Design and Synthesis of Glucagon Partial Agonists and Antagonists†

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ABSTRACT: The hyperglycemia and ketosis of diabetes mellitus are generally associated with elevated levels of glucagon in the blood. This suggests that glucagon is a contributing factor in the metabolic abnormalities of diabetes mellitus. A glucagon-receptor antagonist might provide important evidence for glucagons's role in this disease. In this work we describe how we combined structural modifications that led to glucagon analogues with partial agonist activity to give glucagon analogues that can act as competitive antagonists of glucagon-stimulated adenylate cyclase activity. Using solid-phase synthesis methodology and preparative reverse-phase high-performance liquid chromatography, we synthesized the following seven glucagon analogues and obtained them in high purity: [D-Phe^4,Tyr^5,Arg^{12}]glucagon (**2**); [D-Phe^4,Tyr^5,Lys^{17,18}]glucagon (**3**); [Phe^1,Glu^3,Lys^{17,18}]glucagon (**4**); [Glu^3,Val^5,Lys^{17,18}]glucagon (**5**); [Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon (**6**); I_4-[Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon (**7**); [Pro^3]glucagon (**8**). Purity was assessed by enzymatic total hydrolysis, by chymotryptic peptide mapping, and by reverse-phase high-performance liquid chromatography. The new analogues were tested for specific binding, for their effect on the adenylate cyclase activity in rat liver membranes, and for their effect on the blood glucose levels in normal rats relative to glucagon. Analogues showing no adenylate cyclase activity were examined for their ability to act as antagonists by displacing glucagon-stimulated adenylate cyclase dose–response curves to the right (higher concentrations). The binding potencies of the new analogues relative to glucagon (=100) were respectively 1.0 (**2**), 1.3 (**3**), 3.8 (**4**), 0.4 (**5**), 1.3 (**6**), 5.3 (**7**), and 3 (**8**). Glucagon analogues **3**–**5** and **8** were all weak partial agonists with EC_{50} values of 500 (**3**), 250 (**4**), 1600 (**5**), and 395 nM (**8**), respectively. None of these analogues were able to fully stimulate in vitro adenylate cyclase activity relative to glucagon (=100), and they displayed partial agonist activities of 5 (**3**), 10 (**4**), 10 (**5**), and 40 (**8**), respectively, in this assay. All showed weak glycogenolytic activity in vivo. Glucagon analogues **2**, **6**, and **7** showed no adenylate cyclase activity at concentrations up to 10–100 μM in vitro, and none showed glycogenolytic activity in vivo at concentrations up to 2000 μg/kg. These latter compounds could act as glucagon antagonists in the in vitro liver plasma membrane adenylate cyclase system by shifting glucagon-stimulated adenylate cyclase dose–response curves to higher values. Glucagon antagonists such as those described here will be useful tools for investigating the mechanisms of glucagon action and for the further development of glucagon antagonists.

Glucagon is a 29 amino acid peptide hormone, produced in the α cells of the pancreas. Although the effects of glucagon on the liver to produce glucogenolysis and gluconeogenesis are well-known, the importance of glucagon in the pathogenesis of diabetes mellitus remains a matter of considerable controversy (Unger, 1978; Sherwin et al., 1978; Lefebvre & Luyckx, 1979; Vranic et al., 1981; Unger, 1985).

Glucagon activates the adenylate cyclase of liver plasma membranes and hepatocytes (Rodbell et al., 1974). It is generally accepted that cAMP[1] activates an enzymatic cascade that leads to the stimulation of glycogenolysis (Rodbell, 1980). On the other hand, Fain and Shepherd (1977), using an excess of 2′,5′-dideoxyadenosine, showed that the glucagon-activated stimulation of glycogenolysis is cAMP independent under these conditions. Glucagon also stimulates amino acid and lipid catabolism, which results in gluconeogenesis and ketosis (Farah, 1983). In diabetes mellitus, hyperglycemia and ketosis are generally associated with elevated levels of glucagon in the blood. This suggests that excessive glucagon is a contributing factor in the metabolic abnormalities of diabetes mellitus (Bartusch-Marrain, 1983). According to the bihormonal hypothesis regarding diabetes mellitus (Unger, 1978), insulin deficiency causes impairment of glucose utilization, but overproduction of glucose and ketones by the liver is primarily mediated by glucagon.

Several investigators have tried to develop glucagon-receptor antagonists that would provide direct evidence for glucagon's role in diabetes mellitus (Hruby et al., 1986). Most glucagon structure–activity studies are based on receptor binding and adenylate cyclase activity. The basic approach to design antagonists is to dissociate the structural, conformational, and dynamic properties necessary for binding from those necessary for transducing the message into the cell (Hruby, 1981; Hruby, 1982). It is well established that most of the residues 1–29 contribute to the binding of glucagon to its hepatic receptor,

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; Bzl, benzyl; cAMP, adenosine cyclic 3′,5′-monophosphate; Chx, cyclohexyl; DCC, dicyclohexylcarbodiimide; For, formyl; GTP, guanosine 5′-triphosphate; HF, hydrofluoric acid; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; LAP, leucine aminopeptidase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. The standard abbreviations and nomenclature for amino acids, peptides, and peptide derivatives of IUB/IUPAC are used throughout. Amino acids except glycine are of the L configuration unless otherwise stated.

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whereas single modifications in positions 1–5 and 12 result in partial agonists (Hruby, 1982). These and many other studies (Hruby et al., 1986) suggest that the C-terminal region of glucagon is primarily important in binding, while the N-terminal region mediates transduction. By application of Chou and Fasman prediction rules (Chou & Fasman, 1978), the following conformational potentials have been predicted for glucagon: an α-helical or β-sheet conformation in the sequence 19–29 and a β-turn in positions 2–5, 10–13, and 15–18. The three-dimensional conformation of micelle-bound glucagon has been examined by ^{1}H NMR spectroscopy (Braun et al., 1983). The secondary structure on a micelle/water interface subdivides the sequence into similar domains as predicted by the Chou and Fasman rules: the sequence 17–29 forms an amphiphilic helix, sequence 10–14 is helical, and sequence 1–5 is extended into the water phase. The above results suggest that the sequence 1–5 of glucagon is the most critical part of the molecule with respect to transduction, whereas the conformation of sequence 6–18 might affect the proper fit of sequence 1–5 to the "active site" of the receptor.

Bregman et al. (1980) prepared semisynthetically [N^{α}-(trinitrophenyl)-His^1,homo-Arg^{12}]glucagon (THG), which was shown to have in vitro antagonist activites. This analogue also was able to lower the blood glucose levels of diabetic rats (Johnson et al., 1982). Subsequently, THG was found to possess extremely weak, partial agonist activity for cAMP accumulation (Corvera et al., 1984). However, THG was a full agonist for the stimulation of glycogenolysis and gluconeogenesis in rat hepatocytes (Corvera et al., 1984). The above results stress the need to develop fully synthetic analogues to explore in more detail the structural and conformational properties of glucagon necessary to obtain glucagon-receptor antagonist analogues. In particular, we have sought to develop glucagon analogues that would bind to glucagon receptors in liver membranes, would not stimulate adenylate cyclase, and would competitively inhibit glucagon-stimulated adenylate cyclase activity.

In this paper we describe the total synthesis of six new glucagon analogues by solid-phase methodology and the conversion of one of these analogues to its tetraiodo derivative. In our design approach we obtained antagonists by combining structural features that led to partial agonist properties. The new analogues were tested for their effects on adenylate cyclase activity and on binding to glucagon receptors in rat liver membranes and for their effects on glucose levels in normal rats.

Experimental Procedures

Materials. N^{α}-Boc-amino acids were purchased from Vega Biotechnologies (Tucson, AZ) or were prepared by standard literature methods. Boc-Asp(Chx) was obtained from Peptides International (Louisville, KY). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA) (Halocarbon Products, NJ); anisole, 1,2-ethanedithiol, 1-hydroxybenzotriazole (HOBt), *N*-acetimidazole, and diisopropylethylamine (Aldrich, Milwaukee, WI); 3 *N* mercaptoethanesulfonic acid, ninhydrin, and dicyclohexylcarbodiimide (DCC) (Pierce, Rockford, IL); chloromethylated polystyrene resin 1% cross-linked with divinylbenzene (Lab Systems, San Mateo, CA); purified acetonitrile (Burdick & Jackson, Muskegon, MI); bovine serum albumin, chromatographic alumina (type WN_3, neutral), cAMP, ATP, GTP, and all enzymes (Sigma, St. Louis, MO); $Na^{125}I$, carrier free (Amersham, Arlington Heights, IL); [^{3}H]cAMP (New England Nuclear, Boston, MA); [α-^{32}P]ATP (ICN Radiochemicals, Irvine, CA); crystalline porcine glucagon (Elanco, Indianapolis, IN); dialysis membranes (Spectrum Medical Ind., Los Angeles, CA); Dowex AG 50-W4 cation-exchange resin (Bio-Rad, San Diego, CA).

A Vega-Coupler 1000 peptide synthesizer (Tucson, AZ) was used for peptide synthesis. Amino acid analyses were performed with a Beckman Model 120C analyzer. A Perkin-Elmer HPLC system was used for analysis and purification of peptides. For the binding experiments, a Millipore (Bedford, MA) filtration apparatus and 0.45-μm filters (Cellulose acetate, Oxoid, Columbia, MD) were used. The glucose concentration was measured with a Beckman glucose analyzer. Male Sprague-Dawley rats were used for the isolation of liver membranes and Wistar rats for all in vivo experiments.

Preparation of Resin. *tert*-Butoxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl resin (PAM resin) was prepared as previously described (Tam et al., 1979) with a chloromethylated poly(styrene-co-1% divinylbenzene) resin. When working with the standard Merrifield resin, the first amino acid was coupled by the CsCl method (Gisin, 1973). The resin substitutions were 0.1–0.2 mM/g and were determined by amino acid analyses of Thr.

Synthesis of Glucagon Analogues. All amino acids were coupled as their N^{α}-Boc derivatives. Reactive side chains were protected as follows: Ser and Thr, *O*-benzyl; Lys, 2,4-dichlorobenzyloxycarbonyl; His and Arg, tosyl; Glu, *O*-benzyl; Asp, *O*-cyclohexyl; Tyr, *O*-(2-bromobenzyloxycarbonyl); Trp, formyl. Except for Asn, protected amino acids were coupled to the growing peptide chain as their symmetric anhydrides (Hagenmaier & Frank, 1972). Asn was coupled as its *p*-nitrophenyl ester (3 equiv) in the presence of an equimolar amount of 1-hydroxybenzotriazole, and any unreacted chains were terminated by *N*-acetylimidazole (10 equiv in methylene chloride, 6 h). The same termination reaction was performed if the ninhydrin test was still positive after double coupling.

The synthesis was begun by loading 3 g of Boc-threonyl-oxymethyl-PAM resin into a 60-mL reaction vessel. All the metered solvent volumes were 40 mL, except for amino acid reagents that were 12 mL. One cycle of the synthesis consisted of the following operations: (1) CH_2Cl_2 washes and rinses (3 × 1 min); (2) deprotection with 48% trifluoroacetic acid/2% anisole/CH_2Cl_2 (1 × 2-min prewash, 1 × 20 min); (3) CH_2Cl_2 washes (5 × 1 min); (4) neutralize with 10% diisopropylethylamine in CH_2Cl_2 (3 × 3 min); (5) CH_2Cl_2 washes (3 × 2 min); (6) filter preformed symmetric anhydride of Boc-amino acid into reaction vessel (3 equiv in CH_2Cl with DMF, if necessary), couple 30 min; (7) repeat step 6 using DMF as solvent; (8) CH_2Cl_2 wash (1 × 2 min); (9) ethanol wash (1 × 2 min); (10) CH_2Cl_2 wash (1 × 2 min). Following the washing steps after each coupling and deprotection, an aliquot of the resin was monitored qualitatively by the ninhydrin method described by Kaiser et al. (1970).

Cleavage and Purification. Hydrofluoric acid (HF) cleavage was performed with 15 mL of liquid (HF/anisole/1,2-ethanedithiol, 85/10/5) per gram of resin and was allowed to proceed for 30 min at 4 °C. The reaction product was washed extensively with ether and then stirred in 20 mL of 30% acetic acid for 1 h. The filtrate was dialyzed 3 times against 4 L of 2% acetic acid for 2 h or longer and lyophilized.

Purification by ion-exchange chromatography was carried out on a 2.5 × 30 cm (150-mL) SP-Sephadex G-25 column. The dialyzed and lyophilized HF cleavage product was dissolved in 15 mL of starting buffer (10% acetic acid, 6 M urea, pH 3.3). After prewash with 150 mL of starting buffer, a sodium chloride gradient (0–1 M NaCl, 2 × 300 mL) was applied. Fractions of 4.3 mL were collected; the flow rate was

Table I: Physical Data of New Glucagon Analogues

compd	no.	E_{280} (mol^{-1} cm^{-1})[a]	yield (%)[b]	HPLC, k'[c]
glucagon	1	8210		3.44
[D-Phe^4,Tyr^5,Arg^{12}]glucagon	2	8800	1.6[d]	3.91
[D-Phe^4,Tyr^5,Lys^{17,18}]-glucagon	3	9100	3.7	4.27
[Phe^1,Glu^3,Lys^{17,18}]glucagon	4	8600	6.2	3.93
[Glu^3,Val^5,Lys^{17,18}]glucagon	5	8100	3.0	3.55
[Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]-glucagon	6	7700	7.0	3.32
I_4-[Asp^3,D-Phe^4,Ser^5,-Lys^{17,18}]glucagon[e]	7	11500		5.70
[Pro^3]glucagon	8	9200	1.2[d]	3.56

[a] Absorbance at 280 nM, measured in 10% acetic acid; the concentration was determined by amino acid analysis (SD ±5%). [b] Based on initial amino acid substitution. [c] In 0.1% trifluoroacetic acid, gradient 30–50% acetonitrile in 20 min. [d] These syntheses were carried out on phenylacetamidomethyl (PAM) resin. [e] Semisynthetic product from iodination of **6** with an average of four iodines incorporated (see Experimental Procedures).

30 mL/h. Peak fractions, eluted by 0.3 M chloride, were collected and dialyzed.

The final purification was carried out by preparative reverse-phase high-performance liquid chromatography (RP-HPLC), with a 15-mL semipreparative VYDAC 218 TPB-16 column. The following gradient system was used: 30–50% acetonitrile in 0.1% trifluoroacetic acid (1%/min). The flow rate was 5 mL/min. The results from the HPLC and the ϵ_{280} for the glucagon analogues prepared in this work are given in Table I.

By use of the above methodologies for synthesis and purification, the following totally synthetic glucagon analogues were prepared: (a) [D-Phe^4,Tyr^5,Arg^{12}]glucagon (**2**); (b) [D-Phe^4,Tyr^5,Lys^{17,18}]glucagon (**3**); (c) [Phe^1,Glu^3,Lys^{17,18}]-glucagon (**4**); (d) [Glu^3,Val^5,Lys^{17,18}]glucagon (**5**); (e) [Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon (**6**); (f) [Pro^3]glucagon (**8**). Purity was assessed by RP-HPLC as shown in Table I (single homogeneous compounds were obtained—98% purity by amino acid analysis after hydrolysis in 3 *N* mercaptoethanesulfonic acid and after enzymatic hydrolysis and also by peptide mapping).

Amino Acid Analysis. Purified peptides were hydrolyzed by 3 *N* mercaptoethanesulfonic acid at 120 °C for 24 h. Enzymatic total hydrolysis was carried out as follows: 0.1 mg (1.33 units) of microsomal leucine aminopeptidase and 1 mg of peptide were incubated in 1 mL of Tris-HCl buffer (50 mM, pH 8.5; $MgCl_2$, 5 mM) for 15 h at 40 °C. In cases where the peptide contained D-Phe, the pH was adjusted to 6.8, and 0.01 mg of carboxypeptidase Y was added; then the sample was incubated again at 40 °C for 15 h. Amino acid analyses were carried out on a Beckman 120C amino acid analyzer. The results for all the new compounds are shown in Table II.

Peptide Mapping. Glucagon or glucagon analogue (0.3 mg) and chymotrypsin (100/1 w/w) were incubated in 1 mL of sodium bicarbonate buffer (10 mM, pH 8.2) for 15 h at room temperature. The same treatment was repeated 2 more times. After lyophilization, the sample was dissolved in 0.1% trifluoroacetic acid. The peptide mixtures were analyzed by RP-HPLC (0.1% TFA, acetonitrile gradient 5–30% in 25 min, flow rate 2 mL/min). Patterns for the glucagon analogues were compared with those obtained with glucagon in each case. Individual peaks were isolated and subjected to amino acid analysis.

Preparation of Rat Liver Membranes. Rat liver membranes were prepared as described (Neville, 1968) and modified (Pohl et al., 1971). The livers of six animals were usually processed. The membrane preparation was stored in fractions (0.25 mL of buffer with 1.5 mg of protein) in liquid nitrogen. Membrane protein was determined as previously described (Markwell et al., 1978).

Iodination of Glucagon and of [Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon. To 3 nM of glucagon (20 μL of a 0.15 mM solution in 2 mM Na_2CO_3, OD_{280} = 1.25) and 2.17 mCi of ^{125}I (1 nM, 22 μL) in 20 μL of phosphate buffer (0.6 M, pH 7.2) 0.5 nM of chloramine T (2.8 mg/100 mL) was added 3 times at time intervals of 30 s. The reaction was terminated with 20 μL of $NaHSO_3$ (0.5%) and 20 μL of Trizma base (0.5 M); then 1 mL of elution buffer was added. The purification was carried out with QAE-Sephadex A-25 as previously described (Jorgensen & Larsen, 1972). The yield was 20 mL of solution with 4.5×10^8 dpm. A fraction was analyzed by RP-HPLC, and the main peak contained 70% of the total radioactivity. The specific binding was 90%. I_4-[Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon (**7**) was prepared from [Asp^3,D-Phe^4,Ser^5,Lys^{17,18}]glucagon (**6**) by methods similar to those previously described for glucagon (Desbuquois, 1975) and then purified by RP-HPLC.

Receptor Binding Assay. A total of 400 μL of Tris-HCl buffer (25 mM, pH 7.5, bovine serum albumin, 1%) containing

Table II: Amino Acid Analysis of New Glucagon Analogues[a]

	1	2[b]		3[b]		4		5		6[b]		8	
		A	E	A	E	A	E	A	E	A	E	A	E
Lys	1			2.7	3.2	3.3	3.0	3.0	3.1	2.9	3.2	0.9	1.0
His	1	1.0	0.9	0.9	1.0			0.9	1.0	0.9	1.0	0.8	0.7
Arg	2	3.0	2.9									2.1	2.0
Asp	3		3.0		2.5		3.5		2.8		3.1		2.8
Asx	4	4.0		3.8		3.9		3.9		4.9		4.0	
Thr	3	2.0		1.8		2.8		2.1		2.0		3.0	
Ser	4	3.9	8.2	3.8	8.6	3.7	8.1	3.9	8.0	5.1	8.0*[d]	4.0	8.9
Gln[c]	3												
Glu							1.1		1.1				
Glx	3	3.0		2.6		2.9		3.1		2.0		2.1	
Pro												0.9	0.5
Gly	1					1.4	1.1	1.5	1.1			0.9	1.0
Tyr	2	3.0	2.1*	2.9	2.1*	2.0	2.0	2.0	2.2	2.0	2.1	1.9	2.1
Phe	2	3.1	2.2*	2.9	2.1*	2.6	3.0	1.8	2.0	3.0	1.8*	1.8	1.9

[a] The numbers refer to the analogues as indicated in Table I. Analogues were hydrolyzed by mercaptoethanesulfonic acid (A) and leucine aminopeptidase (E). Analysis results are given only for those amino acids that are either added or deleted relative to native glucagon. Satisfactory analysis results were obtained for all other amino acid residues. [b] Analogues containing D-Phe, hydrolyzed by leucine aminopeptidase and carboxypeptidase Y. [c] As pyrrolidonecarboxylic acid in enzymatic digest; peak overlapped with Thr and Ser peaks. [d] An (*) indicates peptide D-Phe-X was not hydrolyzed by enzymes.

the membrane (50 μg of protein) was incubated with 50 μL of peptide solution (Tris-HCl buffer) and 50 μL of ^{125}I-glucagon (100000 cpm, 25 fM) for 10 min at 30 °C and then cooled in an ice bath for 20 min. The samples were then filtered through Oxoid filters and washed with 4 × 1 mL of Tris-HCl buffer [see McKee et al. (1986) for further details]. The radioactivity retained by the filters was counted. Nonspecific binding measured in the presence of excess unlabeled peptide (1024 nM) was typically 15–20% of the total binding and was subtracted from the total to give specific binding. Assays were run in triplicate and repeated 2 or 3 times. Data analysis was as reported previously (McKee et al., 1986).

Adenylate Cyclase Assay. The assay was carried out as previously described (Lin et al., 1975), and the cAMP was separated by the method of Salomon et al. (1976). The assay volume was 0.10 mL (25 mM Tris-HCl buffer, pH 7.5; 1% bovine serum albumin; 5 mM $MgCl_2$; 10 mM GTP; 1 mM EDTA; 1 mM [α-^{32}P]ATP, i.e., about 50 cpm/pM; 1 mM cAMP containing 10000 cpm [^{3}H]cAMP; 35 μg of membrane protein and an ATP regenerating system that had 20 mM phosphocreatine and 0.72 mg/mL or 100 units/mL creatine phosphokinase). Assays were done in triplicate and repeated 2 or 3 times. Data analysis was as reported previously (McKee et al., 1986).

Glycogenolysis. Male Wistar rats, weighing 260–300 g and fed ad libitum, were anesthesized by intraperitoneal injection of sodium pentobarbital (7 mg/100 g of body weight). Wistar rats were used in the glycogenolytic assay because in our experience they have more reproducible glycogenolytic responses to glucagon than Sprague-Dawley rats. After 30 min, a 0.10-mL blood sample was drawn from the tail vein into a heparinized microhematocrit capillary tube. The capillary tubes were centrifuged to separate the plasma, and the plasma was analyzed for glucose concentration on a Beckman glucose analyzer. Immediately after the base-line blood sample was obtained, glucagon, glucagon analogue, or saline dissolved in 0.5 mL of normal saline containing 0.25% bovine serum albumin was injected subcutaneously. After 20 min, a second 0.10-mL blood sample was obtained from the tail vein to measure the plasma blood glucose concentration. Glucose concentrations were measured in triplicate.

Results and Discussion

The glucagon analogues were synthesized by methods very similar to those already established for glucagon and glucagon analogues (Mojsov & Merrifield, 1981; Krstenansky et al., 1968a). Generally, a 3-fold excess of symmetric anhydride with double coupling on Merrifield or phenylacetamidomethyl (PAM) resin (Tam et al. 1979) was used throughout for the syntheses of the new analogues reported here. The peptides were cleaved from the resin, and the protecting groups were removed by use of anhydrous HF in the presence of scavengers, and the crude product was purified by dialysis, ion-exchange chromatography, and reverse-phase preparative high-performance liquid chromatography (RP-HPLC). The analogues were obtained in a highly purified form in an average yield of 5% on Merrifield resin (Table I). Amino acid analysis after total hydrolysis with mercaptoethanesulfonic acid gave the correct amino acid composition (Table II). Purity and chiral integrity were assessed by hydrolysis with leucine aminopeptidase (LAP) or by both LAP and carboxypeptidase Y (Table II). LAP converted Gln into pyrrolidonecarboxylic acid, which overlapped with the Thr and Ser peaks. In the enzymatic degradation of D-phenylalanine-4-containing peptides, the dipeptide D-Phe-X was left undigested. With the exception of the above restrictions, enzymatic hydrolysis also

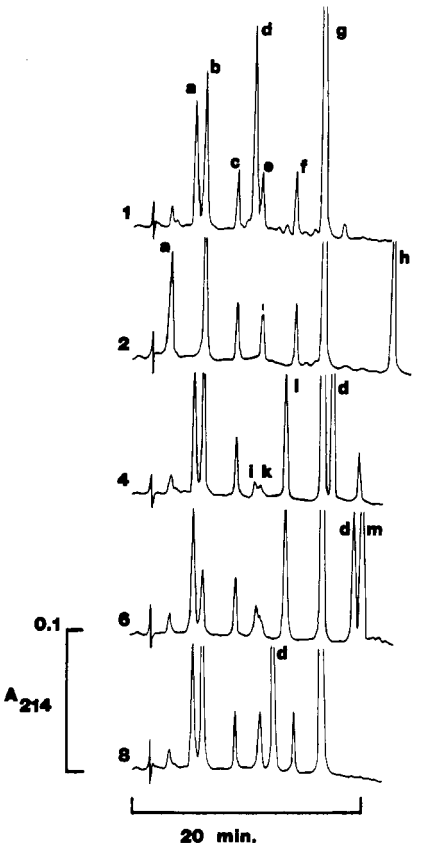

FIGURE 1: Peptide mapping. Glucagon analogues (0.3 mg) were digested with chymotrypsin as outlined under Experimental Procedures, and the digestion products were analyzed by RP-HPLC. The individual peaks were isolated, and the amino acid composition was determined. The numbers refer to the glucagon analogues in Table I. The peaks represent the following partial sequences of glucagon: (a) 10–13, (b) 7–10, (c) 26–29, (d) 1–6, (e) 18–22, (f) 14–17, (g) 23–25, (h) 1–5, (i) 18–22, (k) 7–13, (l) 14–22, and (m) 1–10.

gave the correct amino acid composition. These results show that no (≤3%) racemization occurred during the synthesis and that no modified amino acids are present in the purified analogues. Furthermore, all analogues were analyzed for their purity and sequence fidelity by the extremely useful method of peptide mapping. For this purpose, we have utilized chymotryptic digestion followed by analysis on RP-HPLC. The HPLC patterns that were obtained were directly compared with those obtained with glucagon (Figure 1). Differences from glucagon occurred only in those peaks in which modifications were introduced. Amino acid analysis of these shifted peaks showed the expected composition. The HPLC pattern when combined with amino acid analysis of the peptide fragments for final proof of structure of the appropriate fragment(s) gave unequivocal confirmation of structure for the new analogues.

As already mentioned, single modifications in the N-terminal region previously have led to partial agonist analogues of glucagon. In the approach used in this study, we sought to test if two modifications in this region would result in antagonist analogues. In some of the analogues we exchanged the arginine residues in positions 17 and 18 by lysine. This modification simplified the synthesis and increased the yield and solubility of the final compound (Krstenansky et al. 1986b).

[Phe^1]glucagon (Sueiras-Diaz et al., 1984) was previously shown to be a partial agonist (McKee et al., 1986). We have synthesized [Phe^1,Glu^3,Lys^{17,18}]glucagon (**4**), which was found to be a weak partial agonist with only 10% the maximal

Table III: Biological Activities of Glucagon Analogues[a]

compd	receptor binding, IC_{50} (nM)	relative binding potency[b]	adenylate cyclase, EC_{50} (nM)	adenylate cyclase potency (%)	adenylate cyclase max stimulation (%)	glycogenolysis mg/dL	glycogenolysis μg
glucagon (1)	6.7 (3.1–11)	100	9.6 (4.5–16)	100	100	103	5
[D-Phe[4],Tyr[5],Arg[12]]glucagon (2)	708 (560–1000)	1	ia[c] at 10 μM		0	0	2000
[D-Phe[4],Tyr[5],Lys[17,18]]glucagon (3)	501 (355–630)	1.3	500 (350–630)	1.9	5	60	2000
[Phe[1],Glu[3],Lys[17,18]]glucagon (4)	178 (140–355)	3.8	250 (180–315)	3.8	10	69	500
[Glu[3],Val[5],Lys[17,18]]glucagon (5)	1580 (1260–2510)	0.4	1600 (900–2500)	0.6	10	40	2000
[Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (6)	500 (400–630)	1.3	ia[c] at 100 μM		0	0	2000
I_4-[Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (7)	125 (100–180)	5.3	ia[c] at 10 μM		0	0	2000
[Pro[3]]glucagon (8)	220 (180–315)	3.0	395 (315–500)	2.4	40	40	2000

[a] Receptor binding and adenylate cyclase activities were measured with rat liver membranes (see Experimental Procedures). Values are the mean of at least three independent experiments with 95% confidence limits in parentheses. Glycogenolysis was determined in vivo as an increase of the blood sugar level 20 min after subcutaneous injection, and the values are the mean of at least four animals. [b] Binding potencies are given relative to glucagon = 100 and are calculated as follows: relative binding potency = [(receptor binding EC_{50} for glucagon)/(receptor binding EC_{50} for glucagon analogue)] × 100. [c] ia = inactive.

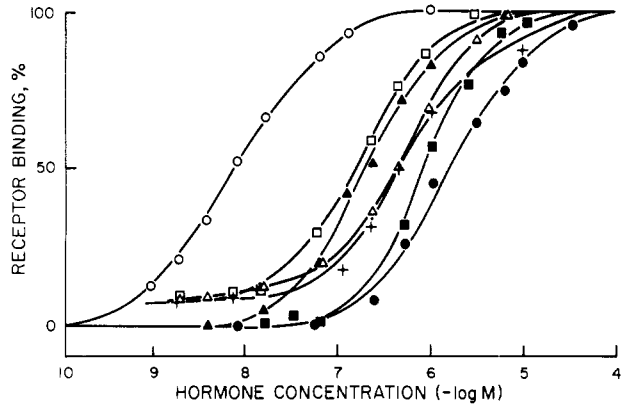

FIGURE 2: Receptor binding of glucagon analogues to rat liver membranes. Binding was determined as ^{125}I-glucagon displacement. Points are corrected for radioactivity (nonspecific binding) that remained bound in the presence of 1 μM glucagon. Each point is the mean of two to three independent experimentts. (○) Glucagon (**1**), (▲) [Pro[3]]glucagon (**8**), (□) [Phe[1],Glu[3],Lys[17,18]]glucagon (**4**), (●) [Glu[3],Val[5],Lys[17,18]]glucagon (**5**), (△) [Asp[3],D-Phe[4],Ser[5],Lys[17,18]]-glucagon (**6**), (■) [D-Phe[4],Tyr[5],Arg[12]]glucagon (**2**) and (+) [D-Phe[4],Tyr[5],Lys[17,18]]glucagon (**3**).

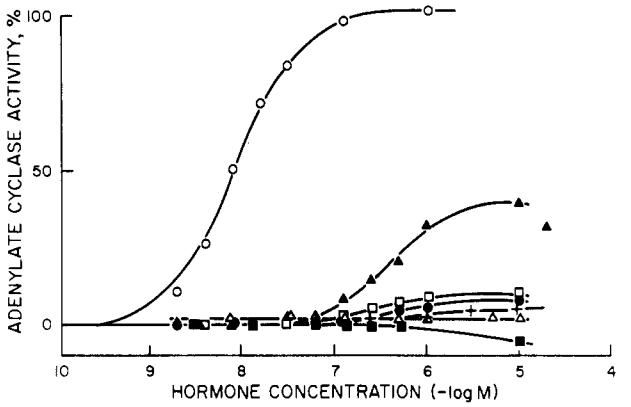

FIGURE 3: Adenylate cyclase activity of new glucagon analogues. cAMP production of rat liver membranes was measured as described under Experimental Procedures. Points are corrected for basal activity and are the mean of two to three independent experiments. Symbols are the same as for Figure 2.

stimulation and 3.8% the potency of glucagon in the adenylate cyclase assay (Figure 2) and in the binding assay (Figure 3). However, this poor partial stimulation of the adenylate cyclase system above basel levels nonetheless was sufficient to significantly increase the blood glucose levels in normal rats (Table III). Pseudoisosteric modifications can change the specific interactions with the "active site" of the receptor, whereas the overall fit of the molecule to the receptor remains intact. [Glu[3],Val[5],Lys[17,18]]glucagon (**5**), an analogue in which the Gln-3 residue of glucagon was replaced by Glu and the Thr-5 residue by Val, retained only 10% partial agonist activity and only 0.6% of the potency of glucagon in the adenylate cyclase assay but nonetheless retained some ability to increase glycogenolysis in vivo. The β-turn potential in sequence 2–5 of compound **5** as measured by Chou and Fasman (1978) analysis is very low. To increase the β-turn potential, we replaced Glu-3 and Val-5 by Asp and Ser, respectively, and in addition put a D-Phe residue in position 4 for a possible increase in the stability of the analogue to enzymatic breakdown and for a possible increase in in vivo potency (Sueiras-Diaz et al., 1984; McKee et al., 1986). The resulting compound, [Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (**6**), had no adenylate cyclase activity at concentrations as high as 100 μM. The binding potency of **6**, however, was increased 3-fold relative to analogue **5** (Table III, Figure 3). No in vivo glycogenolytic activity was found at a dose 400 times higher than those of glucagon. It has been reported that iodination of glucagon increases agonist potency and in vivo half-life time (Desbuquois, 1975). We, therefore, decided to iodinate **6** to see if its binding potency would increase. Indeed, I_4-[Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (**7**) had a 4-fold increase in binding potency relative to **6** to 5.3% that of glucagon but had no detectable adenylate cyclase or glycogenolytic activity (Figures 2 and 3; Table III). To further assess the effet of increased β-turn potential in the 2–5 sequence of glucagon on bioactivity, we replaced the Gln-3 residue with a Pro residue, which likewise increases the β-turn potential. [Pro[3]]glucagon (**8**) retained 3% binding potency of glucagon, but this analogue still showed 40% adenylate cyclase activity and significantly increased blood glucose levels in vivo in normal animials (Table III). It would appear that a high β-turn potential of glucagon in the 2–5 sequence, per se, is not sufficient to produce a glucagon antagonist.

In a slightly different approach to obtaining an antagonist, we introduced a bulkier amino acid into one position instead of introducing two isosteric modifications, while still retaining a high β-turn potential, by synthesizing [D-Phe[4],Tyr[5],Lys[17,18]]glucagon (**3**). The adenylate cyclase activity was quite low, and the analogue showed a very weak partial agonist activity of 5% (Figure 3). However, the weak binding and adenylate cyclase potencies (Table III) were sufficient to produce a considerable increase in blood sugar (Table III) in normal animals. [Arg[12]]glucagon is a partial agonist with 50% the potency of glucagon (Hruby, 1982). Following up on our initial approach, we synthesized [D-Phe[4],Tyr[5],Arg[12]]glucagon

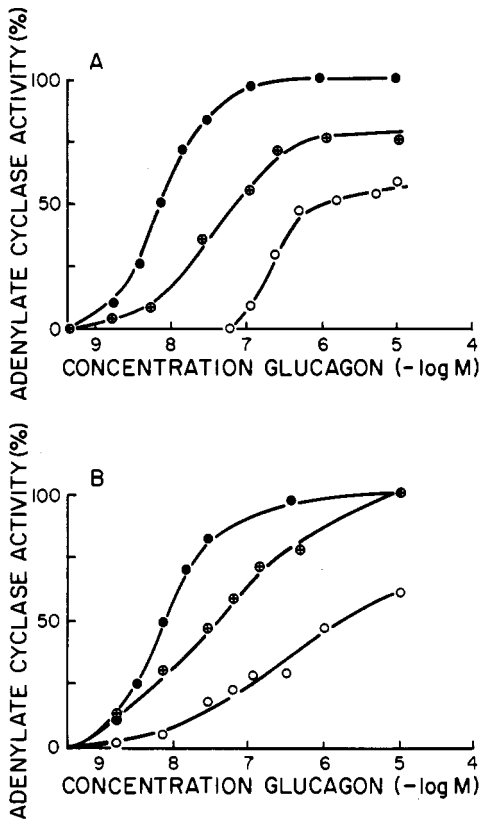

FIGURE 4: Inhibition of glucagon-mediated adenylate cyclase activity in liver plasma membranes by the glucagon analogues [D-Phe[4],Tyr[5],Arg[12]]glucagon (A) and [Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (**6**) (B). In (A), glucagon-stimulated adenylate cyclase activity was measured with glucagon alone (●), glucagon in the presence of 5 μM **2** (⊕), and glucagon in the presence of 10 μM **2** (○). In (B), glucagon-stimulated adenylate cyclase activity was measured with glucagon alone (●), glucagon in the presence of 1 μM **6** (⊕), and glucagon in the presence of 10 μM **6** (○). Data points are corrected for basal activity and are the mean of two to three experiments run in triplicate (see Experimental Procedures for further details).

(**1**). This analogue turned out to have 1% the binding potency of glucagon and no detectable adenylate cyclase or glycogenolytic activity (Table III).

Since the totally synthetic glucagon analogues [D-Phe[4],Tyr[5],Arg[12]]glucagon (**2**) and [Asp[3],D-Phe[4],Ser[5],Lys[17,18]]glucagon (**6**) were found to have no in vitro adenylate cyclase activity or in vivo glycogenolytic activity but both bind reasonably well to liver membrane receptors for glucagon, we decided to investigate whether they could act as glucagon antagonists by displacing the glucagon-stimulated adenylate cyclase activity to the right. The results of some of these competitive experiments are shown in Figure 4. In both cases, appropriate concentrations of the glucagon analogue could clearly displace the glucagon-stimulated adenylate cyclase activity dose–response curves to the right in a dose-dependent manner consistent with that of a receptor antagonist. Interestingly, analogue **2** showed a rather unique biphasic inhibition of the glucagon-stimulated adenylate cyclase dose–response curve. The origins of this biphasic inhibition are unclear, but it is apparent that the analogue can inhibit glucagon-stimulated adenylate cyclase activity. Whether these antagonist analogues can inhibit endogenous glucagon stimulation of glucose production in vivo remains for future investigations.

In this investigation we measured the adenylate cyclase activity of the glucagon analogues on rat liver membrane preparations (Figure 3) and observed monophasic binding curves (Figure 2), which were extended over 3 orders of magnitude. Recently, the glucagon binding to canine (Bonnevie-Nielsen & Tager, 1983) and rat hepatocytes (Musso et al. 1984) has been analyzed in terms of two noninterfering receptor populations. On the other hand, only one receptor protein was found by photoaffinity labeling (Herberg et al., 1984). In plasma membrane vesicles the respective dissociation constants for high- and low-affinity receptors differed by a factor of only 14 (Bonnevie-Nielsen & Tager, 1983). This gives the binding curves a monophasic appearance; apparently, part of the selectivity is lost in the process of preparing the plasma membranes. We think that this is also the case for our data. The binding curves of all analogues were parallel to those of glucagon, with the exception of [D-Phe$_4$,Tyr[5],Arg[12]]glucagon **3** (Figure 2). For the latter analogues, this may reflect a shift of relative affinity between the two receptors, which manifests itself in a biphasic inhibition curve in the adenylate cyclase assay of this analogue. Analogues with no detectable adenylate cyclase activity (**2**, **6**, and **7**) also had no detectable effect on blood glucose levels.

In summary, the totally synthetic glucagon analogues reported in this paper provide insight into those structural features of glucagon, especially in the N-terminal 1–5 region, which can lead to separation of the binding and transduction messages of the hormone (Hruby, 1981) and hence to antagonists analogues. Modification of the β-turn potential in the 1–5 region of glucagon by single- or double-residue substitutions leads to very weak partial agonists (analogues **3–5** and **8**), which, with the exception of [Pro[3]]glucagon (**8**), have only 5–10% partial agonist activity relative to glucagon in the adenylate cyclase assay. In other cases they led to a complete loss of adenylate cyclase activity but can act as glucagon receptor antagonists (Figure 4). Interestingly, even the very low 5% partial agonist activity of **3** (Figure 2) is sufficient to cause significant in vivo glycogenolytic activity (Table III). Clearly, a complete elimination of adenylate cyclase activity is necessary to also eliminate glycogenolytic activity of glucagon analogues that bind to liver plasma membrane glucagon receptors. Alternatively, it is possible that some of the glycogenolyses caused by glucagon and glucagon analogues may be mediated through mechanisms that do not involve adenylate cyclase.

ADDED IN PROOF

In a recent, collaborative study (Wakelam et al., 1986), we have obtained direct evidence for the presence of a second signal transduction system activated by glucagon in hepatocytes.

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