Development of Potent Truncated Glucagon Antagonists

Jung-Mo Ahn, Matthew Medeiros, Dev Trivedi, and Victor J. Hruby*

Department of Chemistry, University of Arizona, Tucson, Arizona 85721

Received October 23, 2000

In pursuit of truncated glucagon analogues that can interact with the glucagon receptor with substantial binding affinity, 23 truncated glucagon analogues have been designed and synthesized. These truncated analogues consist of several fragments of glucagon with 11 or 12 amino acid residues (**1**−**4**), conformationally constrained analogues containing the sequence of the middle region of glucagon (**5**−**15**), and truncated analogues containing the sequence of the C-terminal region (**16**−**23**). Biological assays of these analogues showed that the truncated glucagon analogues with the sequence of the C-terminal region possess significantly better binding affinity compared to the truncated analogues with the sequence of the middle region, and these analogues (**17**−**23**) demonstrated potent antagonistic activity (pA_2 values between 6.5 and 7.5). On the basis of these results, it can be suggested that glucagon interacts with its receptor with two hydrophobic patches located in the middle and the C-terminal regions of glucagon, and both hydrophobic patches are necessary for significant receptor recognition. These two hydrophobic binding motifs, located in two different regions of glucagon, appear to be the reason why the earlier attempts to obtain truncated analogues with good binding affinity did not result in any success. Long peptide hormones such as glucagon seem to require more than one binding pocket on the receptors for maximal interaction.

Introduction

Glucagon is a peptide hormone, which consists of 29 amino acid residues, and is secreted by the α-cells of pancreatic islets (Figure 1). The secretion of glucagon is primarily controlled by blood glucose levels, and during the hypoglycemic state, glucagon is secreted by α-cells of pancreas, released into the blood, and delivered to its target organs, especially hepatocytes and adipocytes.[1] Glucagon has a very important role in glucose homeostasis, stimulating gluconeogenesis and glycogenolysis in hepatocytes[2] and stimulating lipolysis in the adipocytes.[3] Glucagon exerts its activity via a seven-transmembrane G protein-coupled receptor, and the rat and human glucagon receptors have been cloned.[4,5]

Diabetes mellitus is a widespread, degenerative disease that can be divided into two major categories: type I, or insulin-dependent diabetes mellitus (IDDM); and type II, or non-insulin-dependent diabetes mellitus (NIDDM). Although the role of glucagon in pathogenesis of diabetes is controversial, abnormally high concentrations of glucagon are found in diabetic patients.[6] The proposed bihormonal hypothesis in diabetes mellitus postulates that insulin deficiency causes impairment of glucose utilization, and glucagon is a primary mediator of the overproduction of glucose and ketone bodies.

Several glucagon antagonists, such as [N^{α}-trinitrophenyl-His[1], homo-Arg[12]]glucagon[7] (THG; IC_{50} = 15.0 nM; pA_2 = 8.16), [desHis[1], Glu[9]]glucagon amide[8] (IC_{50} = 3.75 nM; pA_2 = 7.25), and [desHis[1], desPhe[6], Glu[9]]-glucagon amide[9] (IC_{50} = 48.0 nM; pA_2 = 8.20) which bind to the plasma membrane receptor and competitively prevent the binding of glucagon and some aspects of glucagon action,[10,11] have been developed. But except for the latter compound, [desHis[1], desPhe[6], Glu[9]]-glucagon amide, THG and [desHis[1], Glu[9]]glucagon amide have been shown to be partial agonists by the cAMP accumulation assay.[11] The glucagon antagonist [desHis[1], desPhe[6], Glu[9]]glucagon amide was tested in vivo, where it lowered the blood glucose levels to 60% in streptozotocin-induced diabetic rats,[11] as the glucagon antagonists THG[10] and [desHis[1], Glu[9]]glucagon amide[12] did. On the other hand, similar normalization of blood glucose concentration was successfully achieved by using a monoclonal glucagon antibody to immunoneutralize endogenous glucagon.[13] These studies demonstrate the potential use of potent glucagon antagonists for therapeutic applications.

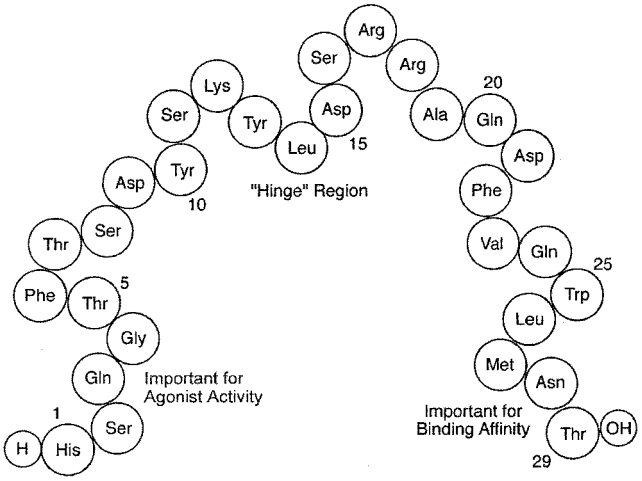

Figure 1. Primary structure of glucagon.

As observed earlier for peptide hormones such as glucagon, obtaining relatively small size ligands with

* To whom correspondence should be addressed. Telephone: (520) 621-6332. Fax: (520) 621-8407. E-mail: hruby@u.arizona.edu.

10.1021/jm000453e CCC: $20.00
Published on Web 03/31/2001

Table 1. Biological Activities of the Truncated Glucagon Analogues Reported Earlier

	receptor binding		adenylate cyclase activity			
compound	IC_{50} (nM)	relative binding (%)	EC_{50} (nM)	relative potency (%)	pA_2	ref
glucagon	1.5	100	8	100	–	
glucagon(1−6)		<0.0001		<0.0001	–	15
glucagon(1−15)		<0.0001		<0.0001	–	15
[Asn[15]]glucagon(1−17)		~0.0001		~0.0001	–	15
glucagon(1−24)		0.0002		0.0002	–	15
[Glu[9]]glucagon(7−29)-NH_2		0.33	–	0	–	19
[Glu[9]]glucagon(9−29)-NH_2		0.04	–	0	–	19

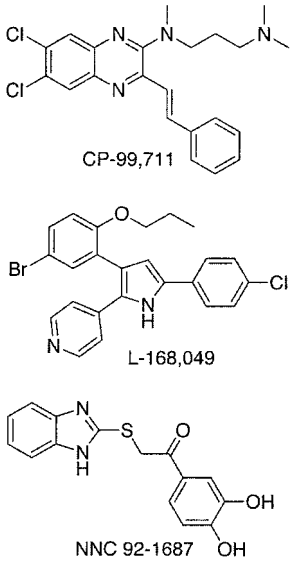

Figure 2. Non-peptide glucagon antagonists reported earlier.

substantial biological activities has been a difficult goal for structure−activity relationship studies. Generally, truncated peptide hormone analogues allow the researchers to synthesize analogues with less effort, materials, and time and to study more systematically structure−activity relationship. This approach would be especially beneficial for longer peptides such as glucagon. In pursuit of potent truncated glucagon analogues, extensive structure−activity relationship studies have been carried out,[14,15] but all the attempts to obtain biologically active glucagon analogues that are substantially truncated have failed to date. Table 1 shows a few of the truncated glucagon analogues reported earlier. Small fragments of glucagon in the C-terminal region, such as glucagon(20−29), glucagon(22−29), glucagon(16−24), and glucagon(19−29), and the N-terminal region, such as glucagon(1−6), glucagon(1−12), glucagon(1−15), and glucagon(1−17), were found to have no biological activity or receptor binding affinities even at millimolar concentrations.[15−18] On the other hand, [Glu[9]]-glucagon(7−29) amide and [Glu[9]]glucagon(9−29) amide showed very weak binding affinity (0.33% and 0.04% relative to glucagon, respectively).[19]

Although a truncated glucagon analogue with potent binding affinity has not been successfully synthesized to date, several non-peptidic glucagon antagonists were reported by pharmaceutical companies (Figure 2).[20−22] However, some of these small molecule glucagon antagonists appeared not to interact with the same glucagon binding site in the glucagon receptor.[20,21] One of these noncompetitive antagonists seems to bind deep into the transmembrane region of the glucagon receptor to inhibit glucagon from binding allosterically.[21]

Therefore, a major goal in this research has been to design and synthesize truncated glucagon analogues and determine the minimum sequence for substantial receptor binding affinity. In this study, 23 glucagon analogues were synthesized (Table 2). These include several glucagon fragments with 11 or 12 amino acid residues in order to determine the structural requirements for the receptor binding of glucagon, as well as conformationally restricted truncated analogues containing the sequence of the middle region of glucagon, and finally truncated analogues containing the sequence of the C-terminal region.

Results and Discussion

Development of truncated analogues of peptide hormones gives a great deal of advantage to the researcher, especially for long peptide hormones such as glucagon, because shortening the length of peptides requires less synthetic time and effort and makes systematic structure−activity relationship studies more readily attained. This approach has been successfully demonstrated in our laboratory in the case of α-melanocyte stimulating hormone.[23−25] Although natural α-melanocyte stimulating hormone (α-MSH) consists of 13 amino acid residues, the sequence has been successfully trimmed down to a heptapeptide without loss of biological activity since residues at position of 1, 2, 3, 11, 12, and 13 are relatively unimportant.

Therefore, in pursuit of truncated glucagon analogues with minimal required sequence which can bind to its receptor with significant binding affinity, a number of truncated glucagon analogues have been designed and synthesized.

Systematic Fragmentation of Glucagon into Undeca- and Dodecapeptide Amides. Aiming to find a novel antagonist with low molecular weight, we hypothesized that when glucagon binds to its receptor, the whole molecule is not necessarily required. Consequently, it was suggested that the essential binding domain in glucagon might be as small as several amino acids. On the basis of this hypothesis, a variety of fragment peptides of glucagon that have 11 or 12 amino acid residues were synthesized (**1**−**4**) (Table 2). These peptides have the sequences of residues 1−11 (**1**), 5−16 (**2**), 10−20 (**3**), and 15−26 (**4**), respectively, and the binding affinities of these peptides were obtained in order to examine the minimum binding domains that are required for recognition by the receptor. Unfortunately, none of these peptides displayed any binding affinity, probably due to the unnatural positive charges developed at the N-terminus ammonium group of the fragment peptides and the flexible nature of these linear peptides which make receptor recognition extremely difficult.

Truncated Glucagon Analogues Containing the Middle Region. Since most of the previously reported efforts to acquire a glucagon fragment with good binding affinity for the glucagon receptor have been focused on N-terminal fragments[15] and C-terminal fragments[16−18] (shown in Table 1), truncated glucagon analogues

Table 2. Biological Activities of the Truncated Glucagon Analogues

	compound	receptor binding		adenylate cyclase activity		
		IC_{50} (nM)	relative binding (%)	EC_{50} (nM)	maximum stimulation (%)	pA_2
	glucagon	1.5	100	5.9	100	–
1	glucagon(1−11) amide	i.a.[a]	0	i.a.	0	–
2	glucagon(5−16) amide	i.a.	0	i.a.	0	–
3	glucagon(10−20) amide	i.a.	0	i.a.	0	–
4	glucagon(15−26) amide	i.a.	0	i.a.	0	–
5	Ac-glucagon(6−14) amide	i.a.	0	i.a.	0	–
6	Ac-[$Cys^{7,11}$]glucagon(6−14) amide	n.d.[b]	30[c]	i.a.	0	–
7	Ac-c[$Cys^{7,11}$]glucagon(6−14) amide	n.d.	62[c]	i.a.	0	–
8	Ac-[$Cys^{7,12}$]glucagon(6−14) amide	n.d.	24[c]	i.a.	0	–
9	Ac-c[$Cys^{7,12}$]glucagon(6−14) amide	n.d.	24[c]	i.a.	0	–
10	Ac-[$Cys^{7,12}$]glucagon(6−15) amide	n.d.	56[c]	i.a.	0	–
11	Ac-c[$Cys^{7,12}$]glucagon(6−15) amide	n.d.	30[c]	i.a.	0	–
12	Ac-[$Cys^{9,14}$]glucagon(6−15) amide	n.d.	32[c]	i.a.	0	–
13	Ac-c[$Cys^{9,14}$]glucagon(6−15) amide	n.d.	43[c]	i.a.	0	–
14	Ac-[$Cys^{9,14}$]glucagon(9−14) amide	i.a.	0	i.a.	0	–
15	Ac-c[$Cys^{9,14}$]glucagon(9−14) amide	i.a.	0	i.a.	0	–
16	Ac-glucagon(17−29) amide	2300 ± 900	0.065	i.a.	0	–
17	Ac-glucagon(10−29) amide	60 ± 3	2.5	i.a.	0	7.44
18	Ac-glucagon(6−29) amide	31 ± 9	4.8	i.a.	0	6.57
19	Ac-[Nal(2)^{10,13}]glucagon(10−29) amide	800 ± 150	0.19	i.a.	0	6.87
20	phenylacetyl-glucagon(10−29) amide	249 ± 68	0.60	i.a.	0	7.37
21	phenylbutyryl-glucagon(10−29) amide	21.2 ± 9.9	7.1	i.a.	0	7.38
22	phenylhexanoyl-glucagon(10−29) amide	131 ± 64	1.1	i.a.	0	7.37
23	phenylbutyryl-[Nal(2)^{13}]glucagon(10−29) amide	781 ± 163	0.19	i.a.	0	6.70

[a] i.a.: inactive up to 100 μM. [b] n.d.: not determined. [c] Maximum percent inhibition of radiolabeled glucagon at 100 μM analogue concentration.

containing the middle region of glucagon were synthesized (**5**−**15**). From previous experience with the glucagon fragments described above, the N-terminal amine groups of the new peptides were acetylated in an attempt to remove undesired electrostatic charges, and also, conformational constraints were introduced in order to reduce the flexibility of the peptides. Another reason for designing these peptides containing the middle region of glucagon is that non-peptidic antagonists of glucagon[20−22] (Figure 2), though they do not appear to interact with the same glucagon binding site, have aromatic rings in common. Therefore, we synthesized the truncated glucagon analogues that contain the aromatic rings of Tyr^{10} and Tyr^{13} in order to investigate the importance of aromatic rings for binding affinity.

Although analogues **6**−**11** (Table 2) demonstrated some modest ability to displace radiolabeled glucagon at high concentrations (100 μM), all of these peptides displayed very weak or no binding affinity. On the other hand, Ac-glucagon(6−14) amide (**5**) showed no receptor binding at all, indicating that no nonspecific binding at these extremely high concentrations of peptide took place either. For analogues **10**−**13**, an aspartic acid at position 15 was added to the sequence with residues 6−14 with the expectation of increasing binding affinity, since Asp^{15} was demonstrated to be an important residue for binding affinity.[26] However, this addition of Asp^{15} did not enhance the binding affinity. On the other hand, reduction of the length of the sequence to a hexapeptide, analogues **14** and **15** (Table 2), resulted in complete loss of binding affinity.

Conformational restrictions between residues 7 and 11 (**7**), 7 and 12 (**9**, **11**), and 9 and 14 (**13**, **15**) were introduced via a disulfide bridge in order to force a turn conformation (Table 2). Despite the weak binding affinities of these peptides, percent displacement of radiolabeled glucagon at 100 μM could be obtained. Although the analogues **6**−**15** bound to the glucagon receptor with weak affinity, differences between the maximum displacement of radiolabeled glucagon at 100 μM by linear and cyclic analogues were observed. The linear analogue (**10**) between cysteines at positions 7 and 12 showed greater displacement (56%) of radioactive glucagon than the cyclic analogue (**11**; 30%) did. On the other hand, the cyclized analogue (**7**) between cysteines at positions 7 and 11 displaced glucagon better (62%) than the linear analogue (**6**) (30%). These results can be interpreted to suggest that the cyclic conformational restrictions (disulfide bridge between positions 7 and 11) led to a conformation that could be more easily recognized by the receptor relative to their linear counterparts.

Truncated Glucagon Analogues Containing the C-Terminal Region. As discussed above, the C-terminal fragments have been reported earlier,[16−18] but none of them were found to bind to the receptor, although the C-terminal region of glucagon is generally known to be essential for maintaining significant binding affinity for glucagon.[14] Interestingly, the most dominant enzymatic byproduct of glucagon, glucagon(19−29), also referred to as miniglucagon, did not show any reasonable binding affinity for the glucagon receptor.[18]

However, in this study, the sequences of truncated glucagon analogues containing the C-terminal region were elongated from the C-terminus gradually (**16**−**18**) (Table 2). The smallest one, Ac-glucagon(17−29) amide (**16**), has the sequence of miniglucagon plus Arg^{17}-Arg^{18}, and the result of binding assays showed that the elongation of the sequence of miniglucagon with only two arginine residues was sufficient for the peptide to be recognized by the receptor, though with poor binding affinity (IC_{50} = 2.3 μM). Encouraged by this result, two more peptides containing residues between 10 and 29 (**17**) and 6 and 29 (**18**) were synthesized. Since these peptides have longer middle-region sequences than

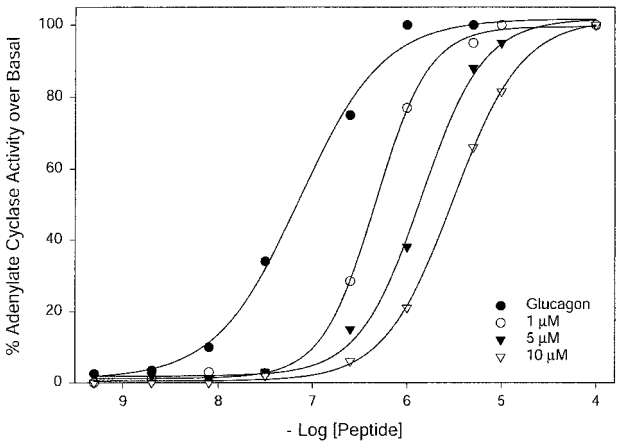

Figure 3. Dose−response curves showing competition of glucagon with 1, 5, and 10 μM of a glucagon antagonist (**18**), Ac-glucagon(6−29) amide.

analogue **16**, they showed better binding affinities (IC_{50} = 60 nM and 31 nM, respectively). The elongation of seven residues from analogue **16** to **17** demonstrated a greater improvement (38-fold) in the binding affinity than the elongation of four residues from analogue **17** to **18** did (2-fold), suggesting that most of the binding motif required for efficient recognition by the receptor already is present in the sequence **17**. The ability of analogue **18** to act as a good competitive antagonist at the glucagon receptor is given in Figure 3.

Since a hydrophobic patch, organized by residues of Phe[6], Tyr[10,13], and Leu[14] and which has been demonstrated by X-ray crystallography[27] and 2D-NMR spectroscopy,[28] is important for substantial binding affinity,[29] the improved binding affinities of the analogues **17** and **18** seem to result from a fortified hydrophobic patch in the middle region, and a more extended hydrophobic patch of **18** resulted in better binding affinity than **17** (Table 2). In accordance with this finding, the tyrosines at position 10 and 13 were replaced with 2-naphthylalanine derivatives for better aromatic interactions in the hydrophobic patch, but this modification disturbed its interaction with the receptor (**19**, IC_{50} = 800 nM), presumably as a result of the removal of the phenolic functional group from tyrosine at position 10, which is important for binding affinity,[14] and the steric hindrance caused by the larger size of the aromatic ring of a naphthyl group than the phenol group of tyrosine.

In an attempt to improve the binding affinity of analogue **17**, several phenyl-substituted acyl groups were added to the N-terminus to replace the missing phenylalanine at position 6 which previously has been found to be necessary to form the full hydrophobic patch in the middle region of glucagon.[29] Phenylacetyl (**20**), phenylbutyryl (**21**), and phenylhexanoyl (**22**) groups were used in order to place the phenyl group in different three-dimensional spaces (Table 2). Of these three peptides, phenylbutyryl-glucagon(10−29) amide (**21**) revealed the best binding affinity (IC_{50} = 21 nM, more than 5-fold better binding affinity than **20** and **22**), indicating that a spacer of three methylene groups is optimum for organizing the important hydrophobic patch properly. With addition of the phenylbutyryl group, analogue **21** interacted with the receptor with

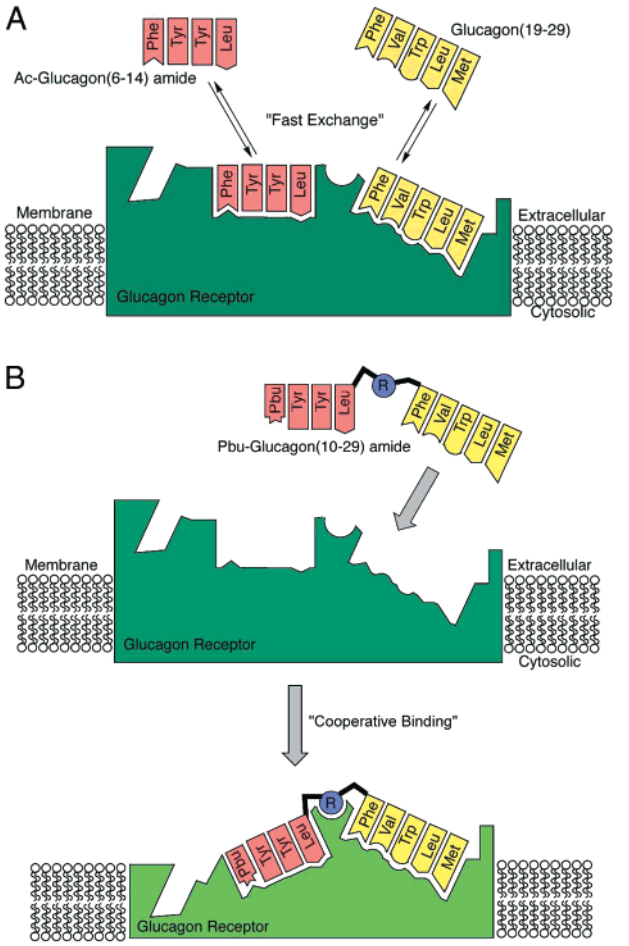

Figure 4. Schematic diagram of the binding of the glucagon fragments that have only one binding element (A) and that have two binding elements (B).

higher affinity than the analogue **18**, which was the peptide that **21** was intended to mimic. Since analogue **21** does not have residues between positions 7−9, it can be presumed that the polar amino acids between residues 6−9 of the analogue **18** increased the flexibility of the N-terminal region of the peptide and disturbed the optimal aromatic interaction between the phenyl ring of phenylbutyryl group and tyrosine side chains, resulting in the slight loss of binding affinity.

In an effort to improve the binding affinity of **21**, 2-naphthylalanine was substituted for tyrosine at position 13 (**23**). Unlike **19**, in which tyrosines at position 10 and 13 were replaced with 2-naphthylalanines, only the tyrosine at position 13 was substituted with 2-naphthylalanine in order to retain a tyrosine at position 10 for receptor recognition. However, steric hindrance of the naphthyl group, in this case as well, greatly reduced (approximately 40-fold) the binding affinity of analogue **23**.

From these results, a conclusion can be drawn that there are at least two different binding domains located separately in the glucagon sequence, and a reasonable receptor interaction is only attained when these two binding domains are present in glucagon analogues (Figure 4). From these findings, we hypothesized that reinforcement of the hydrophobic patches enhances receptor recognition. Hence the main binding elements

would appear to be these hydrophobic patches interacting with their counterparts on the receptor.

This hypothesis explains the reason why relatively small, truncated glucagon analogues that only include one binding domain do not bind to the receptor well enough to be measured by competitive displacement of radiolabeled glucagon. At this point, no complete picture of binding can be given, but it is reasonable to suggest that binding of glucagon to its receptor would be cooperative. If it is not cooperative binding, then peptides with only one of the binding domains should have shown at least moderate receptor affinities. Therefore, we suggest that the C-terminal binding domain initiates the interaction with the receptor, presumably because the secondary structure of the C-terminal region is better defined, and then subsequently a change in ligand and/or receptor structure helps identify a second binding domain for glucagon to enhance further the receptor interaction.

Glucagon, a long linear polypeptide, has two different binding domains, composed of hydrophobic residues and located in the middle and the C-terminal region, and these two hydrophobic patches located apart from each other are required to optimize the receptor recognition. From the crystal structure of glucagon, these two hydrophobic regions appear to be about 20 Å apart. Thus, it can be readily seen why it has been so difficult to obtain glucagon fragments of small molecular weight that can bind to the glucagon receptor.

Potent Truncated Glucagon Antagonists. In terms of adenylate cyclase activity, none of the peptides in this study produced any signal transduction (Table 2). For the analogues **1**−**15**, it was apparently because of lack of substantial binding affinity. On the other hand, all the peptides with significant binding affinity, **16**−**23**, were shown to be antagonists, with varying pA_2 values. Since the truncated analogues containing the C-terminal region (**16**−**23**) do not have the message region that is responsible for triggering G protein to initiate the signal cascade,[15] it is not surprising to observe no adenylate cyclase activity.

It is noteworthy that the pA_2 values of the analogues **17**−**23** do not necessarily correspond to the binding affinity. In general, it is usual to assume that the compounds that bind better will demonstrate a higher degree of antagonism. But in the case of these truncated glucagon analogues, all of them exhibit similar antagonistic potency despite differences in binding affinity, except analogue **18**, which is lower than expected.

For the analogues **19** and **23** with 2-naphthylalanines, the relatively lower pA_2 values can be explained because they did not interact with the receptor well, compared to analogues **17**, **20**, **21**, and **22**. But, the 10-fold higher antagonism of analogue **17** compared to **18**, whose binding affinity is twice that of **17**, is quite surprising. This discrepancy also was observed for the analogues **20**−**22**. These phenyl-substituted acylated glucagon(10−29) amides certainly exhibited different binding affinities. In particular, analogue **21** binds 10-fold better than analogue **20**, while the pA_2 values are the same for these two analogues.

Final Remarks. For the first time, we have obtained glucagon analogues with significantly lower molecular weight and good binding affinity. These potent, truncated glucagon antagonist analogues have been designed and synthesized based on the idea of two binding domains at the glucagon receptor. Apparently nature has designed long peptide hormones, such as glucagon, to interact with several different distinct sites on the receptor. The minimum sequence necessary to attain marginal binding affinity of truncated analogues requires approximately two-thirds the length of the native peptide. For the glucagon receptor, it was demonstrated that there are two separated binding domains in the glucagon sequence, one in the middle region and the other in the C-terminal region, and the evidence suggests that the driving force for receptor recognition is related to specific hydrophobic interactions. Although the best truncated antagonist, phenylbutyryl-glucagon-(10−29) amide, is not as potent as [desHis[1], desPhe[6], Glu[9]]glucagon amide, it can serve as a lead compound to be further optimized for better binding affinity and antagonism. Also, the truncation to two-thirds of the glucagon sequence will help to perform more systematic structure−activity relationship studies for obtaining competitive glucagon antagonists.

Experimental Section

Materials. All peptides designed in this investigation were prepared by solid-phase techniques either by manual synthesis or 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, and acetic anhydride (Aldrich, Milwaukee, WI); dichloromethane (DCM) and *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. The structures of the purified peptides were characterized by electrospray mass spectrometry (Finnigan LCQ ion trap mass spectrometer) and by amino acid analysis (Applied Biosystems model 420A amino acid analyzer). All of these data can be found in the Supporting Information.

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 a Perkin-Elmer LC 90 UV spectrophotometer detector for preparative high-pressure liquid chromatography on 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 a Hewlett-Packard 1100 series fixed-wavelength UV detector or Perkin-Elmer LC 90 UV detector and were integrated with a Hewlett-Packard 3396 series III integrator. The amino acid analysis was done using an Applied Biosystems model 420A amino acid analyzer with automated hydrolysis (vapor phase at 160 °C for 100 min using 6 N HCl).

Peptide Synthesis. (a) General Protocol for Peptide Synthesis with N^{α}-Fmoc/*tert*-Butyl Chemistry. The glucagon analogues used in this investigation were synthesized manually or with Applied Biosystems ABI 431A automated peptide synthesizer using Fmoc/*tert*-butyl chemistry. For the manual synthesis, the Rink amide resin[30] (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, substitution 0.4−0.7 mmol/g) was allowed to be swollen in DMF overnight. The resin was washed with DMF (3 × 2 min), and the 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^{α}-Fmoc amino acid was coupled using preactivated N^{α}-Fmoc amino acid in DMF (3 equiv of N^{α}-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[31] and the 2,4,6-trinitrobenzenesulfonyl acid (TNBS) test[32] 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^{α}-Fmoc amino acid in DMF (prepared in the same manner) and 6 equiv of DIEA for another 2 h. If double coupling did not result in a negative Kaiser ninhydrin test and a 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.

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 the automated peptide syntheses 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.

For N^{α}-acetylation, the resin was placed in the manual peptide synthesis vessel and the N^{α}-Fmoc protection group was removed by 25% piperidine in DMF (1 × 5 min and 1 × 30 min). The resin was washed with DMF (3 × 2 min) and DCM (3 × 2 min) and treated with 10% acetic anhydride in DMF for 30 min. The resin was washed with DMF (3 × 2 min) and DCM (6 × 2 min) and dried in vacuo.

(b) 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 a gentle flow of nitrogen to a volume of approximately 3 mL, and then 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.

(c) 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} or C_4-bonded) HPLC column with gradient elution at a flow rate of 5.0 mL/min. The gradients used for the purification were 0−50% acetonitrile in 0.1% aqueous TFA over 30 min, 10−90% 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 fractions containing the purified peptide were 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, 20−60% over 20 min, and 0−50% over 25 min.

(d) General Procedure for Cyclization To Form a 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 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). 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.

Biological Assays. (a) Isolation of Liver Plasma Membranes. Liver plasma membranes were prepared from male Sprague−Dawley rats weighing between 200 and 250 g.[33,34] 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 was 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[35] and a modified procedure.[36] 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.

(b) Receptor Binding Assay. Glucagon and its analogues were dissolved in 1 mM HCl to a 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 assays were performed according to Wright and

Rodbell[37] 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), was used. The mixture was incubated for 10 min at 30 °C, followed by immediate cooling in an ice bath, and filtered through a 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 a LKB1275 mini-γ 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.

(c) 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.[38] Labeled cAMP was determined by the method of Salomon[39] 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 (40−100 cpm/pmol 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 were used. Results are expressed as a potency (EC_{50}), 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 that were used in calculating the pA_2 values as described by Schild.[40]

Acknowledgment. This work was supported by a grant from the U. S. Public Health Service, DK 21085. The views expressed are those of the authors and do not necessarily reflect those of the U. S. Public Health Service.

Supporting Information Available: Tables summarizing the physicochemical properties of the synthetic glucagon analogues as well as details on amino acid analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hruby, V. J.; Krstenansky, J. L.; McKee, R.; Pelton, J. T. Glucagon Structure−Function Relationships. The Use of Glucagon Analogues in Studies of Glucagon Receptor Interactions. Hormonal Control of Gluconeogenesis. In *Signal Transmission*; Kraus-Friedmann, N., Ed.; CRC Press: Boca Raton, FL, 1986; pp 3−20.

(2) Johnson, M. E. M.; Das, N. M.; Butcher, F. R.; Fain, J. N. The Regulation of Gluconeogenesis in Isolated Rat Liver Cells by Glucagon, Insulin, Dibutyryl Cyclic Adenosine Monophosphate, and Fatty Acids. *J. Biol. Chem.* **1972**, *247*, 3229−3235.

(3) McGarry, J. D.; Foster, D. W. Regulation of Hepatic Fatty Acid Oxidation and Ketone Body Production. *Annu. Rev. Biochem.* **1980**, *49*, 395−420.

(4) Jelinek, L. J.; Lok, S.; Rosenberg, G. B.; Smith, R. A.; Grant, F. J.; Biggs, S.; Bensch, P. A.; Kuijper, J. L.; Sheppard, P. O.; Sprecher, C. A.; O'Hara, P. J.; Foster, D.; Walker, K. M.; Chen, L. H. J.; McKernan, P. A.; Kindsvogel, W. Expression Cloning and Signaling Properties of the Rat Glucagon Receptor. *Science* **1993**, *259*, 1614−1616.

(5) Lok, S.; Kuijper, J. L.; Jelinek, L. J.; Kramer, J. M.; Whitmore, T. E.; Sprecher, C. A.; Mathewes, S.; Grant, F. J.; Biggs, S. H.; Rosenberg, G. B.; Sheppard, P. O.; O'Hara, P. J.; Foster, D. C.; Kindsvogel, W. The Human Glucagon Receptor Encoding Gene: Structure, cDNA Sequence and Chromosomal Localization. *Gene* **1994**, *140*, 203−209.

(6) Unger, R. H.; Orci, L. The Essential Role of Glucagon in the Pathogenesis of Diabetes Mellitus. *Lancet* **1975**, *1*, 14−16.

(7) Bregman, M. D.; Trivedi, D.; Hruby, V. J. Glucagon Amino Groups. Evaluation of Modifications Leading to Antagonism and Agonism. *J. Biol. Chem.* **1980**, *255*, 11725−11731.

(8) Unson, C. G.; Andreu, D.; Gurzenda, E. M.; Merrifield, R. B. Synthetic Antagonists of Glucagon. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4083−4087.

(9) Azizeh, B. Y.; Van Tine, B. A.; Sturm, N. S.; Hutzler, A. M.; David, C.; Trivedi, D.; Hruby, V. J. [desHis^1,desPhe^6,Glu^9]-Glucagon Amide: A Newly Designed "Pure" Glucagon Antagonist. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1849−1852.

(10) Johnson, D. G.; Goebel, C. A.; Hruby, V. J.; Bregman, M. D.; Trivedi, D. Hyperglycemia of Diabetic Rats Decreased by a Glucagon Receptor Antagonist. *Science* **1982**, *215*, 1115−1116.

(11) Van Tine, B. A.; Azizeh, B. Y.; Trivedi, D.; Jason, R. P.; Houslay, M. D.; Johnson, D. G.; Hruby, V. J. Low Level Cyclic Adenosine 3′,5′-Monophosphate Accumulation Analysis of [des-His^1,des-Phe^6,Glu^9]Glucagon-NH_2 Identifies Glucagon Antagonists from Weak Partial Agonists/Antagonists. *Endocrinology* **1996**, *137*, 3316−3322.

(12) Unson, C. G.; Gurzenda, E. M.; Merrifield, R. B. Biological Activities of des-His^1[Glu^9]Glucagon Amide, a Glucagon Antagonist. *Peptides* **1989**, *10*, 1171−1177.

(13) Brand, C. L.; Rolin, B.; Jorgensen, P. N.; Svendsen, I.; Kristensen, J. S.; Holst, J. J. Immunoneutralization of Endogenous Glucagon with Monoclonal Glucagon Antibody Normalizes Hyperglycaemia in Moderately Streptozotocin-Diabetic Rats. *Diabetologia* **1994**, *37*, 985−993.

(14) Hruby, V. J. Structure-Conformation-Activity Studies of Glucagon and Semi-Synthetic Glucagon Analogs. *Mol. Cell. Biochem.* **1982**, *44*, 49−64.

(15) Hruby, V. J.; Krstenansky, J. L.; Gysin, B.; Pelton, J. T.; Trivedi, D.; McKee, R. L. Conformational Considerations in the Design of Glucagon Agonists and Antagonists: Examination Using Synthetic Analogs. *Biopolymers* **1986**, *25*, S135−S155.

(16) Rodbell, M.; Birnbaumer, L.; Pohl, S. L.; Sundby, F. The Reaction of Glucagon with Its Receptor: Evidence for Discrete Regions of Activity and Binding in the Glucagon Molecule. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 909−913.

(17) Edelhoch, H.; Schneider, A. B. Conformational Changes of Protected Glucagon Fragments Bound to Lysolecithin. *Arch. Biochem. Biophys.* **1973**, *157*, 470−475.

(18) Carrey, E. A.; Epand, R. M. Conformational and Biological Properties of Glucagon Fragments Containing Residues 1−17 and 19−29. *Int. J. Pept. Protein Res.* **1983**, *22*, 362−370.

(19) Unson, C. G.; Gurzenda, E. M.; Iwasa, K.; Merrifield, R. B. Glucagon Antagonists: Contribution to Binding and Activity of the Amino-Terminal Sequence 1−5, Position 12, and the Putative α-Helical Segment 19−27. *J. Biol. Chem.* **1989**, *264*, 789−794.

(20) Collins, J. L.; Dambek, P. J.; Goldstein, S. W.; Faraci, W. S. CP-99,711: A Non-Peptide Glucagon Receptor Antagonist. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 915−918.

(21) Cascieri, M. A.; Koch, G. E.; Ber, E.; Sadowski, S. J.; Louizides, D.; de Lazslo, S. E.; Hacker, C.; Hagmann, W.; MacCoss, M.; Chicchi, G. G.; Vicario, P. P. Characterization of a Novel, Non-Peptidyl Antagonist of the Human Glucagon Receptor. *J. Biol. Chem.* **1999**, *274*, 8694−8697.

(22) Madsen, P.; Knudsen, L. B.; Wiberg, F. C.; Carr, R. D. Discovery and Structure−Activity Relationship of the First Non-Peptide Competitive Human Glucagon Receptor Antagonists. *J. Med. Chem.* **1998**, *41*, 5150−5157.

(23) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obeidi, F.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Hintz, M. F.; Riehm, J. P.; Rao, K. R.; Hruby, V. J. Alpha-Melanotropin: The Minimal Active Sequence in the Lizard Skin Bioassay. *Gen. Comput. Endocrinol.* **1989**, *73*, 157−163.

(24) Al-Obeidi, F.; Hruby, V. J.; Castrucci, A. M. L.; Hadley, M. E. Design of Potent Linear α-Melanotropin 4−10 Analogues Modified in Position 5 and 10. *J. Med. Chem.* **1989**, *32*, 174−179.

(25) Al-Obeidi, F.; Hadley, M. E.; Pettitt, B. M.; Hruby, V. J. Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Simulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413−3416.

(26) Unson, C. G.; Wu, C.-R.; Merrifield, R. B. Roles of Aspartic Acid 15 and 21 in Glucagon Action: Receptor Anchor and Surrogates for Aspartic Acid 9. *Biochemistry* **1994**, *33*, 6884−6887.

(27) Sasaki, K.; Dockerill, S.; Adamiak, D. A.; Tickle, I. J.; Blundell, T. X-ray Analysis of Glucagon and Its Relationship to Receptor Binding. *Nature* **1975**, *257*, 751−757.

(28) Braun, W.; Wider, G.; Lee, K. H.; Wüthrich, K. Conformation of Glucagon in a Lipid-Water Interphase by ^{1}H Nuclear Magnetic Resonance. *J. Mol. Biol.* **1983**, *169*, 921−948.
(29) Azizeh, B. Y.; Ahn, J.-M.; Caspari, R.; Shenderovich, M. D.; Trivedi, D.; Hruby, V. J. The Role of Phenylalanine at Position 6 in Glucagon's Mechanism of Biological Action: Multiple Replacement Analogues of Glucagon. *J. Med. Chem.* **1997**, *40*, 2555−2562.
(30) Rink, H. Solid-Phase Synthesis of Protected Peptide Fragments Using a Trialkoxydiphenyl-methylester Resin. *Tetrahedron Lett.* **1987**, *28*, 3787−3790.
(31) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. J. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595−598.
(32) Hancock, W. S.; Battersby, J. E. A New Micro-Test for the Detection of Incomplete Coupling Reactions in Solid-Phase Peptide Synthesis Using 2,4,6-Trinitrobenzenesulphonyl Acid. *Anal. Biochem.* **1976**, *71*, 260−264.
(33) Neville, D. M. Isolation of an Organ Specific Protein Antigen from Cell Surface Membrane of Rat Liver. *Biochim. Biophys. Acta* **1968**, *154*, 540−552.
(34) Pohl, S. L.; Birnbaumer, L.; Rodbell, M. The Glucagon-Sensitive Adenylate Cyclase System in Membranes of Rat Liver. Comparison Between Glucagon- and Fluoride-Stimulated Activities. *J. Biol. Chem.* **1971**, *246*, 1849−1856.
(35) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurement with Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(36) Markwell, M. A. K.; Haas, S. M.; Bieleer, L. L.; Tolbert, N. E. A. A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Anal. Biochem.* **1978**, *87*, 206−210.
(37) Wright, D. E.; Rodbell, M. J. Glucagon$_{1-6}$ Binds to the Glucagon Receptor and Activates Hepatic Adenylate Cyclase. *J. Biol. Chem.* **1979**, *254*, 268−269.
(38) Lin, M. C.; Wright, D. E.; Hruby, V. J.; Rodbell, M. Structure−Function Relationships in Glucagon: Properties of Highly Purified Des-His^1-, Monoiodo-, and [Des-Asn^{28}, Thr^{29}](Homoserine Lactone^{27})-Glucagon. *Biochemistry* **1975**, *14*, 1559−1563.
(39) Salomon, Y.; Londos, C.; Rodbell, M. Highly Sensitive Adenylate Cyclase Assay. *Anal. Biochem.* **1974**, *58*, 541−548.
(40) Schild, H. O. pA, A New Scale for the Measurement of Drug Antagonism. *Brit. J. Pharmacol.* **1947**, *2*, 189−258.

JM000453E