glucagon on the analytical reverse-phase C$_{18}$ HPLC is shown in Fig. 4. Commercial natural glucagon eluted at 21.5 min and contained a small impurity eluting in front of the glucagon peak. When cochromatographed, a single major compound eluting at 21.5 min was obtained in addition to the minor impurity. Therefore, identity of synthetic and natural glucagon in this system was demonstrated.

**Crystallization.** Synthetic glucagon was crystallized with slight modification of the conditions described [6]. Pure synthetic material (1.5 g) was dissolved in 1.5 ml of 0.2 M sodium phosphate buffer, pH 9.2, with slow heating to 50°C, and the solution was allowed to cool to room temperature. After 48 h it was transferred to the cold room (4°C). Typical hexagonal crystals appeared after several days. They were seen under polarized microscope.

**Biological activity.** Biological activity of synthetic glucagon was tested for its activation of the adenylate cyclase of the rat liver membranes. Both the concentration (1 µM) required to achieve the maximum activation in the system and the dose-related concentration for this activation were the same as those observed with the natural hormone (Fig. 5). Half-maximum activation was at a concentration of 7 nM.

**DISCUSSION**

This stepwise solid-phase synthesis of glucagon, which was based on differential acid stability of protecting groups and the ester linkage of the peptide to the solid support, produced material of high homogeneity and good yield. The design of the synthetic protocol incorporated several recent improvements in the methodology [8-10, 16, 20, 22]; the results obtained showed that the following were most important of these. (a) The use of Pam-resin minimized the acidolytic loss of peptide chains from the solid support (about 3.6% total), leading to increased yield of peptide on the resin at the end of the synthesis. (b) Quantitative ninhydrin monitoring facilitated the achievement of almost complete incorporation of each residue (>99.5%) into the peptide chain, increasing the homogeneity of the synthetic material. (c) The modified HF procedure deprotected all of the protecting groups in one step, avoiding the need for the usual base treatment for the removal of the formyl group of tryptophan. Absorption spectra of the synthetic material showed no evidence of oxidation or alkylation of the indole ring. In addition, the application of the new HF conditions was essential in reducing quantitatively methionine sulfoxide that was formed during the assembly of the peptide chain to the desired methionine residue.
The crude cleaved material contained 85–90% glucagon. From this material pure synthetic glucagon was isolated in a single purification step by preparative reverse-phase chromatography. The high-resolution properties of the reverse-phase chromatography were superior to those of CM-cellulose (CM-52) ion-exchange chromatography in separating glucagon from its by-product. The amount of this by-product was much more pronounced (about 50%) in the crude synthetic glucagon obtained from a stepwise solid-phase synthesis in which methionine sulfoxide was incorporated into the peptide chain (our unpublished results). We did not detect the presence of methionine sulfonium salt [23] by either ion-exchange or HPLC. Presumably the sulfonium salt formed during the synthesis but it was effectively decomposed to methionine during the work-up, which included dialysis in acetic acid.

The overall 48% isolated yield of homogeneous glucagon based on the starting C-terminal residues is much higher than the yield obtained in our earlier stepwise solid-phase synthesis of glucagon in which more-acid-labile protecting groups were used [6]. It is also higher than the yield reported for a solid-phase fragment synthesis or for synthesis by solution methods (see [24]).

Since the synthetic glucagon activated adenyl cyclase of rat liver membranes in the same concentration range as natural glucagon, we concluded that our synthesis material had the biological properties expected from the hormone. Considering the ease of purification of synthetic glucagon and the high yield obtained in the synthesis, it seems to be feasible for the first time to approach structure–function studies of the glucagon molecule through the total synthesis of selected analogues.

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