Structure−Function Studies on Positions 17, 18, and 21 Replacement Analogues of Glucagon: The Importance of Charged Residues and Salt Bridges in Glucagon Biological Activity†

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We have designed and synthesized eight compounds **2**−**9** which incorporate various amino acid residues in positions 17, 18, and 21 of the glucagon molecule: **2**, [Lys^{17}]glucagon amide; **3**, [Lys^{18}]glucagon amide; **4**, [Nle^{17},Lys^{18},Glu^{21}]glucagon amide; **5**, [Orn^{17,18}, Glu^{21}]glucagon amide; **6**, [D-Arg^{17}]glucagon; **7**, [D-Arg^{18}]glucagon; **8**, [D-Phe^{17}]glucagon; and **9**, [D-Phe^{18}]glucagon. Compared to glucagon (IC_{50} = 1.5 nM), analogues **2**−**9** were found to have binding affinity IC_{50} values (in nM) of 0.7, 4.1, 1.0, 2.0, 5.0, 25.0, 43.0, and 32.0, respectively. When these compounds were tested for their ability to stimulate adenylate cyclase (AC) activity, they were found to be full or partial agonists having maximum stimulation values of 100, 100, 100, 100, 87, 78, 94, and 100%, respectively. On the basis of the X-ray crystal structure of [Lys^{17,18},-Glu^{21}]glucagon amide reported here, the ability to form a salt bridge between Lys^{18} and Glu^{21} is probably key to their increased binding and second messenger activities. Among the eight analogues synthesized here, only analogue **4** preserves the ability to form a salt bridge between Lys^{18} and Glu^{21}. However, since these modifications are minor they do not seem to change the amphiphilic character of the C-terminus, allowing these analogues to reach 78−100% stimulation in the adenylate cyclase assay. Biological data from analogues **6**−**9** supports the idea that position 18 of glucagon may influence binding only, while position 17 may influence both receptor recognition and transduction.

Introduction

Diabetes mellitus is a widespread, degenerative disease that can be classified into two major types: type I, or insulin-dependent diabetes; and type II, or non-insulin-dependent diabetes. Type I diabetes is characterized by lack of suitable production of insulin by the beta cells of the pancreas, while in type II diabetes insulin levels are near normal, but increased gluconeogenesis and glycogenolysis leading to elevated blood glucose levels are observed. According to Unger[1] and Orci's[1−3] bihormonal hypothesis of diabetes mellitus, insulin deficiency causes impairment of glucose utilization, and glucagon is the primary mediator of the overproduction of glucose and ketone bodies in diabetes. Glucagon is a 29-residue polypeptide (MW = 3482) which interacts with hepatic receptors via a cAMP mediated pathway to stimulate glucose production and release (Figure 1).

1 5 10 15
H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-
20 25 29
Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH

Figure 1. Primary structure of glucagon.

Glucagon circulates in plasma in the free form, and since it does not associate with a transport protein, its plasma half-life is short (<5 min). The liver is the primary target of glucagon action. Glucagon binds to specific receptors in the hepatic cell plasma membrane, and this activates adenylate cyclase. The cAMP generated activates phosphorylase, which enhances the rate of glycogen degradation while inhibiting glycogen synthase and thus glycogen formation. The elevated cAMP levels also stimulate the breakdown of amino acids leading to glucose production by inducing a number of enzymes involved in gluconeogenesis. Glucagon also is a powerful lipolytic agent that activates hormone-sensitive lipase through increased adipose cell cAMP levels. The resulting free fatty acids can be metabolized for energy with the excess being converted to ketone bodies.

To obtain a complete understanding of the mechanism of action of glucagon and its involvement in the diabetic

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† **Abbreviations:** Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB commission on Biochemical Nomenclature. All amino acids except glycine are of the L-configuration unless otherwise stated. Other abbreviations: desHis, no histidine residue; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; DCM, dichloromethane; HPLC, high-pressure liquid chromatography; cAMP, 3′,5′-cyclic adenosine monophosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 3′-triphosphate; tBu, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; Trt, triphenylmethyl; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; C_{l2}-Z, 2,4-dichlorobenzyloxycarbonyl; Bzl, benzyl.

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state, the design and synthesis of glucagon analogues with specific properties is needed. Synthetic glucagon analogues can be used as specific biochemical probes that might enhance and identify those molecular features essential for receptor recognition (the binding message) and those necessary for transduction of the message and a corresponding physiological response (the activity message). A partial picture of the relationship between peptide structure and function has been developed, the basic features of which are summarized here. (1) The C-terminus of glucagon (residues 19−29) which has an α-helical conformation with amphipathic properties appears primarily important for receptor recognition and binding. (2) The N-terminal region (residues 1−5 and residue 9) is intimately involved in transduction of the biological message. (3) An important topographical relationship appears to exist between the N-terminal region and the region at or near Lys^{12} and Ser^{16}. (4) The 10−15 residue middle segment of glucagon seems to act as a hinge region, allowing proper orientation of the C-terminal region, important for receptor recognition, with respect to the N-terminal region, which contains the transduction message. (5) Phenylalanine at position 6 is a key residue in the N-terminal region for glucagon binding and is part of a hydrophobic patch that includes Tyr^{10} and Leu^{14}.

With this knowledge it now is possible to undertake a systematic rational approach to the synthesis of glucagon analogues, particularly those that might have therapeutic value in the treatment of diabetes. Using these approaches the Hruby[4−9] and Merrifield[10−13] groups have synthesized several glucagon antagonists, analogues which bind competitively to the receptor but do not elicit a biological response. One of the most potent antagonists which we have developed, [desHis[1],-desPhe[6],Glu[9]]glucagon amide,[14] which is the first pure glucagon antagonist, has been shown to block glucagon stimulated cAMP production in vitro and to lower blood glucose levels in streptozotocin induced diabetic rats similar to that reported previously for the antagonist, [N^{α}-trinitrophenyl-His[1], homo-Arg^{12}]glucagon (THG glucagon)[6,14,15] which is a partial agonist at higher concentrations.[5] Similar properties have been found for the antagonist [desHis[1],Glu[9]]glucagon amide, synthesized by Merrifield,[10] and this compound also is a partial agonist when measured in a cAMP accumulation assay using isolated hepatocytes in the presence of the phosphodiesterase (PDE) inhibitor Rolipram.[14] These data provides evidence in support of aspects of the Unger and Orci bihormonal hypothesis of diabetes mellitus.

It is generally accepted that cAMP is the major mediator of glucagon action as seen through increased levels of intracellular cAMP which are linked to activation of adenylate cyclase and inhibition of cAMP dependent phosphodiesterase. However some discrepancies between the nanomolar concentrations of glucagon needed to induce a positive agonist response and the micromolar concentrations needed to maximally stimulate cAMP levels exist. It has been found that miniglucagon (glucagon 19−29) can specifically inhibit the Ca^{2+} pump in liver plasma membranes independent of adenylate cyclase activation.[16,17] Miniglucagon may be the product of intracellular tryptic cleavage of the dibasic doublet (Arg^{17}−Arg^{18}) found in the glucagon molecule.

Table 1. Synthetic Glucagon Analogues

compd	structure
1A	glucagon
1B	[$Lys^{17,18}$,Glu^{21}]glucagon
2	[Lys^{17}]glucagon amide
3	[Lys^{18}]glucagon amide
4	[Nle^{17},Lys^{18},Glu^{21}]glucagon amide
5	[$Orn^{17,18}$,Glu^{21}]glucagon amide
6	[D-Arg^{17}]glucagon
7	[D-Arg^{18}]glucagon
8	[D-Phe^{17}]glucagon
9	[D-Phe^{18}]glucagon

We have designed and synthesized five compounds with modifications at Arg^{17}, Arg^{18}, and Asp^{21} aimed at enhancing receptor affinity while still maintaining the ability to transduce the biological message. We also have synthesized four analogues with substitutions at Arg^{17} and Arg^{18} to further study the proposed intracellular miniglucagon activities (Table 1) and to stabilize glucagon from proteolytic degradation. All analogues were tested for their ability to recognize and bind the receptor and for their ability to stimulate adenylate cyclase activity.

Results

Analogues **6**−**9** in this project were synthesized using an Applied Biosystems (ABI) Model 431 automated peptide synthesizer. The 431 performs an optimized protocol for the formation of amino acid HOBt-esters. Amino acids are coupled to the free α-amino groups on the growing peptide chain. This coupling is achieved in an aprotic polar solvent, *N*-methylpyrrolidone, which results in high coupling yields with minimal stoichiometric excesses. For the synthesis of glucagon amide analogues, a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin was used. Analogues **2**−**5** were coupled as their N^{α}-Boc derivatives to standard p-MBHA resin. The remaining amino acids were added to the growing peptide chain as their preformed symmetrical anhydrides. Double coupling was used throughout. Following synthesis all peptides were cleaved from the resin using standard techniques. All peptides were purified using C_{18} Vydac reverse phase HPLC columns. Analogues were characterized by amino acid analysis, electrospray mass spectroscopy, and thin-layer chromatography (see the Experimental Section).

[^{125}I]Glucagon is commonly used in studies of hormone binding. Monoiodoglucagon has been shown to have an identical concentration−activity dependence as native glucagon on hepatic membrane receptors. For receptor binding assays the [^{125}I]glucagon was prepared by the method of Hagopian and Tager.[18] Liver membrane was prepared by the method of Neville[19] and the assay carried out as previously described by Lin.[20] Results are expressed as the percent inhibition of [^{125}I]-glucagon specific binding for analogues **2**−**9** and are shown in Table 2.

Our laboratory uses a highly sensitive adenylate cyclase assay procedure.[21] This method employs sequential chromatography on columns of Dowex cation-exchange resin and aluminum oxide. With the use of α-^{32}P-ATP as a substrate this method allows a nearly complete separation of cyclic ^{32}P-AMP formed from the substrate and other ^{32}P-containing compounds, such as

Table 2. Biological Activities of Glucagon Analogues

	binding[a]		adenylate cyclase[a]		
compd	IC_{50} (nM)[b]	relative binding affinities (%)	EC_{50} (nM)[b]	maximum % stimulation	relative potency
1A, glucagon	1.5 [0.9−1.6]	100	5.6 [3.2−7.9]	100	100
1B, [$Lys^{17,18}$,Glu^{21}][c]	0.3 [0.2−0.4]	500	0.8	100	700
2	0.7 [0.5−0.9]	220	2.2 [1.8−2.7]	100	230
3	4.1 [3.8−4.2]	36	40.8 [24.0−69.4]	100	12
4	1.0 [0.7−1.2]	150	1.7 [1.4−2.1]	100	300
5	2.0 [1.7−2.2]	74	8.7 [4.7−16.2]	100	58
6	5.0 [4.8−5.2]	30	316 [211−420]	87	1.8
7	25.0 [23−28]	6	158 [132−198]	78	3.5
8	43.0 [40−43]	3.5	126 [92−169]	94	4.4
9	32.0 [30−35]	4.7	631 [502−780]	100	0.9

[a] Values given are the mean of at least two independent experiments in triplicate; relative binding affinities or adenylate cyclase potencies are calculated as follows: % binding affinity or potency = [IC_{50} or EC_{50} for glucagon /IC_{50} or EC_{50} for glucagon analogue] × 100. [b] 95% confidence limits are given in brackets. [c] Reference 22.

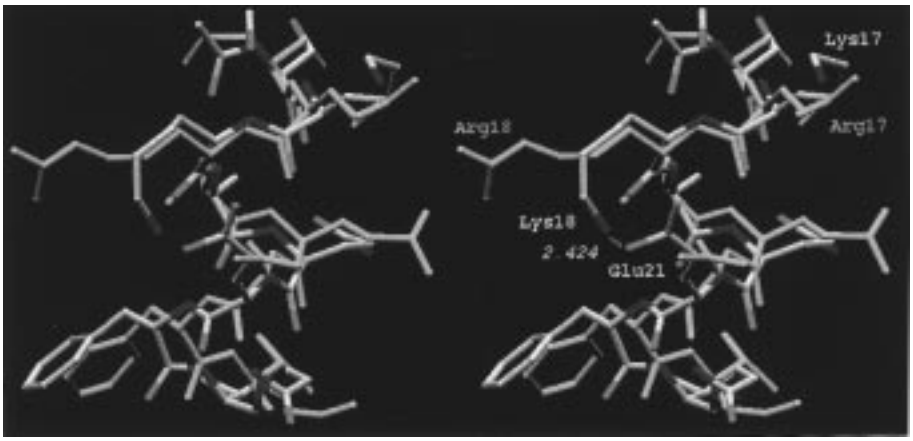

Figure 2. Superimposition of the X-ray crystal structures of glucagon and [$Lys^{17,18}$,Glu^{21}]glucagon amide. Stereoview of residues 16−23, including the region of the proposed salt bridge. Relevant amino acid residues and bond distances are indicated.

^{32}P in the assay blanks. This method permits detection of the small amounts of cyclic AMP formed at low enzyme concentrations or at early time points in kinetic studies. The results (Table 2) are expressed as potency relative to glucagon (100) and in comparison with the maximal stimulation of adenylate cyclase by glucagon (100%).

From the binding affinities and biological activities summarized in Table 2, analogues **2** and **4** show superagonist activity, with analogue **4** being approximately 1.5 times more potent than glucagon in binding affinity and 3 times more potent in adenylate cyclase stimulation. Analogue **2** is 2.2 times more potent in binding affinity and signal transduction. Two other analogues, [$Orn^{17,18}$,Glu^{21}]glucagon amide (**5**) and [Lys^{18}]-glucagon amide (**3**) are less potent in the binding and adenylate cyclase assays, but still give 100% maximum stimulation. With respect to the binding affinities on substitution in positions 18 and 21, [$Lys^{17,18}$,Glu^{21}]-glucagon (a superagonist), is the most potent followed by glucagon, followed by compounds [$Orn^{17,18}$,Glu^{21}]-glucagon amide (**5**) and [Lys^{18}]glucagon amide (**3**). Replacement of Arg^{17} by D-Arg produced analogues with 30% the binding affinity of glucagon, whose percent stimulation in the adenylate cyclase assay was 87%, while replacement at Arg^{18} gave 6% binding affinity and 78% stimulation relative to glucagon. Two other analogues [D-Phe^{17}]glucagon (**8**) and [D-Phe18]glucagon (**9**) are less potent in binding affinity (3−5%) and adenylate cyclase potency, but still give close to 100% maximum stimulation in the adenylate cyclase assay.

Discussion

Earlier the analogue [$Lys^{17,18}$,Glu^{21}]glucagon was reported to be a superagonist, with 5−7 times the receptor affinity and adenylate cyclase activity of similarly substituted compounds.[22] This increased potency was due to a significant increase in overall α-helical content[23] as determined by CD spectroscopy. We further examined this superagonist by comparison to glucagon with emphasis on distance geometry as provided by X-ray crystallography results (Figure 2). These results indicate that the difference between glucagon and the superagonist appears to be, primarily, the formation of a new salt bridge between NZ (the ammonium group of the side chain) of Lys^{18} and OE2 (the carboxylate group of the side chain) of Glu^{21} (distance = 2.4 Å). We believe that this new salt bridge may work as a stabilizing feature, and explain the increase in α-helix content we observed in the C-terminal region. By removing the possibility of a salt bridge formation entirely, as in the case of our earlier reported analogue [$Nle^{17,18}$]glucagon,[4] we produced a weak partial agonist with a binding affinity of 1800 nM (glucagon IC_{50} = 1.5 nM) and a maximum stimulation of only 10%.

The observed salt bridge between Lys^{18} and Glu^{21} may represent a stabilized noncovalent homologue of cyclic α-helical peptides such as those prepared by Taylor and co-workers (e.g., ref 24). Cyclic and multicyclic peptides prepared using this approach demonstrate that lactam bridges generated between Lys and Glu residues separated by three or four residues preferentially stabilize

α-helical conformations. In the context of [Lys[17,18],Glu[21]]-glucagon, a noncovalent interaction between Lys *i* and Glu *i* + 3 increases the potency of this glucagon agonist substantially. This result has two important implications for glucagon and its analogues. First it provides further evidence that the C-terminal portion of glucagon recognizes its receptor in an α-helical conformation. Second, it suggests that it may be possible to synthesize potent glucagon agonists by introducing strategically interleaved *i* to *i* + 3 or *i* + 4 side chain−side chain cross-links in the structure. Peptides with one or more interleaved lactam *i* to *i* + 4 bridges have high propensities for forming α-helices in aqueous solution. In addition, this cyclic or multicyclic analogue could be expected to be relatively resistant to proteolytic degradation.

Due to our interest in understanding the conformational effects of modifications at positions 17, 18, and 21, analogues **2**−**9** were designed, synthesized, and tested for binding affinity and for bioactivity in the adenylate cyclase assay. With the introduction of D-Arg and D-Phe in positions 17 and 18, we further explored the possibility of blocking the formation of miniglucagon (glucagon 19−29), a product of intracellular tryptic cleavage of the dibasic doublet (Arg[17]−Arg[18]). It has been found that miniglucagon can specifically inhibit the Ca^{2+} pump in liver plasma membranes independently of adenylate cyclase activation.[16,17]

The ability to form a salt bridge between positions 18 and 21 depends on the length and flexibility of the charged side chains in these positions. The X-ray crystal structure of [Lys[17,18],Glu[21]]glucagon amide suggests that the Lys[18]−Glu[21] pair is probably optimal for the salt bridge formation as compared to the Arg[18]−Asp[21] pair in the native glucagon (Figure 2). The salt bridge is probably the key feature responsible for the large increase in binding potency of [Lys[17,18],Glu[21]]-glucagon. Among the eight analogues synthesized here, only analogue **4** preserves this structural feature. Furthermore, in analogue **2**, where the native Arg[18]−Asp[21] pair is present, the salt bridge may be less stable. This in turn could explain a drop in potency from 0.3 nM[32] in the case of [Lys[17,18],Glu[21]]glucagon to 0.7 nM for analogue **2** (Table 2). In analogue **4** where the optimal Lys[18]−Glu[21] pair is present, but a neutral hydrophobic residue Nle is substituted at position 17, we observe a similar drop in potency which could be due to a loss of charge at position 17. Though a drop in potency is observed in analogues **2** and **4**, it is interesting to note that their binding remains relatively high compared to rest of the analogues in this study. We suggest that residues (Lys and Nle) might be creating a hydrophobic pocket that may enhance ligand−receptor interaction. Analogues **3** and **5** with Asp in position 21 and Orn in position 18 show a further drop in potency probably due to shortened side chains at these positions which, respectively, may hinder salt bridge formation. The further decreases in potency observed with compounds **7** and **9**, D-Arg[18] and D-Phe[18], are likely due to altered stereochemistry in the case of **7** and lack of charge at position 18 in analogue **9**. All of the above modifications do not seem to seriously alter the amphiphilic character of the C-terminus allowing these analogues to reach 78−100% stimulation in the adenylate cyclase assay.

Table 3. Mass Spectral Data for Tryptic Cleavage Fragments

	MS	
fragment	calculated	analyzed
1−18	2148.29	2148.9
1−12	1357.41	1357.9
13−18	808.90	809.5
13−17	652.71	653.4
18−29	1508.73	1508.9
19−29	1352.54	1353.0

Biological data from analogues **6**−**9** seems to complement the idea that position 18 may influence binding only, while position 17 may influence both receptor binding recognition and adenylate cyclase activation.[25] A similar observation was made in the case of [Nle[17,18]]-glucagon.[26] The substitution of Nle (**4**), D-Arg (**6**), and D-Phe (**8**) at position 17 decreases the potency of these analogues and may suggest an independent role for position 17 in the physiological activity of glucagon. With respect to compounds **2**−**4**, and looking only at position 17, we see a decrease in potency between [Lys[17,18],Glu[21]]glucagon amide with its free ϵ amino group to analogue **4** with an uncharged side chain, to compound **3** with a free guanidine side chain. Since Lys[17] is not directly involved in the salt bridge formation, a possible explanation for the function of position 17 is its direct involvement in receptor binding at Asp[64] on the glucagon receptor[27] or interaction with the N-terminal region of glucagon.

An endopeptidase that cleaves glucagon, producing miniglucagon, was recently isolated from rat liver membranes. The enzyme is inhibited by insulin, glucagon like peptide-1 and glucagon like peptide-1 amide (7−36), but other peptides that contain dibasic sites had no effect on its activity.[28] This indicates that the endopeptidase does not display strict selectivity toward dibasic doublets.[28] By replacing Arg[17] and Arg[18] with D-Arg and D-Phe, respectively, we hope to block the effects of the endopeptidase action on these peptides. The endopeptidase should not recognize D-amino acids, and in particular with D-Arg[18], we should not see any of the previously described biological effects of miniglucagon (19−29). HPLC results from tryptic cleavage of glucagon and analogues **6** and **7** are provided in the Supplementary Information. Results were as expected: glucagon gave four fragments 1−12, 13−17, 18−29, and 19−29, while [D-Arg[17]]glucagon and [D-Arg[18]]-glucagon gave four and three peaks (1−12, 1−18, 13−18, 19−29 and 1−12, 13−17, 18−29), respectively. All peaks were collected and subjected to mass spectral analysis, and results are presented in Table 3.

Given the results of our enzymatic studies, and in particular the fact that [D-Arg[18]]-glucagon did not yield any miniglucagon (residues 19−29) following tryptic cleavage, it is possible that this compound, an inhibitor of miniglucagon's action (personal communication from Dr. F. Pecker, miniglucagon's action on cardiac cells), may shed some light on a recently developed Ca^{2+} pump assay[16,17] and consequently on glycogenolysis. Miniglucagon may be the product of intracellular tryptic cleavage of the dibasic doublet (Arg[17]−Arg[18]) found in the glucagon molecule. The interaction of glucagon with liver cells leads to the cleavage of the hormone by endopeptidase at Arg[17]−Arg[18]. This gives rise to two fragments, glucagon(19−29), or miniglucagon, and glu-

cagon(18−29).[16,17] The miniglucagon fragment itself has been shown to have biological activity and induces glycogenolysis in hepatocytes independent of adenylate cyclase activation.[16,17]

The in vivo actions of glucagon potentially relied not only on the effects due to glucagon itself but also on the effects elicited by its metabolite, miniglucagon. When glucagon is replaced with a nonhydrolyzable analogue [D-Arg^{18}]glucagon, miniglucagon is not available for its biological action. Thus [D-Arg^{18}]glucagon will interfere with calcium cycling of cAMP independent pathway and acts as an inhibitor affecting the overall glycogenolysis.

To summarize our findings, the formation of a salt bridge between residues 18 and 21 is an important feature in enhancing glucagon binding to its receptor and an inhibitor (fragment 18−29) of miniglucagon's cAMP independent action (fragment 19−29) created by introducing D-Arg^{18} may alter Ca^{2+} pump activity to our advantage in controlling of excessive amount of glucose production as observed in diabetics.

Experimental Section

Materials and Methods: Materials. N^{α}-Fmoc and N^{α}-Boc amino acids and Rink acid resin 4-(2′,4′-dimethyoxyphenyl-hydroxymethyl)-phenoxy resin were purchased from Bachem California (Torrance, CA). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid, anisole, 1,2-ethanedithiol, methyl sulfide, diisopropylethylamine (Aldrich, Milwaukee, WI); dimethyl formamide and dichloromethane (Fisher Scientific, Fair Lawn, NJ); 1-hydroxybenzotriazole (Richelieu Biotechnologies, St. Hyacinthe, Canada); *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (Chem-Impex International, Wood Dale, IL); HPLC-quality acetonitrile (J. T. Baker, Phillipsberg, NJ); bovine serum albumin, chromatographic alumina (type WN3, neutral), cAMP, ATP, GTP and all enzymes (Sigma Chemical Co., 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); Dowex AG 50-W4 cation-exchange resin (BioRad, San Diego, CA); *p*-methylbenzhydrylamine resin (p-MBHA resin) (Peptides International, Louisville, KY).

Instrumentation. Purification and chromatographic analyses of purified peptides were performed on a Perkin-Elmer model 410-BIO using a VYDAC C18-reverse phase, 10 × 250 mm, 10 μm, 300 Å semipreparative column at ambient temperature. All traces were recorded with a Perkin-Elmer GP 100 graphics printer. All quantitative amino acid analysis data were obtained from peptide analogue hydrolysates on an Applied Biosystems 420 amino acid analyzer by Wallace Clark at the Macromolecular Structure Facility, University of Arizona in Tucson, AZ. Mass spectral data for compounds **2**−**5** were obtained on a AMD Intectra double focusing (BE) mass spectrometer at the University of Arizona, while mass spectral data for compounds **6**−**9** were obtained on a Finnigan-MAT SSQ700 mass spectrometer by Dr. Sue Weintraub at the University of Texas Health Sciences Center in San Antonio, TX.

Synthesis of Glucagon Analogues 2−5. All amino acids were coupled as their N^{α}-Boc derivatives to *p*-MBHA resin (0.39 mmol/g). Trifunctional amino acids were side chain protected as follows: Lys(2,4-Cl_2-Z), Orn(2,4-Cl_2-Z), Asp(Bzl), Glu(Bzl), Thr(O-Bzl), Ser(O-Bzl) and Trp(For). N^{α}-Boc-Asn-OH and N^{α}-Boc-Gln-OH were coupled with DCC in the presence of an equimolar amount of HOBt. The remaining amino acids were added to the growing peptide chain as their preformed symmetrical anhydrides. Double coupling was used throughout. The synthetic protocol consisted of the following steps: (1) 48% TFA/2% anisole in DCM (2 × 20 min); (2) DCM (3 × 1 min); (3) 10% DIEA in DCM (3 × 2 min); (4) DCM (4 × 1 min); (5) coupling performed as symmetric anhydride in DMF for 30 min or by DIC/HOBt for 2 h; (6) DCM (4 × 1 min); (7) repeat steps (1) through (6). The reactions were monitored by the Ninhydrin test.[29] If the tests gave a positive result, the unreacted peptide chains were acetylated with acetic anhydride. After the final synthetic step, the N-terminal Boc group was removed (steps (1) to (4)) and the resin dried in vacuo.

Cleavage, Purification, and Characterization for Compounds 2−5. The HF cleavage was performed using the method of Tam et al.[30] After removal of the HF the resin was washed with ethyl acetate and filtered; the peptide was recovered by extracting the resin with 30% acetic acid. The peptide solution was lyophilized and the residue subjected to either gel filtration (Sephadex G-15, 10% acetic acid) or dialysis against 2% acetic acid (Spectrapor dialysis tubing, molecular weight cutoff 1000). Further purification was achieved by ion exchange chromatography on a SP-Sephadex G-25 column (2.5 × 30 cm). The sample was eluted with a linear gradient of 0−1 N NaCl in 6 M urea/10% acetic acid (pH 3.3). The major peak was collected, lyophilized, and desalted. Final purification was carried out by semipreparative high-pressure liquid chromatography with a VYDAC C18 column (16 × 250 mm) as discussed above. The sample was eluted by applying a gradient of 30−40% acetonitrile in 0.1% aqueous TFA over 20 min. The product detected by UV monitoring at 280 nm was collected and lyophilized. Initial purity was determined by rechromatography on an analytical VYDAC C18 column (4.6 × 250 mm). Characterization and purity was assessed by thin-layer chromatography, amino acid analysis, and mass spectroscopy.

Synthesis of Glucagon Analogues 6−9. All peptide syntheses of these analogues were accomplished using an ABI 431 automated peptide synthesizer with the HOBt−HBTU Fmoc synthesis protocol. Trifunctional amino acids were side chain protected as follows: Arg(Pmc), Asp(OtBu), Glu(OtBu), Tyr(tBu), Ser(tBu), Thr(tBu), Gln(Trt), Asn(Trt), Lys(Boc), and Trp(Boc). The Rink acid resin (0.25 mmol, substitution 0.35 mmol/g) was placed in the reaction vessel (RV) while one cartridge (1.0 mmol) of the desired Fmoc amino acid was activated in situ as the HOBt/HBTU-ester and subsequently coupled to the resin for 20 min. The Fmoc protecting group on the α-amino acid was removed with piperidine (20%); following deprotection the resin was washed with NMP to remove the piperidine. The peptide resin, which was now ready for coupling, was left in the reaction vessel while the next amino acid in the chain was prepared. The dry, protected amino acid (1.0 mmol) contained in the cartridge was dissolved in a solution of NMP containing HOBt (0.5 M)/HBTU (0.45 M). This solution was transferred to another reaction activator vessel (ACT). After 5 min the active ester was transferred to the RV. Four equivalents of the activated amino acid one per equivalent of the growing peptide chain were employed in the coupling reactions. The deprotection and coupling steps were repeated twice with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid residue was deprotected using piperidine (20%), the resin was washed with NMP and thoroughly dried by vortexing under nitrogen.

Cleavage, Purification, and Characterization for Compounds 6−9. A cleavage mixture consisting of TFA (18.0 mL), methyl sulfide (0.5 mL), 1,2-ethanedithiol (0.5 mL), and anisole (1 mL) was chilled on ice. The resin bound peptide was placed in a disposable 50 mL polystyrene tube. The chilled cleavage mixture was added to the tube, and the resultant solution was bubbled with nitrogen for 2 min. The tube was capped, covered with aluminum foil, and stirred at room temperature for 2 h. The resultant slurry was filtered using a coarse scintered glass filter. The tube and resulting precipitate (resin) were washed with TFA (1 mL) followed by 3 × 5 mL of DCM. The filtrate was transferred to a round-bottom flask and rotary evaporated to a volume of 0.5 mL. Following the addition of cold ether (40 mL) the flask was cooled on dry ice−ethanol. After 1 h the precipitated peptide was filtered using a fine scintered glass filter and washed thoroughly with cold ether. The

Table 4. Amino Acid Analysis of Synthetic Glucagon Analogues

	2	3	4	5	6	7	8	9
Ala	0.95(1)	0.98(1)	1.08(1)	1.02(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
Arg	1.05(1)	0.96(1)			1.82(2)	1.76(2)	0.99(1)	1.00(1)
Asx	4.20(4)	4.24(4)	2.82(3)	2.82(3)	3.85(4)	3.81(4)	3.75(4)	3.91(4)
Glx	2.91(3)	3.03(3)	3.84(4)	4.20(4)	3.75(3)	2.80(3)	2.84(3)	2.93(3)
Gly	0.98(1)	1.05(1)	1.02(1)	0.95(1)	1.20(1)	1.04(1)	1.05(1)	1.08(1)
His	1.05(1)	1.02(1)	1.03(1)	0.91(1)	0.84(1)	0.85(1)	0.84(1)	0.88(1)
Leu	2.04(2)	2.04(2)	2.02(2)	1.86(2)	1.93(2)	1.96(2)	1.90(2)	1.96(2)
Lys	1.96(2)	2.06(2)	1.90(2)	1.04(1)	0.91(2)	0.95(1)	0.91(1)	0.97(1)
Met	1.05(1)	0.86(1)	1.00(1)	1.09(1)	0.84(1)	1.00(1)	1.05(1)	0.91(1)
Nle			1.03(1)					
Orn				1.82(2)				
Phe	2.00(2)	2.00(2)	2.00(2)	2.00(2)	1.80(2)	1.78(2)	2.85(3)	2.86(3)
Ser	4.08(4)	3.96(4)	3.88(4)	4.08(4)	3.87(4)	3.84(4)	3.91(4)	3.95(4)
Thr	2.97(3)	2.91(3)	3.33(3)	2.73(3)	2.75(3)	2.74(3)	2.83(3)	2.86(3)
Tyr	1.96(2)	2.00(2)	2.00(2)	2.04(2)	1.80(2)	1.78(2)	1.79(2)	1.89(2)
Val	0.93(1)	0.97(1)	0.97(1)	0.97(1)	0.84(1)	0.91(1)	0.92(1)	0.86(1)

Table 5. Physical Data for Glucagon Synthetic Analogues

	MS[a]		E_{280}[b]	HPLC[c]
compd	calculated	analyzed	($M^{-1}\,cm^{-1}$)	k′
2	3454.7	3454.8	5306	1.47
3	3454.7	3454.5	5621	1.48
4	3425.8	3425.5	6694	1.75
5	3413.8	3413.9	6473	1.74
6	3483.8	3483.0	5857	1.52
7	3483.8	3483.1	6554	1.52
8	3473.8	3473.8	5187	1.54
9	3473.8	3473.6	9327	1.54

[a] Compounds **2**−**5**: The mass spectra were carried out by FAB mass spectroscopy on a AMD Intectra double focusing (BE) spectrometer. Compounds **6**−**9**: The mass spectra were carried out by Electro Spray mass spectroscopy on a Finnegan-MAT SSQ700 spectrometer. [b] Absorbance was measured at 280 nm, and the concentration was determined by amino acid analysis for all the compounds. [c] k' values were determined under the following conditions: gradient 10−90% acetonitrile over 60 min, UV detection at 280 nm, flow rate 1 mL/min.

Table 6. Thin Layer Chromatography for Synthetic Analogues

	R_f values[a]		
compd	I	II	III
2	0.67	0.79	0.57
3	0.67	0.75	0.57
4	0.69	0.68	0.66
5	0.69	0.70	0.75
6	0.56	0.81	0.63
7	0.53	0.83	0.68
8	0.66	0.74	0.61
9	0.54	0.76	0.65

[a] Solvent compositions for compounds **2**−**5**. I: ethyl acetate (12); pyridine (4); acetic acid (4.2); water (2.2). II: 1-butanol (5); acetic acid (4); pyridine (1); water (5). III: 1-butanol (4); acetic acid (1); pyridine (1); water (3). Solvent compositions for compounds **6**−**9**. I: 1-butanol (15); acetic acid (3); water (12); pyridine (10). II: ethyl acetate (12); acetic acid (4.2); water (2.2); pyridine (4). III: 2-propanol (4); ammonium hydroxide (1); water (1).

peptide was dissolved in a minimum amount of glacial acetic acid/distilled water (25−50%) and lyophilized.

Synthetic peptides were purified by reverse phase high-pressure liquid chromatography (HPLC) using the same methods as discussed above. All samples were eluted using the following conditions and gradients: A = distilled H_2O/0.1% TFA; B = CH_3CN, λ = 280 nm, flow rate = 5 mL/min; time 0, %A 100; %B 0, Time 30, %A 0, %B 100. The extent of purity for each peptide was monitored by rechromatography on an analytical VYDAC 218 TBP-16 column (4.6 × 250 mm) at both 280 and 214 nm, flow rate = 1.0 mL/min. Characterization was performed by thin-layer chromatography and mass spectral and amino acid analysis.

The amino acid analysis for compounds **2**−**9** were consistent with the given sequences (Table 4). Mass spectral values (calculated and analyzed), extinction coefficients, and HPLC k' values are shown in Table 5 and thin-layer chromatography (TLC) values are provided in three different solvent systems (Table 6). Additionally, mass spectrometry analysis of tryptic cleavage fragments (Table 3) was done using electrospray mass spectrometry (low resolution).

The following procedures were the same for all synthetic analogues:

Isolation of Liver Plasma Membranes. Liver plasma membranes were prepared using male Sprague−Dawley rats weighing between 200 and 250 g according to the procedure of Neville[19] with modifications as described by Pohl et al.[31] The protein concentration was determined using the methods of Lowry et al.,[32] and 600−800 mg protein aliquots in 25 mM Tris buffer, pH 7.5, were stored at −80 °C for 1−2 months during which they were used for biological assays.

Adenylate Cyclase Assay. Glucagon and its analogues were dissolved in 5 mM HCl to concentration of 100 μM (stored at −80 °C in the form of a lyophilized powder) and further diluted by 25 mM Tris buffer (pH 7.5 at 25 °C) containing 0.4% BSA to desired concentration range just prior to the assay. Adenylate cyclase activity was measured by the conversion of [α^{32}P]ATP to cyclic-3′,5′-AMP as described by Lin.[20] Labeled cAMP was determined by the method of Solomon[21] using sequential chromatography on columns of Dowex cation-exchange resin and aluminum oxide. Briefly, 0.1 mL of incubation medium consisting of 1 mM [α^{32}P]ATP; 5 mM $MgCl_2$; 10 mM GTP; 1 mM EDTA; 1 mM CAMP containing 10 000 CPM of [^{3}H]cAMP; 25 mM Tris-HCl (pH 7.5); 0.4% BSA; 35 μg of membrane protein and an ATP regenerating system that contained 20 mM phosphocreatine and 0.72 mg/ mL creatine phosphokinase. Results are expressed as potency (IC_{50}), relative to glucagon (100) and in terms of the maximal stimulation of adenylate cyclase by glucagon (maximum being 100%) (Table 2).

Receptor Binding Assay. Radioiodination of glucagon was carried out by the method of Hagopian and Tager.[18] Glucagon (3 nmol) was allowed to react with a 1.0 nmol sample of carrier-free Na^{125}I (2.0 mCi) in the presence of 1.5 nmol of chloramine-T, added at regular intervals of 30 s (0.5 nmol each time). The reaction was terminated by the addition of sodium metabisulfite solution (0.5%). Chromatographic purification of the labeled glucagon was done as described by Jorgensen and Larsen.[33] Two fractions were collected and the mono-iodinated glucagon peak, as determined by reverse phase HPLC, was stored at −20 °C for use within 2 weeks for receptor binding. The receptor binding assay was carried out as described by Lin.[20] The incubation medium had a final volume of 500 μL consisting of liver plasma membrane containing 50 μg of protein, 100 000 CPM of [^{125}I]glucagon and unlabeled glucagon or synthetic analogues at the desired concentration (range 0−10 μM), all in 25 mM Tris-HCl with 0.4% BSA, pH 7.5 at 25 °C. The mixture was incubated for

10 min at 30 °C followed by immediate cooling in an ice bath and then filtered through 0.45 μm cellulose acetate filters previously soaked for 12 h in the Tris-BSA buffer. The filters were washed 4 × 1 mL with ice-cold 25 mM Tris-HCl buffer, pH 7.5, and the amount of radioactivity remaining in the filter was quantitated using an LKB 1275 mini gamma counter. Nonspecific binding, measured in the presence of excess unlabeled peptide (1024 nM), was typically 15−20% of the total binding and was subtracted in each case to give specific binding. Results are expressed as the percent inhibition of [^{125}I]glucagon specific binding.

In both the adenylate cyclase and binding assays, triplicate determinations of each data point were obtained, and all experiments were carried out at least twice. The EC_{50} values and 95% confidence limits for each of the analogues in both assays were determined by the statistical methods given by Bowman and Rand.[34]

Tryptic Cleavage for Analogues 6 and 7.[35] Peptides were digested for 1 h at 37 °C in 50 mM Tris HCl, 1 mM $CaCl_2$, pH 7.5 with tosylphenylethyl chloromethyl ketone (TPCK) treated trypsin (Sigma) at a 1:100 (w/w) enzyme/substrate ratio. After boiling for 3 min the peptide mixture was separated by HPLC on a Perkin-Elmer model 410-BIO using an analytical VYDAC C18 TBP-16 reverse phase (4.6 × 250 mm), 10 μm, 300 A column at ambient temperature run at 1.5 mL/min in 0.1% TFA. The peptides were eluted with a linear gradient (0−20% in 10 min; 20−50% in 60 min) of acetonitrile containing 0.05% ethyl acetate.[36] Peptides were monitored by UV absorption at 214 and 280 nm, and all traces were recorded with a Perkin-Elmer GP 100 graphics printer.

Preparation of [Lys^{17,18},Glu^{21}]glucagon Amide Crystals.[37] In a vial, [Lys^{17,18},Glu^{21}]glucagon amide (4.40 mg) was dissolved in 0.5 mL of 0.2 M phosphate buffer (pH 9.24) at 50 °C. The vial, in a beaker containing 100 mL of distilled water, was placed in a 50 °C oven and allowed to cool to room temperature slowly. Numerous single crystals (∼0.5 mm diameter) were grown. When the crystals were washed with 0.01% NaCl, they became cloudy, so they were redissolved and allowed to cool slowly.

Data Collection and Structure Determination. Cubic crystals with one [Lys^{17,18},Glu^{21}]glucagon amide molecule per asymmetric unit (space group $P2_13$; a = 47.9 Å) and typical dimensions 0.5 × 0.5 × 0.5 mm^3 were used for data collection. Diffraction measurements were carried out to 3 Å resolution, at room temperature using an Enraf-Nonius CAD4 diffractometer. The crystals proved to be isomorphous to those obtained with wild-type glucagon by Blundell and co-workers.[31] Initially the structure of [Lys^{17,18},Glu^{21}]glucagon amide was refined using a partial model in which residues 17, 18, and 20 were treated as alanines. This partial model was subjected to rigid body and positional refinement with X-PLOR (Brunger, AT X-PLOR v. 3.1 manual, Yale University, New Haven, 1992). After simulated annealing, difference Fourier maps permitted location of the omitted side chains, and the refinement was completed using tightly restrained individual temperature factors against all observed reflections between 6 and 3 Å resolution (1F1> 2 <(1F1)). The current crystallographic model consists of amino acid residues 1−29 giving a crystallographic R factor of 25.3%, with root-mean-square deviations from ideal values of 0.025 Å for bond lengths and 3.30 for bond angles (the amide group was not modeled, and no water molecules were included in the refinement). The electron density for the polypeptide backbone is everywhere continuous at 1.0σ in a $(2|F_{observed}| - |F_{calculated}|)$ difference Fourier synthesis except for the first three residues, which are poorly ordered. Comparison of the refined structure of [Lys^{17,18},Glu^{21}]glucagon amide with the structure of wild-type glucagon revealed no significant differences. The root-mean-square deviation of α-carbon atomic positions was 1.00 Å. The structure factors and atomic coordinates have been submitted to the Protein Data Bank (PDB), Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (accession code, 1bh0).

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Supporting Information Available: HPLC trace following tryptic cleavage of glucagon, [D-Arg^{17}]glucagon, and [D-Arg^{18}]-glucagon including expertimental conditions (4 pages). Ordering information is given on any current masthead page.

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