Design and Synthesis of Conformationally Constrained Glucagon Analogues[§]

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Glucagon was systematically modified by forming lactam bridges within the central region of the molecule to give conformationally constrained cyclic analogues. Six cyclic glucagon analogues have been designed and synthesized. They are c[Asp^{9},Lys^{12}][Lys^{17,18},Glu^{21}]glucagon-NH$_2$ (**1**), c[Asp^{9},Lys^{12}]glucagon-NH$_2$ (**2**), c[Lys^{12},Asp^{15}]glucagon-NH$_2$ (**3**), c[Asp^{15},Lys^{18}]glucagon-NH$_2$ (**4**), [Lys^{17}-c[Lys^{18},Glu^{21}]glucagon-NH$_2$ (**5**), and c[Lys^{12},Asp^{21}]glucagon-NH$_2$ (**6**). The receptor binding potencies and receptor second messenger activities were determined by radio-receptor binding assays and adenylate cyclase assays, respectively, using rat liver plasma membranes. Most interestingly, analogues **1**, **2**, **3**, and **4** were antagonists of glucagon stimulated adenylate cyclase activity, whereas analogues **5** and **6** were partial agonists in the functional assay. All of the cyclic analogues were found to have reduced binding potencies relative to glucagon. The structural features that might be responsible for these effects were studied using circular dichroism spectroscopy and molecular modeling. These results demonstrated the significant modulations of both receptor binding affinity and transduction (adenylate cyclase activity) that can accompany regional conformational constraints even in larger polypeptide ligands. These studies suggest that the entire molecular conformation, including the flexible middle portion, is important for molecular recognition and transduction at the hepatic glucagon receptor.

Introduction

Glucagon is a polypeptide hormone that consists of 29 amino acid residues and is best known for its key role in maintaining glucose homeostasis in mammalian systems. It is secreted by the α cells of the pancreas and exerts its major effects on hepatocytes by stimulating glucose production and release during the hypoglycemic state. Glucagon action is mediated by a G protein coupled receptor[1] that stimulates cyclic AMP accumulation as a result of adenylate cyclase activation. It is this increase in intracellular cAMP that leads to glycogen hydrolysis and glucose synthesis, with a rapid rise in glucose output by the liver. The physiological and pathogenic role of glucagon has been linked to diabetes mellitus and diabetic ketoacidosis, and this coincides well with the bihormonal hypothesis of Unger and Orci.[2] Several investigators[3−5] have suggested intracellular signaling pathways other than cAMP that might be activated by glucagon and thus explain the complex interplay of signaling systems.

Several antagonists of glucagon bioactivity have been reported by the laboratories of Hruby and Merrifield, but only recently have antagonists without partial agonist properties been developed as has been discussed in detail by Van Tine et al.[6] The development of potent and stable glucagon antagonists will have enhanced therapeutic value in the treatment of diabetes and its symptoms.[7] With the view of obtaining a potent glucagon antagonist, extensive structure−function studies have been done and a partial picture is slowly emerging with regard to key residues involved in the glucagon's mechanism of action. The first series of partial antagonists that were synthesized had no histidine or some modified form of it at the N-terminus.[8,9] Earlier, it was reported that electrostatic interactions of the negatively charged side chain groups of aspartic acid 9, 15, and 21 were essential in glucagon function.[10,11] It has been previously hypothesized that residues 1, 9, and 16 may form an active site catalytic triad much like that seen in the serine proteases.[12] Recently Unson et al.[13] has shown that the positively charged (Lys^{12}, Arg^{17}, and Arg^{18}) residues of glucagon have very large effects on receptor binding and transduction of the hormonal signal.

Hruby and co-workers[14] have looked extensively at the role of Phe^{6} in glucagon bioactivity and have proposed a hydrophobic cluster model in glucagon's structure, which also involves the N-terminal residue His^{1}, for glucagon's bioactivity model. In this it was further emphasized that the proper stacking of Phe^{6}, Tyr^{10}, and Tyr^{13} is very important for binding and for transduction. Hruby et al.[15,16] also explored structural and topographical requirements of the glucagon receptor to the amino acid in position 10 of glucagon, and recently they suggested that the superagonist, [Lys^{17,18},Glu^{21}]-glucagon, analogue may form an intramolecular salt bridge between charged side chains at positions 18 and 21.[17]

[§] Abbreviations: Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB commission on Biochemical Nomenclature. All amino acids are of the L-configuration unless otherwise stated. Other abbreviations: DIC, diisopropylcarbodiimide; pMBHA, *p*-methylbenzhydrylamine; TFE, trifluoroethanol; TFA, trifluoroacetic acid; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; HPLC, high-pressure liquid chromatography; cAMP, 3′,5′-cyclic adenosine monophosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; tBu, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl.

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Table 1. Mass Spectra Data[a] of Glucagon Analogues Using Endoproteinase Asp-N Digestion

	FAB-MS[M + 1]$^+$		fragment 1[c]		fragment 2		fragment 3		fragment 4	
peptide[b]	calcd	found	calcd	found	calcd	found	calcd	found	calcd	found
glucagon	3483.8	3483.5	864.4	864.4	788.4	788.5	732.3	732.4	1153.5	1153.7
1	3422.8	3422.6	1616.7	1616.2	1825.1	1825.5	-	-	-	
2	3464.8	3465.2	1616.7	1616.7	732.3	732.4	1152.5	1152.6	-	
3	3464.8	3464.2	864.4	864.2	1484.6	1484.6	1152.5	1152.8	-	
4	3436.8	3436.8	864.4	864.3	1456.6	1456.8	1152.5	1152.8	-	
5	3422.8	3422.4	864.4	864.2	788.4	788.2	1807.0	1807.0	-	
6	3464.8	3464.6	864.4	864.3	2618.9	2618.4	-	-	-	

[a] The mass spectra were carried out by fast atom bombardment mass spectrometry (see text). [b] Peptides: **1** c[Asp^9,Lys^{12}][Lys^{17,18},Glu^{21}]-glucagon-NH_2; **2** c[Asp^9,Lys^{12}]glucagon-NH_2; **3** c[Lys^{12},Asp^{15}]glucagon-NH_2; **4** c[Asp^{15},Lys^{18}] glucagon-NH_2; **5** [Lys^{17}-c[Lys^{18}, Glu^{21}]glucagon-NH_2; **6** c[Lys^{12},Asp^{21}]glucagon-NH_2. [c] Reference to Table 2 for the structures of the fragments.

Overall, it has been found that the C-terminal region of glucagon is primarily important for receptor binding, whereas the N-terminal region is essential for signal transduction. However, not much is known about the middle portion of the hormone. In this work we examine this region using the conformational constraint approach.[18,19] Efforts to determine the three-dimensional properties of glucagon that might be of biological importance have been difficult. The only definitive structure available for glucagon is the X-ray crystal structure.[20] It shows an α-helix conformation in the 10−25 region and extending toward both ends with a less regular right-handed helix for the 6−9 and 26−29 residues and no discernible conformation in the 1−5 region. By applying Chou−Fasman calculations[21] and other protein secondary structure prediction methods,[22−26] the following conformational properties have been predicted for glucagon: an α-helical conformation in the sequence 17−29, *β*-turns in positions 2−5, and 8−11, and/or 10−13.[25,26] ^{1}H NMR spectroscopy has been used to examine the conformation of micelle-bounded glucagon, and the results indicated that the 17−29 sequence formed an amphiphilic helix, the 10−14 sequence was helical, and the 1−5 region extends into the water phase.[27] However, the circular dichroism (CD) results were inconsistent. Some studies found 10−15% α-helix in aqueous solution,[28] whereas others showed very little stable α-helical structure.[29] This is very different from the X-ray crystal structure.

To obtain further insights into the structural and conformational requirements of glucagon action, we have used covalent conformational constraints in the glucagon molecule by cyclizing amino acid side chain groups along the peptide chain to form a series of lactam bridges.[18,19,30] We have previously used lactam bridges as constraints with considerable success in the design of potent oxytocin antagonists[31−33] and superagonist MSH analogues.[34,35] We report in this paper the results of a study on the conformational contributions of the central regions of glucagon, because of our previous evidence[26] that this region might serve as a 'hinge" between the C-terminal binding region and the N-terminal transduction region. Therefore, six analogues with cyclic constraints in the middle region of the molecule from residues 9 to 21 were systematically designed and synthesized. They are c[Asp^9,Lys^{12}][Lys^{17,18},Glu^{21}]-glucagon-NH_2 (**1**), c[Asp^9,Lys^{12}]glucagon-NH_2 (**2**), c[Lys^{12},Asp^{15}]glucagon-NH_2 (**3**), c[Asp^{15},Lys^{18}]glucagon-NH_2 (**4**), [Lys^{17}-c[Lys^{18},Glu^{21}]glucagon-NH_2 (**5**), and a more globally cyclized analogue, c[Lys^{12},Asp^{21}]glucagon-NH_2 (**6**). Bioassays of these analogues suggest that this approach can provide a new tool for the design of glucagon antagonists. Some aspects of this work have appeared in preliminary form.[36]

Results

The side chain cyclic lactam glucagon analogues were prepared by solid-phase synthesis using a Boc strategy on *p*-methylbenzhydrylamine (pMBHA) resins.[30,35] The side chain protecting groups for the Lys and Asp residues involved in the cyclization are N$^\epsilon$-Fmoc and OFm, respectively. The BOP-mediated cyclization procedure[30,35] with some modifications was used for cyclic lactam ring formation (see Experimental Section). The cleavage of the peptide from the resin and removal of the side chain protecting groups were done by the low−high HF procedure of Tam et al.[37] The cyclic glucagon analogues were then purified using a combination of dialysis, gel filtration on Sephadex G-15, and semi-preparative reverse phase high-performance liquid chromatography (RP-HPLC) with a VYDAC C_4 or C_{18} column.

The purity of the final products was assessed by HPLC, amino acid analysis, and thin-layer chromatography. The final products were further analyzed with enzymatic peptide mapping (and sequencing) using FAB mass spectroscopy, a procedure we have developed[38] using endoproteinase Asp-N digestion followed by FAB mass spectroscopy (Table 1). This provided unequivocal confirmation of the presence and the location of the lactam bridges (Table 2).

Biological activities of the synthetic analogues were examined in vitro using the rat liver plasma membrane binding and adenylate cyclase bioassays,[39] and Table 3 summarizes the results of these assays. Among all the cyclic analogues, those with the cyclization at residues 9 to 12 (**1** and **2**) and those cyclized at 18 and 21 or 12 and 21 (**5** and **6**) bind relatively better (5−30 times) compared to those cyclized at the 12 and 15 and 15 and 18 positions (**3**, **4** − Table 3). Note that all of the cyclic analogues with the cyclic moiety residues in the 9 to 18 region of glucagon (**1**−**4**) were found to be antagonists. Their pA_2 values (Table 3) range from 6.0 to 6.5. On the other hand, analogue **5** and the more globally constrained cyclic analogue **6** show partial agonist activities. Analogue **5** has 1.6% the potency of glucagon and could stimulate adenylate cyclase to 60% of the level reached by glucagon. Analogue **6** shows 0.6% of the potency of glucagon and a maximum stimulation of 40%.

CD spectroscopy was employed to evaluate the conformational features of glucagon and its cyclic analogues using α-helical inducing solvent, trifluoroethanol (TFE). As expected, based on our previous studies,[40] analogue

Table 2. Predicted Fragments of Glucagon and Its Analogues after Endoproteinase Asp-N Digestion

peptides		fragments	MW
glucagon	1−8	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-OH	864.4
	9−14	H-Asp-Tyr-Ser-Lys-Tyr-Leu-OH	788.8
	15−20	H-Asp-Ser-Arg-Arg-Ala-Gln-OH	732.3
	21−29	H-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH	1153.5
1 c[Asp^{9},Lys^{12}][Lys^{17,18},Glu^{21}]-glucagon-NH$_2$	1−14	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-c[Asp-Tyr-Ser-Lys]-Tyr-Leu-OH	1616.7
	15−29	H-Asp-Ser-Lys-Lys-Ala-Gln-Glu-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$	1825.1
2 c[Asp^{9},Lys^{12}]glucagon-NH$_2$	1−14	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-c[Asp-Tyr-Ser-Lys]-Tyr-Leu-OH	1616.7
	15−20	H-Asp-Ser-Arg-Arg-Ala-Gln-OH	732.3
	21−29	H-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$	1152.5
3 c[Lys^{12},Asp^{15}]glucagon-NH$_2$	1−8	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-OH	864.4
	9−20	H-Asp-Tyr-Ser-c[Lys-Tyr-Leu-Asp]-Ser-Arg-Arg-Ala-Gln-OH	1484.6
	21−29	H-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$	1152.5
4 c[Asp^{15},Lys^{18}]glucagon-NH$_2$	1−8	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-OH	864.4
	9−20	H-Asp-Tyr-Ser-Lys-Tyr-Leu-c[Asp-Ser-Arg-Lys]-Ala-Gln-OH	1456.6
	21−29	H-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$	1152.5
5 [Lys^{17}-c[Lys^{18},Glu^{21}]glucagon-NH$_2$	1−8	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-OH	864.4
	9−14	H-Asp-Tyr-Ser-Lys-Tyr-Leu-OH	788.4
	15−29	H-Asp-Ser-Lys-c[Lys-Ala-Gln-Glu]-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$	1807.0
6 c[Lys^{12},Asp^{21}]glucagon-NH$_2$	1−8	H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-OH	864.4
	9−29	H-Asp-Tyr-Ser-c[Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp]-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$ (**I**)	2618.9
	9−29	H-Asp-Tyr-Ser-c[Lys-Tyr-Leu-OH- H$_2$N-Asp-Ser-Arg-Arg-Ala-Gln-Asp]-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH$_2$ (**II**)	2636.9

Table 3. Biological Activities of Glucagon Analogues[a]

	receptor binding		adenylate cyclase		
compound	IC$_{50}$ (nM)	EC$_{50}$ (nM)	potency (%)	maximum stimulation (%)	pA$_2$
glucagon	1.5 (1.13−1.99)	5.0 (3.2−7.9)	100	100	
1	250 (235−269)	ia[b]	ia up to 50 μM	none	6.3
2	130 (100−164)	ia	ia up to 20 μM	none	6.5
3	1200 (904−1570)	ia	ia up to 50 μM	none	6.1
4	1500 (1180−1890)	ia	ia up to 20 μM	none	6.0
5	54 (36−82)	320 (184−555)	1.6	60	
6	190 (144−258)	794 (481−1310)	0.63	40	

[a] Receptor binding and adenylate cyclase activities were measured with rat liver membranes (see Experimental Section). Values are the mean of at least three independent experiments with 95% confidence limits in parentheses. 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] ia = inactive.

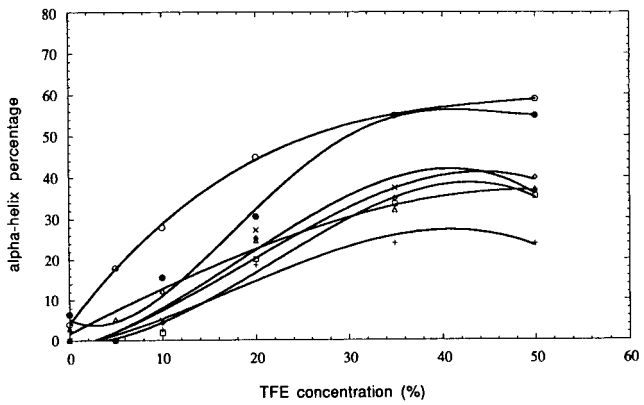

Figure 1. The effects of TFE concentration on the α-helix formation of cyclic glucagon analogues: (●) glucagon; (○) c[Asp^{9},Lys^{12}][Lys^{17,18},Glu^{21}]glucagon-NH$_2$ (**1**); (◊); c[Asp^{9},Lys^{12}]-glucagon-NH$_2$ (**2**); (×) c[Lys^{12},Asp^{15}]glucagon-NH$_2$ (**3**); (△); c[Asp^{15},Lys^{18}]glucagon-NH$_2$ (**4**); (□) [Lys^{17}-c[Lys^{18},Glu^{21}]-glucagon-NH$_2$ (**5**); (+) c[Lys^{12},Asp^{21}]glucagon-NH$_2$ (**6**).

1 with modifications at 17, 18, and 21 showed a significant increase in α-helix in comparison with glucagon, even at the low TFE concentration. Generally, for the other analogues, the α-helical content increased with an increase in TFE concentration and then remained virtually constant beyond 35% TFE (Figure 1). While glucagon could reach a maximum α-helical content of 58%, the four-residue cyclic glucagon analogues had maxima of only 35−39%. Interestingly, the more globally cyclized analogue **6** had 30% less α-helical content than glucagon even at high TFE concentrations.

Discussion

Designed synthetic peptide analogues, particularly with conformational constraints to identify and evaluate those molecular features essential to its bioactivities, have provided a powerful tool for increasing selectivity and specificity of peptide ligands for receptors, enzymes, and other macromolecular acceptor systems.[18,19] The conformational constraints used in this work utilize cyclization as a three-dimensional constraint. Since the biologically relevant three-dimensional structure of glucagon is unknown, the cyclic amide analogues were designed partly to examine the importance of the central region of the peptide with respect to receptor recognition and signal transduction.

The decreased binding potencies and loss of adenylate cyclase activities (antagonists were obtained) are quite significant. They undoubtedly are the results of alternations in the conformation (and perhaps loss of the charged groups) as a result of cyclic lactam formation. Unson et al.[10−13] also has suggested that charged residues in the glucagon sequence make major contributions in the stabilization of the binding interaction with the glucagon receptor that results in maximum biological potency.

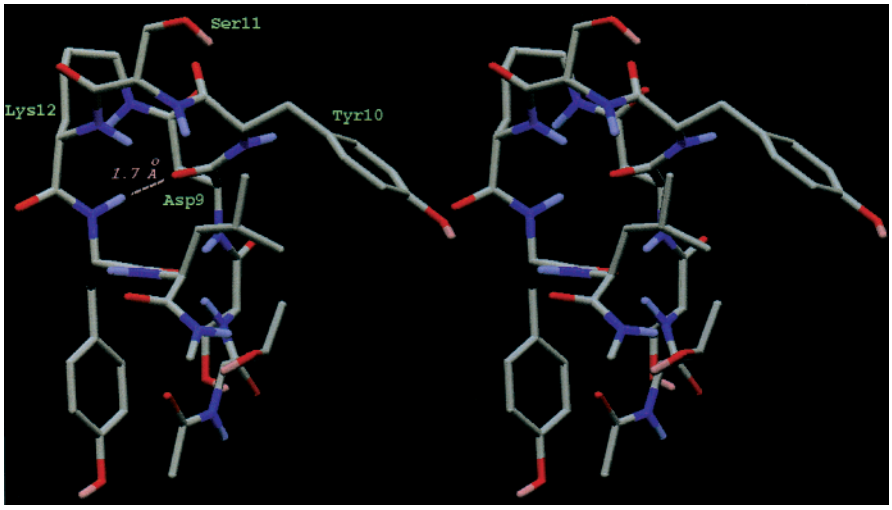

Figure 2. Stereoview of the lowest energy conformation of Ac-c[Asp^9,Lys^{12}]glucagon(7−14)-NH-Me.

Table 4. Dihedral Angles (deg) from Lowest Energy Conformation of Ac-c[Asp^9,Lys^{12}]glucagon(7−14)-NH-Me

	Thr^7	Ser^8	Asp^9	Tyr^{10}	Ser^{11}	Lys^{12}	Tyr^{13}	Leu^{14}
ϕ	−152.0	−150.7	−159.9	−61.1	73.9	−132.3	−133.2	−150.7
ψ	141.7	−9.6	113.7	131.5	10.9	−53.3	95.1	146.4

The constraints for analogues **1**, **2**, and **5** were designed to be located in the 9−12 and 18−21 regions of glucagon, which is near either the N-terminal or the C-terminal of this central region of glucagon. These two regions have been suggested to be conformationally more defined in glucagon. For example, as previously discussed, the C-terminal region has been found to be mostly α-helical, whereas the 8 to 11 or 10 to 13 region were predicted to have β-turn structures.[25,26] Because the residues involved in the cyclization are brought close to one another in space, these constraints would tend to stabilize certain turn conformations in the local region of the constraint.

To evaluate the secondary structure formed by the conformational constraint in analogues **1** and **2**, part of the sequence, including the region cyclized, was studied by molecular modeling using methods described in the Experimental Section. The lowest energy conformation, shown in Figure 2, suggests the formation of β-turn conformations between residues 9 and 12 (Table 4). Though there is a considerable drop in binding potency when compared with glucagon, both compounds **1** and **2** are antagonists without partial agonist properties. It is apparent that the cyclic conformation constraint induced in these peptides leads to a structure that is incompatible with transduction on binding to the glucagon receptor. This is reminiscent of oxytocin antagonist development,[41] where a local constraint imposed by replacing a Cys^1 residue with penicillamine (Pen, β,β-dimethylcysteine) converted the cyclic peptide from an agonist to an antagonist.

From the previous structure−activity relationship studies,[9,10] histidine at position 1 is very important for triggering a biological response, and all potent antagonists, such as [desHis^1,Glu^9]glucagon amide[10] and [desHis^1,desPhe^6,Glu^9]glucagon amide,[6] were designed based on this hypothesis. Interestingly, cyclic analogues **1** and **2** showed antagonist activity though these analogues possess a histidine residue at position 1. This result suggests that cyclization between Asp^9 and Lys^{12} not only induces a β-turn conformation in the middle region of the peptide but also positions the N-terminal (known to be responsible for biological response in glucagon analogues) in such a way that it cannot interact with the glucagon receptor for conversion of the receptor to its agonist conformation. This suggests that the middle part of glucagon, originally proposed as a "hinge region", is important for the proper relative orientation between binding elements (amphiphilic α-helical C-terminal) and signal elements (N-terminal) of glucagon, and it indicates that a β-turn conformation between residues 9 and 12 may not be the conformation preferred for agonist activity. This finding is also consistent with our earlier results[15] where the importance of side chain orientation at position 10 was demonstrated with β-MePhe substitution for Tyr^{10}. Improper orientation of side chain by β-MePhe substitution at position 10 would drive the peptide backbone to a conformation in which glucagon receptor does not prefer to interact, resulting in loss of binding affinity.

On the other hand, the cyclic analogue **6** with a much larger ring (35 membered ring vs 17 membered ring in analogues **1**−**4**) allows sufficient flexibility for partial agonist activity. In analogue **3**, the small ring size formed by the lactam bridge between Lys^{12} and Asp^{15} restricted the flexibility considerably, and as a result this conformational constraint diminished the molecule's ability to be recognized by the receptor. The conformation which this molecule is forced to assume results in poor binding and poor signal transduction. This explains the reduced binding affinity and antagonist activity for analogue **3**. On the contrary, the larger ring size of analogue **6** gives higher degrees of freedom in conformational space which allows the molecule more of a chance to obtain the conformations required for effective receptor recognition and signal transduction. As explained above, increased flexibility of the constraint imposed by cyclization in analogue **6** might be the reason for the restoration of the proper relative orientation of binding elements and signal transduction elements that are responsible for the observed partial agonism.

Constraining the 12 to 18 region obviously also has a profound effect on binding and transduction. The weak

binding potency for analogues **3** and **4** certainly reflect the effect of the constraint in this region. Apparently a reverse turn structure in this region of glucagon is not compatible with the analogue assuming a conformation necessary for transduction. Cyclization in **3** and **4** results in weak binding to the glucagon receptor, suggesting the involvement of this region (particularly, the negatively charged Asp^{15} residue) in the binding process, which agrees well with the findings of Unson et al.[10,11] In this regard it is interesting to note that the cyclic analogue [Lys^{17}-c[Lys^{18},Glu^{21}]glucagon-NH_2 (**5**) retained fairly good binding when compared with **3** and **4** though the cyclization is in the region of the C-terminal which is thought to be critically important for glucagon binding. It is noteworthy that this is in contrast to what was observed by Sturm et al.[17] where the formation of salt bridge in this region leads to binding potency even better than glucagon. This strongly suggests that a proper ring size on cyclization is very important to maximize receptor ligand interaction. Generally, lactam bridges generated between Lys and Glu residues separated by three or four residues preferentially stabilize α-helical conformations, which appears to be a requirement for receptor recognition in the case of glucagon.[17] Interestingly, this analogue retains modest partial agonist activity in the functional assay system. The results obtained are thus very intriguing.

Since the N-terminal region of glucagon is known to be primarily responsible for signal transduction, and thereby critical for determining agonist vs antagonist activity, the current results would suggest that constraints in the central region of glucagon can modulate the N-terminal conformation sufficiently that the glucagon analogue can no longer attain a conformation suitable for interacting with the glucagon receptor in an agonist mode. Studies in Merrifield's group have shown that the Asp^9 residue can be directly involved in development of glucagon antagonist activity.[42] It would appear that a rather extended sequence of the central region of glucagon can effect the overall N-terminal conformation in such a way as to prevent transduction in the glucagon receptor complex. By introducing constraints and forcing structural changes in analogues **1** through **4**, the required bioactive conformation becomes unachievable and, therefore, one ends up with antagonists. On the other hand, the constraint in analogue **5** is some distance from the N-terminus, and since the C-terminal region is not directly involved in the signal transduction process, the effect of the constraint is relatively less severe, resulting in a partial agonist.

In this work, circular dichroism (CD) spectroscopy was used to obtain more detailed information about the conformational features of the synthetic cyclic glucagon analogues. Glucagon has about 55% α-helical content at high TFE concentrations (Figure 1). Presumably this reflects the maximum α-helix forming potential of glucagon in solution. Previous studies have shown that the C-terminal region of glucagon has the highest α-helical forming potential.[40] Therefore, we assume that the 55% α-helix structure begins at the C-terminus and extends through the middle region, probably to residue 14. Except for compound **6** which has a much larger ring, the ring size found in analogues **1**−**5** are not compatible with an α-helical structure and thus they would be expected to disrupt continuation of such a structure and in turn inhibit α-helical potential. Consistent with this thinking, the maximum α-helix obtainable for analogues **2**−**5** drops to between 30% and 40% (Figure 1). The small variation among them probably reflects the different positions of cyclization and the experimental error in such determinations. Interestingly, the more globally cyclized analogue **6** appears to have the least α-helical content under all conditions. The CD spectrum shows the maximum α-helical content of analogue **6** at high TFE concentration to be 30% less than glucagon. On the contrary, analogue **1**, c[Asp^9,-Lys^{12}][$Lys^{17,18}$,Glu^{21}]glucagon, has a high α-helical content even at low TFE concentrations. This results from the α-helix inducing effect of the $Lys^{17,18}$ and Glu^{21} substitutions.[29,40] Nonetheless, this glucagon analogue is an antagonist, suggesting that an extended C-terminal α-helix in glucagon is not sufficient for agonist activity. Thus, specific conformational relationships between the N-terminal and central regions of glucagon are essential for agonist activity. To affect the conformation of the N-terminal of glucagon sufficiently to prevent the analogue from interacting with the glucagon receptor in its agonist conformation is a key in designing glucagon antagonists. We suggest that slowly relaxing the conformational constraint in the globally constrained c[Lys^{12},Asp^{21}]glucagon-NH_2 analogue may lead to better correlation between constraint and binding affinity. Continued structure−function studies in this direction will provide a better understanding of the mechanism of action of glucagon.

Experimental Section

General Methods for Synthesizing Cyclic Lactam Glucagon Analogues. All of the cyclic glucagon analogues were synthesized by using solid-phase peptide synthesis procedures developed in our laboratory[14,30] for glucagon analogues and cyclic lactam-containing peptides with modifications. The resin was *p*-methylbenzhydrylamine-polystyrene cross-linked with 1% divinylbenzene (0.35 mmol/g or 0.64 mmol/g) (Bachem, Torrance, CA). N^{α}-Boc-protected amino acids of the L-configuration were either purchased from Bachem or prepared by standard methods. Before use, the amino acid derivatives were tested by the ninhydrin test.[43] These amino acids were coupled to the growing peptide chain using diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) (Aldrich, Milwaukee, WI). The process was monitored by the ninhydrin test. Cyclizations were accomplished on the resin using benzotriazolyloxytris[dimethylamino]-phosphonium hexafluorophosphate (BOP reagent) (Peptides International, Louisville, KY). After being cleaved from the resin using the low−high HF procedure,[37] the peptides were purified by dialysis, gel filtration on Sephadex G-15 using 10% acetic acid, and RP-HPLC (Spectra Physics model 8700 instrument equipped with a model 8400 variable wavelength detector). Both VYDAC 214TP1010 C_4 semipreparative (10 mm × 250 mm) columns and VYDAC 218TP1010 C_{18} semipreparative (10 mm × 250 mm) columns were used in the purification. Detection was done by monitoring the UV absorbence at 214 nm for the peptide backbone and at 280 nm for tyrosine and tryptophan side chains. The HPLC conditions were as follows: a linear gradient elution starting with 30% CH_3CN and going to 55% CH_3CN in 0.1% TFA (1%/min for 25 min) at a flow rate of 3 mL/min, followed by the elution with 90% CH_3CN in 0.1% TFA for 5 min. The column was equilibrated for 10 min with 30% CH_3CN in 0.1% TFA before the next injection.

General Analytical Methods. The purity of the peptides was checked by the analytical HPLC using a VYDAC 218TBP-

Table 5. Analytical Properties of Synthetic Peptide Analogues (R_f Values of TLC and R_t Values of HPLC)

	TLC[a]			HPLC[b]		UV[c]
peptide	A	B	C	C_4	C_{18}	ϵ_{279}
1 c[Asp^9,Lys^{12}][Lys^{17,18},Glu^{21}]glucagon-NH_2	0.66	0.78	0.53	1.27	1.84	8217
2 c[Asp^9,Lys^{12}]glucagon-NH_2	0.70	0.74	0.64	1.21	1.77	6554
3 c[Lys^{12},Asp^{15}]glucagon-NH_2	0.85	0.77	0.85	0.97	1.82	8475
4 c[Asp^{15},Lys^{18}]glucagon-NH_2	0.51	0.76	0.63	0.90	1.43	6996
5 [Lys^{17}-c[Lys^{18},Glu^{21}]glucagon-NH_2	0.67	0.76	0.63	0.85	1.02	6665
6 c[Lys^{12},Asp^{21}]glucagon-NH_2	0.72	0.86	0.68	0.91	1.57	5854

[a] Kodak analytical glass TLC plates were used. Solvent systems: (a) ethyl acetate/pyridine/acetic acid/water 6:2:2.1:1.1; (b) butanol/acetic acid/pyridine/water 5:4:1:5; (c) butanol/acetic acid/pyridine/water 4:1:1:3. [b] VYDAC 214TP1010 C_4 reversed phase (25 × 0.46 cm) and VYDAC 218TP1010 C_{18} reversed phase (25 × 0.46 cm) with 0.1% trifluoroacetic acid/acetonitrile (75/25 v/v); flow rate 3.0 mL/min. All peptides were monitored at 214 and 280 nm. [c] Molar absorptivity: glucagon at 279 nM wavelength $\epsilon_{279} = 8310\ M^{-1}\ cm^{-1}$.

16 (4.6 mm × 250 mm) column as well as a TLC on 2.5 × 10 cm glass-backed silica gel G plate. For TLC, the following solvent systems were used: (a) ethyl acetate/pyridine/acetic acid/water (6:2:2.1:1.1); (b) 1-butanol/acetic acid/water/pyridine (5:1:4:5); (c) 1-butanol/acetic acid/water/pyridine (4:1:1:3). Detection was made by iodine vapors and ninhydrin spray. Amino acid analysis was performed on model 420A amino acid analyzer (ABI) at the Biotechnology Center Facility of the University of Arizona. The mass spectral $(M + 1)^+$ molecular ions and fragmentation patterns were obtained by endoproteinase Asp-N digest FAB-MS. Each peptide fragment was also sequenced by MS/MS sequencing. The CD spectra were taken with an AVIV model 60DS spectropolarimeter (Lakewood, NJ).

c[Asp^9,Lys^{12}][Lys^{17,18},Glu^{21}]Glucagon-NH_2 (1). The title compound was prepared by the solid-phase methods outlined above, starting with 3.67 g of *p*-methylbenzhydrylamine resin (pMBHA-resin, substitution = 0.64 mmol/g, 2 mmol scale). Acetic anhydride was used to block the free amine groups on the resin after 1 equiv of the first amino acid was coupled. The following 20 protected amino acids, from Asn^{28} to Asp^9, were coupled in stepwise fashion to the growing peptide chain, and the substitutions at 17, 18, and 21 positions were done as desired. Instead of the normally used N^α-Boc-β-carboxycyclohexyl-Asp, position 9 was substituted with N^α-Boc-Asp(β-OFm) and position 12 was substituted with N^α-Boc-Lys (N^ϵ-Fmoc). After removing the side chain Fm and Fmoc protecting groups of Asp^9 and Lys^{12} by 20% piperidine for 40 min, 3 equiv of BOP reagent (2.68 g) and 0.7 mL of DIEA in DMF were added. After 3 h, the ninhydrin test gave a light blue color indicating that the cyclization reaction was not complete. Another portion of the same cyclization reagent was added, and after another 3 h, the cyclization was complete.

TFA solution was used to deprotect the N^α-Boc group of Asp^9. It took three deprotections of 20, 40, and 60 min, respectively, before the Kaiser ninhydrin test showed no trace of blue color, indicating quantitative removal of the free amine group. The rest of amino acids were added in order using the N^α-Boc strategy; 6.95 g of protected peptide-resin was obtained at the completion of the synthesis.

To compare the efficiencies of peptide cleavage and the best approach for side chain deprotection in the HF cleavage procedures, two methods were examined for this analogue. The first used a separate step to deprotect the Trp N^α-formyl group before the traditional high HF cleavage procedure. The second method used the low−high HF procedure.[37]

In the first method, 2.5 g of the resin was stirred in 50 mL of 1 M piperidine in DMF at 0 °C for 2 h. After filtration, the resin was washed with 2 × 10 mL of DMF, 2 × 10 mL of dichloromethane (DCM), and 3 × 10 mL of methanol and dried overnight, yielding 2.3 g. The resin then was cleaved by HF in the following way: 1 g of the resin was placed in a reaction vessel along with 1 mL of anisole, 1 mL of dimethyl sulfide, and 110 mg of mercaptopyridine as scavengers for ions produced by the cleavage of side chain protecting groups. The vessel was cooled with liquid nitrogen and evacuated. Then 15 mL of anhydrous HF was distilled into the reaction vessel. At 0 °C, the mixture was stirred for about 1 h. The HF was removed by vacuum distillation, and the mixture of the resin and the free peptide was washed with anhydrous ethyl ether (3 × 20 mL) and filtered. The resin and peptide were next rinsed with 3 × 20 mL of ethyl acetate and 3 × 20 mL of DCM. Then the peptide was extracted with 5 × 40 mL of 10% acetic acid into a clean flask. The filtrate was frozen in an acetone/dry ice bath and lyophilized overnight to give the desired product.

In the low−high HF procedure, 1.5 g of the peptide-resin was placed into an HF reaction vessel along with 20 mL of dimethyl sulfide, 2.25 mL of *p*-cresol, and 0.8 mL of thiocresol. The mixture was chilled to liquid nitrogen temperature, 7.5 mL of HF was then distilled into the reaction vessel, and the mixture was stirred at 0 °C for 1 h. After HF and dimethyl sulfide were evaporated off in vacuo, the resin was washed with anhydrous ether 3 × 20 mL (yield 1.2 g). This resin was then treated with high HF procedure as described above. The only difference is that the reaction time was 40 min instead of 1 h.

The crude peptide mixture obtained from both methods was dissolved in 10% acetic acid solution and dialyzed overnight in a cold room at 5 °C in a 10% aqueous acetic acid solution with constant stirring. The dialysis membrane had a 1000 to 2000 MW cutoff (Spectrum, Los Angeles, CA: 50 mm × 5 m Sulfur-Free EDTA treated cellulose dialysis tubing in 0.05% sodium azide, record #132118). After dialysis, the peptide solution was lyophilized and a fluffy white solid was obtained. The lyophilized product was again dissolved in 10% acetic acid and purified with RP-HPLC. One major peak was obtained with κ' value of 1.24. This major peak, which is 18.8% of total peak area of the injection, was collected and lyophilized to yield peptide **1** as its TFA salt. The overall yield was approximately 3%. Amino acid analysis for **1**: Ala 0.97 (1.00), Asp/Asn 2.85 (3.00), Glu/Gln 4.00 (4.00), Gly 0.96 (1.00), His 1.02 (1.00), Leu 2.10 (2.00), Lys 3.12 (3.00), Met 1.05 (1.00), Phe 2.00 (2.00), Ser 4.16 (4.00), Thr 3.18 (3.00), Tyr 2.12 (2.00), Val 0.96 (1.00). See Table 5 for analytical data of the purified products.

Mass Spectral Analysis. The determination of the structure of the cyclic glucagon analogues was accomplished by FAB peptide mapping using endoproteinase Asp-N digestion by the procedures given below. Glucagon or glucagon analogue (0.2 mg) was dissolved in 250 μL of 1 M NH_4HCO_3 buffer (pH 8.1), and 2 μg of endoproteinase Asp-N (sequencing grade) was added to this solution, giving an enzyme/substrate ratio of 1:100 (w/w). The mixture was incubated at 37 °C for 30 h and lyophilized.

The lyophilized mixture was analyzed by FAB mass spectrometry on a double focusing BE mass spectrometer designed and built by AMD Intectra (Harpstedt, Germany).[38] The data acquisition and instrument control were accomplished using two AM 68K computers (KWS Computer Systems GmbH, Ettlingen, Germany) and software developed by AMD Intectra. The ionization involved the use of a 2.0 mA primary beam of Cs^+ ions accelerated to 7.0 kV. The samples were placed on the target of the inlet probe, and the target temperature was maintained at 12 °C using chilled methanol/water 1:1 v/v. The mass range 100−2500 was scanned at 60 s/decade with a resolution of 2000 (10% valley definition). The digest mixtures were dissolved in 20 mL of 10% CF_3COOH. Then 1 mL of this solution was mixed with 1 mL of glycerol on the target. The mass spectra obtained are the result of five accumulated scans.

The molecular weights of the glucagon analogues were measured by scanning the magnet from *m/z* 3000 to *m/z* 4000 at 60 s/decade at a resolution of 2000. The peptide mapping results are listed in Table 1. The mass values are mono-isotopic mass except where noted. Spectra are not subtracted.

c[Asp^{9},Lys^{12}]Glucagon-NH$_2$ (2). This compound was prepared from 2 mmol of the pMBHA-resin as analogue **1** above except that positions 17, 18, and 21 were not substituted. The substitution of the resin was 0.35 mmol/g, and thus 5.7 g of the resin was needed to start the synthesis on a 2 mmol level. Cyclization was achieved in the same way as with compound **1**. After cyclization, the next amino acid N$^{\alpha}$-Boc-Ser(O-Bzl)^{8} required three TFA treatments for deprotection, similar to Asp^{9} in compound **1**; 9 g of the protected peptide-resin was obtained. The peptide-resin was then treated with the low−high HF procedure, isolated, and purified as for **1**. The yield of the synthesis was 3%. Amino acid analysis for **2**: Ala 0.97 (1.00), Arg 2.02 (2.00), Asp/Asn 4.32 (4.00), Gly/Gln 3.06 (3.00), Gly 1.04 (1.00), His 1.03 (1.00), Leu 2.02 (2.00), Lys 1.03 (1.00), Met 0.96 (1.00), Phe 2.00 (2.00), Ser 4.08 (4.00), Thr 3.00 (3.00), Tyr 1.96 (2.00), Val 1.06 (1.00). Analytical data for the products are given in Table 5. Peptide mapping results are provided in Table 1.

c[Lys^{12},Asp^{15}]Glucagon-NH$_2$ (3). pMBHA-resin (5.7 g with a substitution of 0.32 mmol/g) was used to carry out the 2 mmol scale synthesis. The method used for this synthesis was the same as that for **1**, except that the position of cyclization was at 12 to 15 instead of 9 to 12. Each of the coupling and deprotecting steps was monitored by the ninhydrin and chloranil tests as usual. Cyclization took three treatments with BOP reagent and a total of 18 h to complete. The protected peptide-resin weighed 9.5 g. After being cleaved from the resin, the peptide mixture was isolated and purified as described previously. Amino acid analysis for **3**: Ala 1.06 (1.00), Arg 1.96 (2.00), Asp/Asn 3.84 (4.00), Glu/Gln 2.94 (3.00), Gly 1.01 (1.00), His 0.96 (1.00), Leu 1.88 (2.00), Lys 0.92 (1.00), Met 0.98 (1.00), Phe 2.00 (2.00), Ser 3.76 (4.00), Thr 2.79 (3.00), Tyr 1.90 (2.00), Val 0.99 (1.00). Analytical data for the product are given in Table 5. Peptide mapping results are shown in Table 1.

c[Asp^{15},Lys^{18}]Glucagon-NH$_2$ (4). The peptide was synthesized starting from 5.7 g of the pMBHA-resin with a 0.35 mmol/g substitution. The procedures were similar to the previous ones, except that Arg^{18} is substituted by Lys^{18} with N$^{\epsilon}$-Fmoc protection on the side chain and the cyclization was between Asp^{15} and Lys^{18}. Again, the BOP-mediated cyclization had to be repeated three times over 18 h for completion. A portion of the peptide-resin (4 g) was treated with low−high HF and the peptide extracted and purified as previously described. A total of 30 mg of pure **4** was obtained. Amino acid analysis for **4**: Ala 1.05 (1.00), Arg 1.07 (1.00), Asp/Asn 4.08 (4.00), Glu/Gln 3.24 (3.00), Gly 1.08 (1.00), His 1.04 (1.00), Leu 2.10 (2.00), Lys 1.96 (2.00), Met 1.12 (1.00), Phe 2.00 (2.00), Ser 3.88 (4.00), Thr 3.03 (3.00), Tyr 2.08 (2.00), Val 1.05 (1.00). Analytical data for the products are given in Table 5. Peptide mapping results are shown in Table l.

[Lys^{17}-c[Lys^{18},Glu^{21}]Glucagon-NH$_2$ (5). This analogue was synthesized starting from 5.7 g of pMHBA-resin with 0.35 mmol/g substitution. The process of the synthesis was similar to those for analogues **1**−**4**. The cyclization reaction for Lys^{18} and Glu^{21} residues took only two BOP-mediated treatments and less than 8 h to complete. After synthesis, the resin weighed 9.1 g. The low−high HF procedure was used to cleave the peptide from the resin. The same system was used for HPLC purification. The yield of the total synthesis was 4%. The amino acid analysis for **5**: Ala 0.99 (1.00), Asp/Asn 3.12 (3.00), Glu/Gln 3.92 (4.00), Gly 1.02 (1.00), His 0.98 (1.00), Leu 2.02 (2.00), Lys 2.91 (3.00), Met 1.06 (1.00), Phe 2.00 (2.00), Ser 3.72 (4.00), Thr 3.12 (3.00), Tyr 2.08 (2.00), Val 0.83 (1.00). Analytical data for the products are given in Table 5. Peptide mapping results are listed in Table l.

c[Lys^{12},Asp^{21}]Glucagon-NH$_2$ (6). A sample of 4 g of pMBHA-resin with 0.35 mmol/g substitution (1.4 mmol) was used to start the synthesis. Again, the cyclization took three BOP-mediated treatments and 18 h to complete; 6.9 g of the protected peptide-resin was recovered at the end of the synthesis. HF cleavage and purification procedure were the same as described above. The overall yield was about 1%. Amino acid analysis for **6**: Ala 0.96 (1.00), Arg 1.84 (2.00), Asp/Asn 3.80 (4.00), Glu/Gln 2.82 (3.00), Gly 0.90 (1.00), His 0.98 (1.00), Leu 1.90 (2.00), Lys 2.02 (2.00), Met 0.80 (1.00), Phe 2.00 (2.00), Ser 3.92 (4.00), Thr 2.91 (3.00), Tyr 1.82 (2.00), Val 1.01 (1.00). Analytical data for the products are given in Table 5. Peptide mapping results are shown in Table 1.

Biological Methods. The glucagon used in this work was purchased from Sigma and used without further purification. Glucagon and its analogues were initially dissolved in 10% acetic acid containing 1% BSA to a concentration of 100 μM. Then 200 μL of this solution was transferred to several tubes and lyophilized. The desired concentrations for adenylate cyclase and receptor binding assays were achieved by combining or diluting of the sample tube with 25 mM Tris-HCl, pH 7.5 at 25 °C, containing 1% BSA.

The rat liver membranes were isolated from male Sprague−Dawley rats weighing between 175 and 200 g according to the methods of Neville[44] with modifications described by Pohl et al.[39] The final membrane preparation was suspended in 10 mL of 25 mM Tris-HCl buffer with pH 7.5 at 25 °C. Aliquots containing approximately 1−2 mg of protein, determined by a modified method for membrane proteins,[45] were stored in liquid nitrogen for use within three months.

Glucagon was radioiodinated with Na^{125}I in the presence of chloramine T following the method of Tager et al.[46] To 3 nmol of glucagon (20 μL of a 0.15 nM solution in 2 mM Na_2CO_3) and 2.17 mCi of Na^{125}I (1 nM, 20 μL) in phosphate buffer (20 μL of 0.6 M, pH 7.2) was added 1.5 nmol of chloramine T (28 mg/100 mL) three times at 30 s time intervals. The reaction was terminated with 20 μL of $Na_2S_2O_5$ (0.5%) and 20 μL of Trizma base (0.5 M). Then 1 mL of elution buffer (0.08 M Tris, 0.02 M HCl, 0.08 M NaCl, and 1% BSA) was added to the reaction mixture. The [^{125}I]-glucagon was purified as described by Jorgensen and Larsen[47] on a QAE-sephadex A-25 column, previously washed with elution buffer, pH 8.6.

Receptor Binding Assay. The binding of glucagon and analogues **1** to **6** to liver plasma membranes were assayed using a method similar to that of Lin et al.[48] The incubation medium had a final volume of 500 μL consisting of liver plasma membrane (50 μg of protein), 125 000 cpm of [^{125}I]-glucagon, and unlabeled glucagon or glucagon analogues at concentrations ranging from 0 to 10 μM, all in 25 mM Tris-HCl, pH 7.5, at 25 °C, containing 1% BSA. The mixture was incubated for 10 min at 30 °C, followed by immediate cooling in an ice bath, and then filtered in a Millipore (Bedford, MA) filtration apparatus with 0.45 μm Oxoid filters previously soaked in Tris-BSA buffer for 12 h. The filters were washed four times with 1 mL of ice-cold 25 mM Tris-HCl buffer, pH 7.5, and transferred to a test tube. The remaining radioactivity of the receptor-hormone complex bound to the filter was then counted by a LKB 1275 minigamma counter. The nonspecific binding, measured in the presence of the excess unlabeled peptide (1 μM), was typically 15−20% of the total binding. It was subtracted from the total to give specific binding. The results were expressed as the percentage inhibition of [^{125}I]-glucagon specific binding. Triplicate determinations were made in all binding assays and each experiment was carried out at least twice, i.e., total of 6 determination/point.

Adenylate Cyclase Assay. Adenylate cyclase activity was determined by the procedure of Lin et al.[48] The standard incubation medium contained the following in a final volume of 0.1 mL including the glucagon and the analogue samples: 1 mM [α-^{32}P]ATP (40−100 cpm/pmol ATP); 5 mM $MgCl_2$; 1 mM cAMP; 10 mM GTP; 1 mM EDTA; 30 μg of liver plasma membrane, and an ATP regenerating system consisting of 20 mM creatine phosphate and 0.72 mg/mL (100 units/mL) creatine phosphokinase. The mixture was incubated for 10 min and termination of the reaction proceeded via addition of 100 μL of stopping solution which consisted of 0.01 Ci/100 mL [^{3}H]-cAMP, 2% sodium dodecyl sulfate, 5 mM ATP, and 2 mM cAMP followed by heating in a boiling water bath. The labeled

cAMP was then purified with Dowex 50 and alumina chromatography as described by Salomon et al.[49] The radioactivity was measured with a liquid scintillation counter, and the results were expressed as the percentage of stimulation of cAMP production above basal. Triplicate determinations of all the data points were obtained for various peptide concentrations, and all the experiments were repeated at least twice.

CD Spectroscopy. Concentrations of the glucagon and glucagon analogues (0.2 mg in each tube) were monitored accurately by UV molar absorption. The desired concentration was achieved by diluting the samples in one or several tubes with different solvents.

The CD spectra of glucagon and the 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.[50] The CD data were expressed in terms of mean residue ellipticity in deg cm^2 $dmol^{-1}$. The mean residue weight was calculated using the relevant amino acid compositions.[51]

Molecular Modeling. To save computational time and effort, only partial sequences of the analogues **1** and **2**, Ac-[Asp^9,Lys^{12}]-glucagon(7−14)-NH-Me, were studied by molecular modeling. The sequence was sketched with MacroModel[52] program (version 4.5), and Monte Carlo conformational search (5000 steps) was performed using united atom AMBER force field[53] implemented into MacroModel program with the generalized Born/surface area model[54] used for calculation of hydration energies. Conformations saved after the Monte Carlo conformational search were energy minimized using the AMBER force field with conjugate gradient.

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