A New Approach to Search for the Bioactive Conformation of Glucagon: Positional Cyclization Scanning

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In search for the bioactive conformation of glucagon, "positional cyclization scanning" was used to determine secondary structures of glucagon required for maximal interaction with the glucagon receptor. Because glucagon is flexible in nature, its bioactive conformation is not known except for an amphiphilic helical conformation at the C-terminal region. To understand the conformational requirement for the N-terminal region that appears to be essential for signal transduction, a series of glucagon analogues conformationally constrained by disulfide or lactam bridges have been designed and synthesized. The conformational restrictions via disulfide bridges between cysteine *i* and cysteine *i* + 5, or lactam bridges between lysine *i* and glutamic acid *i* + 4, were applied to induce and stabilize certain corresponding secondary structures. The results from the binding assays showed that all the cyclic analogues with disulfide bridges bound to the receptor with significantly reduced binding affinities compared to their linear counterparts. On the contrary, glucagon analogues containing lactam bridges, in particular, c[Lys^5, Glu^9]glucagon amide (**10**) and c[Lys^{17}, Glu^{21}]glucagon amide (**14**), demonstrated more than 7-fold increased receptor binding affinities than native glucagon. These results suggest that the bioactive conformation of glucagon may adopt a helical conformation at the N-terminal region as well as the C-terminal region, which was not evident from earlier biophysical studies of glucagon.

Introduction

Most peptide hormones exert their biological activities by interacting with their G protein-coupled receptors (GPCRs).[1] Interactions between peptide hormones and their GPCRs have been studied by many investigators, but it is not yet fully understood how these hormones interact with their receptors. Over the years, several biophysical techniques, in particular, X-ray crystallography and multidimensional nuclear magnetic resonance spectroscopy, have been utilized to study noncovalent interactions between large hosts and small ligands, such as enzymes and their substrates.[2−4] However, these biophysical techniques have not been successful to examine interactions between peptide hormones, neurotransmitters, and their GPCRs, because GPCRs, as membrane proteins, are extremely difficult to isolate and manipulate in large quantities and are not easy targets to be investigated by these biophysical methods even if they were in our hands.

Glucagon, which consists of 29 amino acid residues (Figure 1), is a peptide hormone secreted by the α-cells of pancreas islets and has a critical function for glucose homeostasis by stimulating gluconeogenesis and glycogenolysis in hepatocytes[5,6] and stimulating lipolysis in adipocytes[7] during the hypoglycemic state. The finding that hyperglycemia generally accompanied by elevated glucagon levels has led Unger to suggest the bihormonal hypothesis, which postulates that both insulin and glucagon contribute to the diabetic state.[8,9] Therefore, glucagon receptor antagonists would be the most valuable tool for testing this hypothesis and might eventually lead to new therapeutic approaches for the treatment of diabetes mellitus.[10] In order to develop more potent glucagon antagonists, an understanding of how native glucagon interacts with the glucagon receptor would be extremely beneficial for rational design. On the other hand, the conversion of the native peptide (agonist) into an antagonist by multiple modifications in the N-terminal sequence of glucagon from previous studies[11,12] made us interested in the conformation of the N-terminal region of glucagon.

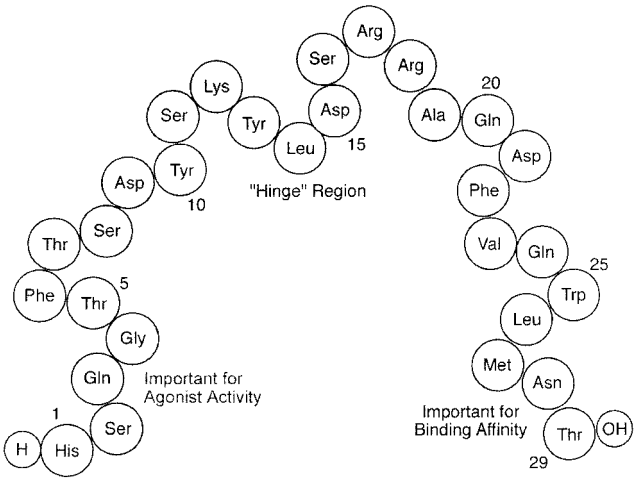

Figure 1. Primary structure of glucagon.

The conformation of glucagon has been examined by many researchers. The X-ray crystal structure[13] of trimeric glucagon revealed that the residues between positions 10 and 25 existed in an α-helical conformation. On the other hand, conformational studies of glucagon by 2D-NMR spectroscopy[14] in a pseudo-membrane

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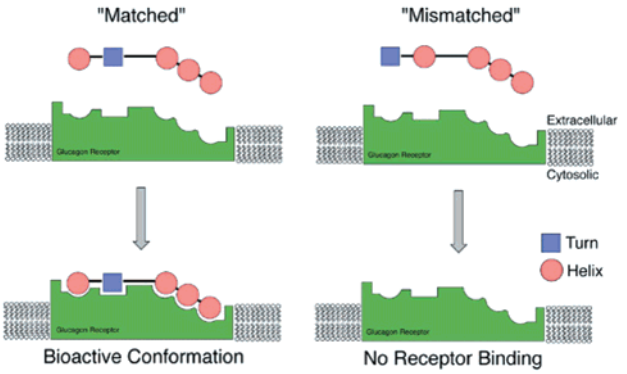

Figure 2. Schematic diagram of positional cyclization scanning. Matched conformation stabilized by cyclization is recognized by the glucagon receptor, while mismatched conformation is rejected.

environment showed substantially different results. In solution, the conformation of the N-terminal end could not be observed with 2D-NMR spectroscopy due to the high degree of mobility in the N-terminal region. Another different model of glucagon was proposed by Chou-Fasman[15] empirical procedure by Korn and Ottensmeyer,[16] and they suggested β-turns located at residues 2−5, 10−13, and 15−18. Although the glucagon conformations discussed above would be useful to be considered, the X-ray crystal structure of glucagon might have been influenced by the unphysiologically high concentration and pH (pH = 9.2) of crystallization condition.[13] Also, the complicated and well-organized interaction between glucagon and the glucagon receptor cannot be mimicked by the micelles used in the NMR study.[14]

Therefore, in an attempt to obtain information of bioactive conformation of glucagon, we have used a new approach, "positional cyclization scanning", to determine secondary structures in the bioactive conformation of glucagon. Positional cyclization scanning (Figure 2) consists of series of structure−activity relationship studies using conformational restriction to help stabilize secondary structures by conformational restriction at defined regions of glucagon. These studies and the result of the receptor binding assay determine whether this secondary structure is required by the glucagon receptor. By introducing secondary structures throughout the sequence of glucagon and examining the affinity of these ligands in receptor binding assays, the bioactive conformation of glucagon can be studied indirectly. The conformational restrictions used in this investigation are disulfide bridges between cysteines at position *i* and cysteines at position $i + 5$ and lactam bridges between lysines at position *i* and glutamic acids at position $i + 4$. The cyclization via the disulfide bridges between Cys^i and Cys^{i+5} was used to induce and stabilize turn conformations, while the cyclization via the lactam bridges between Lys^i and Glu^{i+4} was designed for helical conformation. The choice of the residues and their positions for the conformational restrictions was based on the previous studies of peptides, which demonstrated each type of cyclization for the corresponding secondary structure.[17−20] To induce turn and helical conformations into a certain region of glucagon, two different peptides were synthesized with cyclization via a disulfide bridge and a lactam bridge, respectively. If a certain secondary structure in this region corresponds to the bioactive conformation of glucagon, only the peptide which possesses the required secondary structure will have significant binding affinity, while significantly reduced binding affinity will be observed for the peptide with improper conformation (Figure 2). The design of the cyclic peptides used in this study was based on the following assumptions. First, although almost all of the residues in the glucagon sequence seem to be essential to attain significant binding affinity, some residues can be substituted without substantial loss of binding affinity, whereas other residues appear to be essential to receptor recognition.[10,21,22] Thus, residues such as His^1, Phe^6, Ser^8, Tyr^{10}, Asp^{15}, Arg^{18}, and residues 22−29 were not replaced for cyclization. Second, cyclizations via disulfide bridges[17,18] between Cys^i and Cys^{i+5} and via lactam bridges[19,20] between Lys^i and Glu^{i+4}, respectively, are assumed to define turn and helical conformation regardless of the differences for individual sequences of the peptides.

Therefore, to evaluate the hypothesis and to achieve the objective of discovering the bioactive conformation of glucagon, four conformationally constrained glucagon analogues via disulfide bridges (**2**, **4**, **6**, **8**) that would induce and stabilize turn conformations and four of their linear counterparts (**1**, **3**, **5**, **7**) were designed and synthesized (Table 1). For helical conformations, six cyclic glucagon analogues via lactam bridges (**9**−**14**) were synthesized and evaluated (Table 1).

Results and Discussion

Glucagon Analogues Cyclized via Disulfide Bridges between Cys^i and Cys^{i+5}. Four conformationally restricted glucagon analogues via disulfide bridges between cysteine residues 2 and 7 (**2**), 4 and 9 (**4**), 7 and 12 (**6**), and 14 and 19 (**8**) were synthesized by an N^{α}-Fmoc/*tert*-butyl strategy of solid-phase peptide chemistry. The disulfide bridges between cysteines at position *i* and cysteines at position $i + 5$ were designed for turn conformations. On the other hand, their linear counterparts with the free sulfhydryl groups of cysteines also were prepared in order to evaluate the effect of substitution of these residues for cysteines.

All of the linear glucagon analogues (**1**, **3**, **5**, **7**) with free sulfhydryl group-containing cysteines showed relatively good binding affinity compared to the native glucagon (27%, 42%, 23%, and 11%, respectively). It is arguable whether 10−40% of receptor binding can be considered either as substantial binding affinity or as poor receptor interaction. However, the receptor binding of the linear analogues did not suffer significantly compared to that of their cyclic analogues (**2**, **4**, **6**, **8**). Of the four glucagon analogues cyclized via disulfide bridges, three analogues (**2**, **4**, **6**) (Table 1) contain the conformational restrictions in the N-terminal region, and a disulfide bridge was introduced in the middle of the glucagon sequence for analogue **8**. In these cases, the linear glucagon analogues (**1**, **3**, **5**, **7**) and the cyclic glucagon analogues via disulfide bridges (**2**, **4**, **6**, **8**) all demonstrated very poor binding affinities for the glucagon receptor (1.5%, 1.7%, 0.27%, and 0.27%, respectively). The poor binding affinity of these cyclized analogues, compared to their linear counterparts and

Table 1. Biological Activities of the Conformationally Restricted Glucagon Analogues

		receptor binding		adenylate cyclase activity		
	compound	IC_{50} (nM)	relative binding (%)	EC_{50} (nM)	max stimultn (%)	pA_2
	glucagon	1.5	100	5.9	100	
1	[$Cys^{2,7}$]glucagon amide	5.6	27	41.8	50[a]	
2	c[$Cys^{2,7}$]glucagon amide	101	1.5	9200	23[b]	
3	[$Cys^{4,9}$]glucagon amide	3.6	42	295	18[c]	
4	c[$Cys^{4,9}$]glucagon amide	84.5	1.7	295	7.5[c]	
5	[$Cys^{7,12}$]glucagon amide	6.4	23	59	100[a]	
6	c[$Cys^{7,12}$]glucagon amide	558	0.27	5900	53[b]	
7	[$Cys^{14,19}$]glucagon amide	13.7	11	236	100[c]	
8	c[$Cys^{14,19}$]glucagon amide	562	0.27	7500	30[b]	
9	c[Lys^{3}, Glu^{7}]glucagon amide	6.0	25	i.a.[d]		8.28
10	c[Lys^{5}, Glu^{9}]glucagon amide	0.20	760	390	12	8.26
11	c[Lys^{7}, Glu^{11}]glucagon amide	13.6	11	47	17	
12	c[Lys^{9}, Glu^{13}]glucagon amide	652	0.23	23	19	
13	c[Lys^{13}, Glu^{17}]glucagon amide	1.7	87	6.3	85	
14	c[Lys^{17}, Glu^{21}]glucagon amide	0.24	630	0.27	98	

Maximum stimulation at the concentration of [a] 10 μM, [b] 20 μM, and [c] 1 μM. [d] i.a. = inactive up to 10 μM.

to glucagon, can be considered to arise from either improper conformations resulting from cyclizations or undesirable interactions of disulfide functional groups with the glucagon receptor. Therefore, the results can be interpreted to suggest that turn conformations in the N-terminal region and between residues 14−19 may not be the conformations that are appropriate for maximized interaction with the receptor. In general, the exact conformation of a turn structure is critical for a peptide to efficiently bind to a receptor, and use of L/D-Cys/Pen combination for disulfide bridges may be able to optimize turn conformations for better recognition by the glucagon receptor. However, the approximately 100-fold decreases in binding affinities by cyclizations suggest that reverse turns are not suitable for glucagon receptor interaction in the regions where cyclizations were applied.

These results would appear to be inconsistent with the theoretically derived conformations by Korn and Ottensmeyer,[16] who proposed β-turn conformations between residues 2−5 and 15−19. The poor receptor binding affinity displayed by the cyclic analogues (**2**, **4**, **6**, **8**) suggests that these predicted β-turn conformations in the regions where the disulfide bridges were incorporated for the cyclic analogues do not seem to be the compatible conformations with the receptor. This newly discovered information also confirms the results from earlier experiments that were performed to reinforce the β-turn potentials between residues 2−5 by Unson and co-workers.[23] In their experiment, the residues 2−5 were replaced with amino acids that may enhance the β-turn potentials, but the glucagon analogues with these multiple substitutions did not efficiently bind to the glucagon receptor. The middle region of glucagon, encompassing residues 15−19, is known as the "hinge" region and is theoretically expected to adopt a turn conformation by Korn and Ottensmeyer.[16] However, the lack of binding affinity of analogue **8** suggests that a turn conformation between residues 15−19 is not compatible with the glucagon receptor, and instead, the helical conformation seems to be extended at this middle region from the C-terminus (vide infra).

Glucagon Analogues Cyclized via Lactam Bridge between Lys^{i} and Glu^{i+4}. With the same rationale used to design the cyclic glucagon analogues with disulfide bridges for turn conformations, cyclic glucagon analogues with lactam bridges between lysines at position i and glutamic acids at position $i + 4$ were prepared in order to induce and stabilize helical conformations. In these cases, the linear peptides containing free side chain functional groups of Lys and Glu were not synthesized because the linear analogues may have distinctively different physicochemical properties, compared to the cyclic analogues. The substitution of Lys and Glu in the linear analogues generates undesirable positive and negative charges in the side chain functional groups, while the cyclized analogues do not possess these unwanted electrostatic properties. Possible suitable linear analogues would be ones with substitutions of acetylated lysines and glutamines for lysines and glutamic acids, respectively, but synthesis of these analogues was not pursued.

The synthesis of the cyclic analogues with lactam bridges was performed using allyl side chain protection groups. The allyloxycarbonyl (Alloc) group, first introduced by Stevens and Watanabe,[24] is used for protection of amines and alcohols.[25] The allyl ester group is used to protect the glutamic acid moieties which will be used for cyclic lactam formation.[25] Due to the mild conditions used for deprotection of Alloc and allyl groups and their stability under the conditions used for deprotection of N^{α}-Fmoc and N^{α}-Boc groups, Alloc and allyl groups are a very useful alternative for orthogonal protection in peptide synthesis.[26,27] Because of their properties that Alloc and allyl groups can be deprotected under mild and neutral conditions, they can be used to prepare partially deprotected glucagon analogues that are subsequently cyclized on the solid support to produce conformationally constrained glucagon analogues (Figure 3). Fully protected glucagon analogues were synthesized with a standard N^{α}-Fmoc/*tert*-butyl strategy, while Lys and Glu side chain groups were protected by Alloc and allyl groups, respectively. Then, Alloc and allyl groups were deprotected, and the resulting free amine and carboxylic function groups of Lys and Glu were coupled to form a lactam bridge to produce cyclic glucagon analogues.

Compared to the weak binding affinity demonstrated by the cyclic glucagon analogues with disulfide bridges, most of the cyclic glucagon analogues with lactam bridges between residues 3 and 7 (**9**), 5 and 9 (**10**), 7 and 11 (**11**), 9 and 13 (**12**), 13 and 17 (**13**), 17 and 21 (**14**) displayed significant binding affinities for the glucagon receptor (25%, 760%, 11%, 0.23%, 87%, and

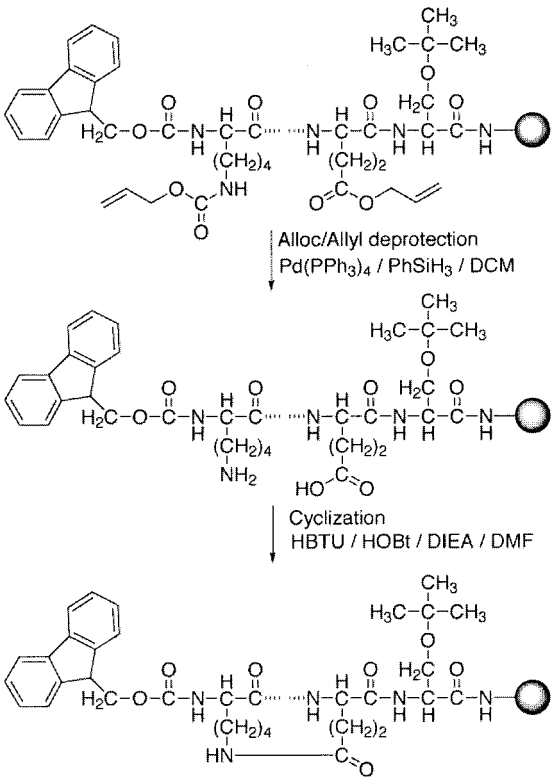

Figure 3. A scheme for synthesis of conformationally constraint glucagon analogues with allyloxycarbonyl and allyl protected peptides.

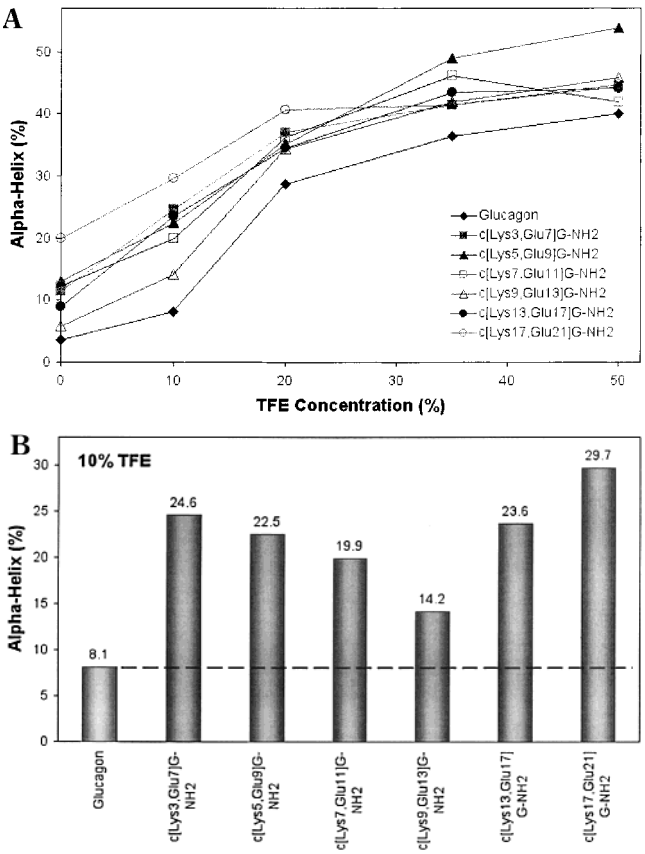

Figure 4. (A) Effects of TFE concentration on the α-helix formation of glucagon and cyclized glucagon analogues by lactam bridges. (B) Calculated α-helical contents of glucagon and cyclized glucagon analogues by lactam bridges in 10% TFE solution.

626%, respectively). In particular, the analogue **14**, c[Lys^{17}, Glu^{21}]glucagon amide, showed extremely good binding affinity (6-fold greater compared to glucagon). This is not surprising because the superagonist, [Lys,^{17,18} Glu^{21}]glucagon,[28] was observed to possess very potent binding affinity (5-fold greater compared to glucagon), and the extended α-helical conformation due to a new salt bridge between Lys^{18} and Glu^{21} of the superagonist, observed in X-ray crystal structure,[29] appears to be responsible for the increased binding affinity. This result strongly suggests that the cyclization via lactam bridge between Lys^{i} and Glu^{i+4} actually induced and stabilized helical conformations. In addition, the increased binding affinity of the analogue **14** suggests that the salt bridge formed in the superagonist can account for the enhanced receptor binding affinity by the superagonist, [Lys^{17,18}, Glu^{21}]glucagon.

The increased helical conformation induced by the lactam bridge between Lys^{i} and Glu^{i+4} also was observed by CD spectroscopy (Figure 4A). CD spectra of the lactam bridge cyclic glucagon analogues we designed (Table 1) were obtained in various concentrations of TFE in aqueous solutions. All cyclic lactam bridged analogues showed increased helical contents compared to native glucagon, although some cyclic peptides displayed more increased helical conformations than other cyclic peptides (Figure 4B). The extent of increased helical content was highly dependent on the position of the lactam bridges, which may indicate that stabilization of a helical conformation by a lactam bridge is dependent on the sequence around the lactam bridge. Although the significantly poor binding affinity of analogue **12** may be contributed to by the lower degree of helix stabilization by the lactam bridge between Lys^{9} and Glu^{13}, the CD spectra of this compound still showed a higher amount of helical content than native glucagon. Therefore, the greatly reduced binding affinity of analogue **12** seems to result from a mismatch of secondary structure between residues 9−13 of glucagon with the glucagon receptor.

Except for analogue **12**, c[Lys^{9}, Glu^{13}]glucagon amide, the potent binding affinities of the other analogues **10**−**11**, **13**−**14** resulted from the induced helical conformations. Since the conformational restrictions were introduced in the N-terminal region for analogues **9**−**11** (Table 1) and the middle region, proximate to the C-terminal region, for analogues **12-14** (Table 1), the enhanced binding affinities of these analogues suggest that the bioactive conformation of glucagon consists of helices in the N-terminal region and the half of the middle region that is close to the C-terminal region, in addition to the well-known amphiphilic helix located in the C-terminal region. The significantly increased binding affinity (ca. 8 times more potent than native glucagon) of analogue **10**, c[Lys^{5}, Glu^{9}]glucagon amide, strongly supports the presence of a helical conformation in the N-terminal region of glucagon that is critical for agonist interaction with the glucagon receptor. On the other hand, when helical conformation was induced in the front half of the middle region in analogue **12**, greatly reduced receptor binding suggests that an α-helical structure is detrimental to receptor recognition by glucagon. These results are consistent with the weak receptor recognition by the glucagon analogues cyclized

via disulfide bridges discussed earlier and successfully demonstrate the possibility of identifying bioactive conformations required for maximum receptor interactions for larger polypeptide hormones using positional cyclization scanning. The conformational study of glucagon by 2D-NMR spectroscopy[14] in lipid/water environment demonstrated an extended conformation between residues 5−9, a helix-like turn for residues 10−14, and another extended conformation between 14−17. Contrary to the conformations observed by NMR spectroscopy[14] and suggested from theoretical calculation[16] (vide supra), the results from the glucagon analogues cyclized via lactam bridges led us to propose a new bioactive conformation of glucagon that consists of helical conformations between residues 1−10 and residues 13−29 as well as some other (not helical) conformation between residues 10−13.

The cyclic glucagon analogues used in this study have all the key residues that are known to be essential for signal transduction, including His^1, Phe^6, Ser^8, Asp^9, Tyr^{10}, Asp^{15}, and Arg^{18}, and therefore, most of the peptides except for analogues **9** and **10** are found to be agonists. In general, lower adenylate cyclase activities have been observed for the glucagon analogues with disulfide bridges compared to their linear counterparts, probably because of their lower binding affinities. Surprisingly, analogue **14**, c[Lys^{17}, Glu^{21}]glucagon amide, displayed extremely potent adenylate cyclase activity (a 21-fold increase compared to glucagon), and this enormous increase seems to result from the improved binding affinity as a result of the formation of the lactam bridge between positions 17 and 21. On the other hand, the glucagon analogues with lactam bridges in the N-terminal region (analogues **9** and **10**) showed no or very minor adenylate cyclase activity, and these analogues were demonstrated to be potent antagonists ($pA_2 = 8.28$ and 8.26, respectively). Especially, analogue **10** is an extremely potent glucagon antagonist with dramatically high receptor binding affinity, although it retains a histidine residue at position 1. Therefore, it would be logical to speculate that c[desHis^1, Lys^5, Glu^9]-glucagon amide will be the most potent glucagon antagonist with extremely high binding affinity. Although it would be presumptuous to make any final conclusion based on the role of glucagon's secondary structures on its overall functional effects, the high binding affinities of analogues **9** and **10** and their antagonist activities suggests that a helical conformation in the N-terminal region seems to promote antagonist activity, although the key residue for signal transduction, His^1, is still present.

Conclusions. In an attempt to search for the bioactive conformation of glucagon, a series of conformationally restricted glucagon analogues via disulfide bridges and lactam bridges have been designed and synthesized. Cyclization via disulfide bridges between Cys^i and Cys^{i+5} was introduced in order to induce and stabilize turn conformations between the cysteine residues that were involved in the cyclization, while cyclization via lactam bridges between Lys^i and Glu^{i+4} were introduced to stabilize helical conformations. The decreased binding affinities of all of the cyclic disulfide-containing glucagon analogues suggest that turn conformations in the N-terminal region and between residues 14−19 in glucagon are unlikely in the bioactive conformations. These results indicate that the earlier prediction[16] of β-turn in these regions by the propensity of amino acids for the secondary structures seems to be invalid. On the other hand, the predominant helical conformations in the N-terminal region and in residues 13−29 can be proposed by the increased receptor binding affinities of the cyclic lactam glucagon analogues. Helical conformations in the N-terminal region were not observed in any conformational studies performed previously. As a result, the bioactive conformation of glucagon determined in this study consists of two helical segments between residues 1−10 and 13−29. These two helical regions are connected by a nonhelical region between residues 10−13. This proposed structure of glucagon in the glucagon receptor may be similar to a helix-turn-helix conformation that is commonly observed in numerous proteins.[30,31]

Experimental Section

Materials. All peptides designed in this investigation were prepared by solid-phase methods either by manual synthesis or by using an Applied Biosystems ABI 431A automated peptide synthesizer with N^{α}-Fmoc/*tert*-butyl chemistry. N^{α}-Fmoc protected amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin were purchased from Advanced Chemtech (Louisville, KY), Bachem (Torrance, CA), and American Peptide Company (Sunnyvale, CA). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA; Halocarbon Products, NJ); *N*,*N*-diisopropylethylamine (DIEA), anisole, 1,2-ethanedithiol, dimethyl sulfide, piperidine, potassium ferricyanide, acetic anhydride, tetrakis(triphenylphosphine)palladium(0), and phenylsilane (Aldrich, Milwaukee, WI); dichloromethane (DCM); *N*,*N*-dimethylforamide (DMF; Fischer Scientific, Pittsburgh, PA); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt; Chem-Impex International, Wood Dale, IL); HPLC-quality acetonitrile (J. T. Baker, Phillipsberg, NJ); bovine serum albumin (BSA), chromatographic alumina (type WN3, neutral), cAMP, ATP, GTP, and all enzymes (Sigma Chemicals, St. Louis, MO); [^{125}I]glucagon, [^{3}H]cAMP, and [α-^{32}P]ATP (New England Nuclear, Boston, MA); Dowex AG 50-W4 cation-exchange resin and weakly basic anion-exchange resin, IRA-68 (BioRad, San Diego, CA). All amino acids were of the L-configuration unless otherwise stated. The purity of the peptides was checked by thin-layer chromatography (TLC) in three different solvent systems and analytical reverse-phase high-pressure liquid chromatography (HPLC) using VYDAC 218 TBP-16 column (4.6 × 250 mm) at 214, 254, and 280 nm (Table 2), and the structures of the purified peptides were characterized by electrospray mass spectrometry (Finnigan LCQ Ion Trap Mass Spectrometer; Table 2).

TLC was performed using Merck silica gel 60 F-254 plates (0.25 mm layer thickness), and the following solvent systems were used: (A) 1-butanol/acetic acid/pyridine/water (5:4:1:4); (B) 1-butanol/acetic acid/pyridine/water (4:1:1:3); (C) ethyl acetate/pyridine/acetic acid/water (12:4:4.2:2.2). The peptides were detected on the TLC plates using iodine vapor. The purification of the peptides was achieved using a Hewlett-Packard 1100 series HPLC instrument, or a Perkin-Elmer Binary LC Pump 250 with Perkin-Elmer LC 90 UV spectrophotometer detector for preparative high-pressure liquid chromatography on a C_{18}-bonded silica columns (VYDAC, 10 × 250 mm, 10 μm, 300 Å, semipreparative columns, Cat. No. 218TP1010) unless otherwise stated. The peptides were eluted with a linear acetonitrile in 0.1% aqueous TFA gradient at a flow rate of 5.0 mL/min. The separations were monitored at 280 nm with Hewlett-Packard 1100 series fixed-wavelength UV detector or Perkin-Elmer LC 90 UV detector, and integrated with Hewlett-Packard 3396 series III integrator.

Table 2. Physicochemical Properties of Glucagon Analogues

	average molecular mass		HPLC	R_f, TLC eluents[c]		
peptide	calcd	found[a]	k'[b]	A	B	C
1	3499.9	3499.8	3.25[d]	0.72	0.31	0.07
2	3497.9	3497.7	3.11[d]	0.74	0.43	0.11
3	3516.0	3515.7	3.27[d]	0.77	0.33	0.08
4	3514.0	3513.6	3.21[d]	0.78	0.32	0.06
5	3458.8	3458.8	3.52[d]	0.82	0.37	0.18
6	3456.8	3456.3	3.41[d]	0.74	0.44	0.12
7	3503.9	3503.4	2.75[d]	0.75	0.35	0.05
8	3501.9	3501.9	2.65[d]	0.74	0.24	0.06
9	3491.9	3491.4	4.92[e]	0.74	0.42	0.12
10	3504.9	3504.8	4.88[e]	0.75	0.41	0.09
11	3532.9	3532.8	4.99[e]	0.75	0.32	0.07
12	3442.8	3443.2	4.79[e]	0.74	0.29	0.06
13	3401.7	3402.0	4.93[e]	0.76	0.33	0.05
14	3449.8	3450.0	5.03[e]	0.76	0.42	0.07

[a] Molecular weight found by electrospray mass spectrometry. [b] HPLC k' = [(peptide retention time − solvent retention time)/(solvent retention time)] under the following conditions: gradient 20−60% acetonitrile in 0.1% trifluoroacetic acid over 20 min[d]; 10−90% over 40 min[e] (flow rate 1.0 mL/min). [c] TLC: silica gel 60 F-245, 0.25 mm layer thickness; eluent systems: A1—butanol/acetic acid/pyridine/water (5:4:1:4); B1—butanol/acetic acid/pyridine/water (4:1:1:3); C—ethyl acetate/pyridine/acetic acid/water (12:4:4.2:2.2).

Peptide Synthesis. General Protocol for Peptide Synthesis with N$^{\alpha}$-Fmoc/*tert*-Butyl Chemistry. The glucagon analogues used in this investigation were synthesized manually or using an Applied Biosystems ABI 431A automated peptide synthesizer using N$^{\alpha}$-Fmoc/*tert*-butyl chemistry. For the manual synthesis, the Rink amide resin[32] (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, substitution 0.4−0.7 mmol/g) was swollen in DMF overnight. The resin was washed with DMF (3 × 2 min), and N$^{\alpha}$-Fmoc protecting group was deprotected with 25% piperidine in DMF (1 × 5 min and 1 × 30 min). Then, the resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min), and the first N$^{\alpha}$-Fmoc amino acid was coupled using preactivated N$^{\alpha}$-Fmoc amino acid in DMF (3 equiv of N$^{\alpha}$-Fmoc amino acid, 3 equiv of HBTU, and 3 equiv of HOBt in DMF solution was stirred for 30−60 min) and 6 equiv of DIEA until the Kaiser ninhydrin test[33] and the 2,4,6-trinitrobenzenesulfonyl acid (TNBS) test[34] became negative. If the tests were still positive 2 h after the coupling reaction started, the resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min), and the amino acid was recoupled with preactivated N$^{\alpha}$-Fmoc amino acid in DMF (prepared in same manner) and 6 equiv of DIEA for another 2 h. If double coupling did not result in a negative Kaiser ninhydrin test and negative TNBS test, the resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min), and the unreacted amino group was capped with 10% acetic anhydride in DMF for 30 min. When the coupling reaction was finished, the resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min), and the same procedure was repeated for the next amino acid until all the amino acids in the sequence were coupled. After the peptide was synthesized on the resin, the resin was washed with DCM (3 × 2 min) and dried in vacuo.

On the other hand, a typical automated peptide synthesis was accomplished using an ABI 431A automated peptide synthesizer with the HOBt−HBTU−Fmoc synthesis protocol (ABI version No. 1.01B). The Rink amide resin (0.25 mmol, substitution 0.4−0.7 mmol/g) was placed in the reaction vessel 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% in DMF, 1 × 3 min and 1 × 6 min), and following deprotection the resin was washed with DMF (4 × 1 min) to remove the piperidine. The resin-bound peptide, which was now ready for coupling, was left in the reaction vessel while the next amino acid in the chain was prepared. The dry, Fmoc-protected amino acid (1.0 mmol/g) contained in the cartridge was dissolved in a solution of DMF and HOBt (0.50 M)/HBTU (0.45 M). This solution was transferred to the reaction vessel with DIEA. Four equivalents of the activated amino acid (one per equivalent of the growing peptide chain) were employed in the coupling reactions with 8 equiv of DIEA. The deprotection and coupling steps were repeated with the addition of each subsequent amino acid until the peptide synthesis was completed. The final amino acid was deprotected with piperidine (20% in DMF, 1 × 3 min and 1 × 6 min), and the resin was washed with DMF, followed by DCM. The resin was dried thoroughly in vacuo. Although most of automated peptide synthesis followed the procedure described above, some modifications (e.g., elongated deprotection and coupling reaction times, and capping of unreacted amino groups with acetic anhydride) were employed for improved peptide synthesis.

General Procedure for Cleavage and Final Deprotection of Peptides. A cleavage mixture consisting of trifluoroacetic acid (18.0 mL), dimethyl sulfide (0.5 mL), 1,2-ethanedithiol (0.5 mL), and anisole (1.0 mL) was chilled on ice. The resin bound peptide (0.25 mmol) 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 and covered with aluminum foil, and the reaction mixture was stirred at room temperature for 2 h. The solution was filtered, and the resin was washed with trifluoroacetic acid (5 mL) and DCM (2 × 5 mL). The combined solution was concentrated with gentle stream of nitrogen to a volume of approximately 3 mL, and the peptide was precipitated with cold diethyl ether (40 mL). The peptide was centrifuged to remove the ether and washed with another 40 mL of diethyl ether. The peptide was centrifuged and dried in vacuo.

General Procedure for the Purification of Peptides. The crude peptide was dissolved in 50% aqueous acetic acid, and insoluble materials were centrifuged out. The peptide was purified with HPLC using a semipreparative VYDAC reverse-phase (C_{18}-bonded) HPLC column with gradient elution at a flow rate of 5.0 mL/min. The gradients used for the purification were 10−90% acetonitrile in 0.1% aqueous TFA over 40 min, 20−50% over 30 min, 20−60% over 40 min, 25−55% over 30 min, and 35−55% over 20 min. Approximately 5 mg of the crude peptide was injected to the column each time, and the fraction containing the purified peptide was collected, followed by lyophilization. The extent of purity for each peptide was monitored by analytical HPLC, using an analytical VYDAC 218TPB-16 C_{18}-bonded column (4.6 × 250 mm) at 214, 254, and 280 nm, flow rate 1.0 mL/min with gradients such as 10−90% acetonitrile in 0.1% aqueous TFA over 40 min and 20−60% over 20 min.

General Procedure for the Cyclization via Disulfide Bridge. The purified peptide was dissolved in 20−25 mL of water, and acetonitrile was added to help dissolution. In the meantime, the solution of potassium ferricyanide was prepared by mixing of 1 mmol of $K_3Fe(CN)_6$ in 100 mL of water, 20 mL of acetonitrile, and 20 mL of saturated ammonium acetate and adjusting the pH to 8.5 with concentrated ammonium hydroxide. Then, the solution of the peptide was slowly added to the solution of potassium ferricyanide with a syringe pump (infusion rate of 1 mL/h).[35] After addition was completed, the reaction mixture was acidified to pH 4 with acetic acid. Then, weakly basic anion-exchange resin, IRA-68, was used to remove ferricyanide anion. The anion-exchange resin was filtered, and the volatiles were evaporated off to give the cyclized peptide.

General Procedure for the Cyclization via Lactam Bridge. The fully protected resin-bound peptide with allyloxycarbonyl (Alloc) and allyl (OAll) protection groups for Lys and Glu was placed in a glass vessel, fitted with a sintered glass filter and Teflon stoppers at both ends of the vessel. At the top outlet of this glass vessel, a mineral oil trap was used to prevent air from entering into the reaction vessel. This system was allowed to be maintained at all times with an argon atmosphere inside the vessel, which is critical for success in the catalytic allyl deprotection reaction.[27] During the

deprotection, washing, and coupling steps, the mixture was always bubbled with argon. The solvents and all other chemicals were introduced into the reaction vessel through the top of the vessel, while argon gas was continuously purged through the reaction vessel. Used reaction mixtures and washing solvents were removed from the resin by filtration through the glass filter at the bottom of the reaction vessel into a waste container, and in the course of filtration, argon was used to push the solutions or solvents through the filter.

In a typical example of cyclic lactam formation on the resin, following assembly of the fully protected peptide on the solid support, the resin was washed with DCM (3 × 2 min) in the presence of argon, and a solution of $PhSiH_3$ (24 equiv) in DCM, and a solution of $Pd(PPh_3)_4$ (0.25 equiv) in DCM was added as argon was bubbled continuously through the resin. The reaction mixture was bubbled under argon for 30 min. Then, the resin was washed with DCM (3 × 2 min), DMF (3 × 1 min), and DCM (4 × 2 min), and the process was repeated. Then, the peptide resin was suspended in DMF, followed by cyclization of the peptide via the free carboxylic acid side chain of Glu and the free amino side chain group of Lys by addition of HBTU (6 equiv), HOBt (6 equiv), and DIEA (12 equiv) for 2 h. This process was repeated until a negative Kaiser ninhydrin test and TNBS test resulted. Finally, the peptide resin was washed up in the usual way to obtain the cyclic peptide.

Biological Assays. Isolation of Liver Plasma Membranes. Liver plasma membranes were prepared from male Sprague Dawley rats weighing between 200 and 250 g.[36,37] The isolated livers were homogenized with 1 mM sodium bicarbonate solution containing 0.5 mM $CaCl_2$ and filtered at 4 °C to remove all large organelles. Next, filtered liver homogenate is centrifuged at 1500*g* to collect a clear pallet. Rat liver plasma membranes were further purified by ultracentrifugation using 42.3%/69% (w/w) sucrose gradient at 100000*g* for 2 h. The final pellets were resuspended with 10 mL of 25 mM Tris buffer (pH 7.5 at 25 °C). The amount of protein was determined by the Lowry method[38] and a modified procedure.[39] Subsequently, 1−2 mg protein aliquots in 25 mM Tris buffer (pH 7.5 at 25 °C) were stored in liquid nitrogen for use within 1−3 months.

Receptor Binding Assay. Glucagon and its analogues were dissolved in 1 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) to the desired concentration range just prior to the assay. The binding assay was performed according to Wright and Rodbell[40] in which competition for glucagon receptors between [^{125}I]glucagon and the glucagon analogue was measured. Briefly, an incubation medium that had a volume of 500 μL consisting of liver plasma membrane containing 50 μg of protein, 150 000 CPM of [^{125}I]-glucagon, and unlabeled glucagon or glucagon analogues at a desired concentration, all in 25 mM Tris 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 filtered through 0.45 μm cellulose acetate filter previously soaked for 12 h in a Tris-BSA buffer. Four milliliters of ice-cold Tris-BSA buffer was used for washing, and the amount of radioactivity remaining on the filter was quantitated using LKB1275 minigamma 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 the specific binding. Results were expressed as the percent inhibition of [^{125}I]glucagon specific binding. Assays were performed in triplicate and repeated twice.

Adenylate Cyclase Assay. Adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to cyclic-3′,5′-AMP as described by Lin.[41] Labeled cAMP was determined by the method of Salomon[42] 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 (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 a potency (EC_{50}), or as relative to glucagon (defined as 100%), and in terms of the maximal stimulation of adenylate cyclase by glucagon (defined as 100%).

For the pA_2 values, a dose/response plot was obtained by determining the response when the concentration of glucagon was varied while the concentration of the antagonist was kept the same. The plots were obtained by using different concentrations (range between 100 nM to 10 μM) of antagonists. The dose/response curves exhibit shifts in the EC_{50} values which were used in calculating the pA_2 values as described by Schild.[43]

CD Spectroscopy. Solutions (20 μM) of glucagon and the cyclized glucagon analogues by lactam bridges containing 0, 10, 20, 35, and 50% trifluoroethanol were prepared, and the concentration of glucagon analogues were examined by amino acid analysis. The CD spectra of glucagon and the cyclized glucagon analogues were taken with an AVIV model 60DS spectropolarimeter under constant nitrogen flush. A standard cell of 1 cm size was used for all the measurements, and the temperature was maintained at 26 °C. The instrument was calibrated with *d*-10-camphorsulfonic acid.[44] The CD data was expressed in terms of mean residue ellipticity in deg cm^2 $dmol^{-1}$. The mean residue weight was calculated using the relevant amino acid composition.[45]

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