The Role of Phenylalanine at Position 6 in Glucagon's Mechanism of Biological Action: Multiple Replacement Analogues of Glucagon

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Extensive evidence gathered from structure-activity relationship analysis has identified and confirmed specific positions in the glucagon sequence that are important either for binding to its receptor or for signal transduction. Fifteen glucagon analogues have been designed and synthesized by incorporating structural changes in the N-terminal region of glucagon, in particular histidine-1, phenylalanine-6, and aspartic acid-9. This investigation was conducted to study the role of phenylalanine at position 6 on the glucagon mechanism of action. These glucagon analogues have been made by either deleting or substituting hydrophobic groups, hydrophilic groups, aromatic amino acids, or a D-phenylalanine residue at this position. The structures of the new analogues are as follows: [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2 (**1**); [des-His^1,Ala^6,Glu^9]glucagon-NH_2 (**2**); [des-His^1,Tyr^6,Glu^9]glucagon-NH_2 (**3**); [des-His^1,Trp^6,Glu^9]-glucagon-NH_2 (**4**); [des-His^1,D-Phe^6,Glu^9]glucagon-NH_2 (**5**); [des-His^1,Nle^6,Glu^9]glucagon-NH_2 (**6**); [des-His^1,Asp^6,Glu^9]glucagon-NH_2 (**7**); [des-His^1,des-Gly^4,Glu^9]glucagon-NH_2 (**8**); [desPhe^6,-Glu^9]glucagon-NH_2 **(9)**; [des-Phe^6]glucagon-NH_2 **(10)**; [des-His^1,des-Phe^6]glucagon-NH_2 (**11**); [des-His^1,des-Phe^6,Glu^9]glucagon (**12**); [des-Phe^6,Glu^9]glucagon (**13**); [des-Phe^6]glucagon (**14**); and [des-His^1,des-Phe^6]glucagon (**15**). The receptor binding potencies IC_{50} values are 48 (**1**), 126 (**2**), 40 (**3**), 19 (**4**), 100 (**5**), 48 (**6**), 2000 (**7**), 52 (**8**), 113 (**9**), 512 (**10**), 128 (**11**), 1000 (**12**), 2000 (**13**), 500 (**14**), and 200 nM (**15**). All analogues were found to be antagonists unable to activate the adenylate cyclase system even at concentrations as high as 10^{-5} M except for analogues **6** and **8**, which were found to be weak partial agonists/partial antagonists with maximum stimulation between 6−12%. In competitive inhibition experiments, all the analogues caused a right shift of the glucagon-stimulated adenylate cyclase dose−response curve. The pA_2 values were 8.20 (**1**), 6.40 (**2**), 6.20 (**3**), 6.25 (**4**), 6.30 (**5**), 6.30 (**7**), 6.05 (**8**), 6.20 (**9**), 6.30 (**10**), 6.25 (**11**), 6.10 (**12**), 6.20 (**13**), 6.20 (**14**), and 6.35 (**15**).

Introduction

Glucagon is a 29-amino acid polypeptide hormone, secreted by the A cells of the pancreas and binds to specific receptors in the liver plasma membrane. This event triggers a series of sequential biological signals that result in stimulating glycogenolysis and gluconeogenesis in the liver, leading to the rise of glucose levels into the bloodstream.[1,2] Glucagon plays a crucial role in the pathophysiology of diabetes mellitus, where maintaining the normal glucose concentrations is the role of glucagon in conjunction with insulin. The bihormonal hypothesis proposed by Unger and coauthors states that the insulin deficiency causes an impairment of glucose utilization, while the overproduction of glucose is due to excess circulation of glucagon.[3−5]

Evidence that blocking endogenous glucagon with glucagon receptor antagonist can noticeably lower blood glucose concentration in streptozotocin-induced diabetic animals without the addition of any exogenous insulin has been obtained from our previous studies[6] utilizing [1-N^{α}-(trinitrophenyl)histidine,12-homoarginine]glucagon (THG),[7] and more recently, a similar observation[8] was made using the pure glucagon antagonist [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2.[9] These two glucagon antagonists reduced the blood glucose levels to ∼55−65% of the initial concentration in diabetic animals,[6,8] and similar observations were made by others using [des-His^1,Glu^9]glucagon-NH_2.[10] Our laboratory has recently developed a very sensitive cAMP accumulation assay, using isolated hepatocytes and phosphodiesterase (PDE) inhibitors that can differentiate between weak partial agonists and pure antagonists and redefine the major characteristics of glucagon antagonists.[8] Hence pure glucagon receptor inhibitors that bind to the plasma membrane receptor for glucagon without activating the adenylate cyclase system or any other second messenger systems activated by glucagon are the most valuable tools in testing the bihormonal hypothesis which might lead to potential therapeutic drugs either alone or with insulin for the treatment of this metabolic disorder.

Recent isolation of the glucagon receptor by Kindvogel and co-workers[11] has indicated that the glucagon receptor belongs to a family of G-protein-coupled hormone receptors that consists of seven membrane spanning domains. It also has been shown that the glucagon receptor is related to those of secretin, parathyroid hormone (PTH), vasoactive intestinal peptide (VIP), and calcitonin (CT). The cloned receptor bound glucagon caused an increase in the intracellular concentrations of 3′,5′-cyclic AMP and also transduced a signal that caused an increase in Ca^{2+} concentration.[11]

Structure−activity,[12] NMR,[13] and X-ray crystallographic[14] studies of glucagon have indicated the importance of phenylalanine at position 6 for the three-dimensional structure and receptor binding of glucagon. The Phe^6 amino acid residue is a highly conserved

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Table 1. Comparison of the Glucagon Sequence to the Sequence of GLP-1, GLP-2, Secretin, and Other Peptides in the GRF Superfamily

glucagon	H-S-Q-G-T-**F**-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-D-F-V-Q-W-L-M-N-T
GLP-1	H-A-E-G-T-**F**-T-S-D-V-S-S-Y-L-E-G-Q-A-A-K-E-F-I-A-W-L-V-K-G-R amide
GLP-2	H-A-D-G-S-**F**-S-D-E-M-N-T-I-L-D-N-L-A-A-R-D-F-I-N-W-L-I-Q-T-K-I-T-D-R
secretin	H-S-D-G-T-**F**-T-S-E-L-S-R-L-R-D-S-A-R-L-Q-R-L-L-Q-G-L-V amide
VIP	H-S-D-A-V-**F**-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N amide
PHI	H-A-D-G-V-**F**-T-S-D-F-S-R-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-I amide

throughout the glucagon family that also includes glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and gastric inhibitory peptide (GIP) which form one branch of the growth hormone-releasing factor (GRF) and are believed to be derived from a common ancestor (Table 1).[15] The other branch of the (GRF) superfamily includes secretin, vasoactive intestinal peptide (VIP), and peptide histidine isoleucine amide (PHI) (Table 1). Phe^6 is assumed to be one of the key residues in the N-terminal region for glucagon binding interaction and is part of a hydrophobic patch that involves also Tyr^{10} and Leu^{14}. Our previous work had indicated the importance of [des-His^1]glucagon, which was found to be a weak partial agonist.[16] Structure−activity studies[12] provided evidence that the N-terminal region of glucagon was important for the transduction message, whereas the C-terminal part was crucial for binding to the receptor.[17,18] Aspartic acid residue at position 9 of glucagon was identified to be important for the transduction effect and operates in conjunction with His^1 for the activation mechanism that follows the binding to the glucagon receptor.[16,19,20]

There is a considerable interest in developing new insights that will lead to more potent, stable, and prolonged-acting glucagon receptor antagonists. In this investigation we have concentrated on the N-terminal region of glucagon, in particular on modifications of phenylalanine at position 6 using the antagonist sequence [des-His^1,Glu^9]glucagon-NH$_2$ as a template.[21] All the glucagon analogues in this work were synthesized by N^{α}-Fmoc chemistry procedures[22] utilizing either 4-[(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)]phenoxy resin to obtain the carboxamide C-terminal or 4-alkoxybenzyl alcohol resin (Wang resin) to get the carboxylate C-terminal. The analogues were designed and synthesized by using the template with the deletion of Phe^6 and substituting Phe^6 with a D-Phe^6, other aromatic amino acids (Tyr^6 and Trp^6), nonaromatic hydrophobic groups (Ala^6 and Nle^6), and a hydrophilic residue (Asp^6).

Results

Chemical Synthesis and Characterization. The 15 new glucagon analogues (Table 2) **1**−**15** were synthesized by solid-phase methodology using N^{α}-Fmoc procedures.[22,23] To obtain the carboxamide C-terminal, 4-[(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)]phenoxy resin was used, and 4-alkoxybenzyl alcohol resin was introduced to obtain the carboxylate C-terminal. Most coupling reactions employed 0.5 M HOBt/0.45 M HBTU in NMP or DMF. The peptides were cleaved from the resin using a cleavage mixture of 90% trifluoroacetic acid, 5% anisole, 2.5% 1,2-ethanedithiol, and 2.5% methyl sulfide. The crude peptides were isolated and purified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The purity of the glucagon analogues was characterized by electrospray mass spectroscopy, thin layer chromatography (TLC), and amino acid analysis (see the Experimental Section).

Table 2. The Primary Sequence of Glucagon and the Structures of Glucagon Analogues

peptide	structure
glucagon	H-His-Ser-Gln-Gly-Thr^5-Phe-Thr-Ser-Asp-Tyr^{10}-Ser-Lys-Tyr-Leu-Asp^{15}-Ser-Arg-Arg-Ala-Gln^{20}-Asp-Phe-Val-Gln-Trp^{25}-Leu-Met-Asn-Thr^{29}-OH
1	[des-His^1,des-Phe^6,Glu^9]glucagon-NH$_2$
2	[des-His^1,Ala^6,Glu^9]glucagon-NH$_2$
3	[des-His^1,Tyr^6,Glu^9]glucagon-NH$_2$
4	[des-His^1,Trp^6,Glu^9]glucagon-NH$_2$
5	[des-His^1,D-Phe^6,Glu^9]glucagon-NH$_2$
6	[des-His^1,Nle^6,Glu^9]glucagon-NH$_2$
7	[des-His^1,Asp^6,Glu^9]glucagon-NH$_2$
8	[des-His^1,des-Gly^4,Glu^9]glucagon-NH$_2$
9	[des-Phe^6,Glu^9]glucagon-NH$_2$
10	[des-Phe^6]glucagon-NH$_2$
11	[des-His^1,des-Phe^6]glucagon-NH$_2$
12	[des-His^1,des-Phe^6,Glu^9]glucagon
13	[des-Phe^6,Glu^9]glucagon
14	[des-Phe^6]glucagon
15	[des-His^1,des-Phe^6]glucagon

Biological Studies. The agonist and antagonist properties of the glucagon analogues were investigated in the adenylate cyclase assay, and the results obtained are shown in Table 3. The receptor binding potency for these compounds was determined using liver plasma membranes in which the displacement of ^{125}I-labeled glucagon was measured, and the results are also shown in Table 3.

The glucagon analogue [des-His^1,des-Phe^6,Glu^9]-glucagon-NH$_2$ (**1**) was designed by the removal of two aromatic amino acid residues His^1 and Phe^6 and the replacement of Asp^9 by Glu^9. This compound was found to be a potent glucagon antagonist with a pA_2 value of 8.20 (Table 3) in classical adenylate cyclase assays and previously was shown to be a pure antagonist in cAMP accumulation assay using isolated hepatocytes in the presence of phosphodiesterase inhibitor Rolipram,[9] unable to activate the system up to a concentration of 10^{-5} M. In the receptor binding assay, the binding potency IC$_{50}$ value was 48 nM compared to glucagon's 1.5 nM. Note that the parent [des-His^1,Glu^9]glucagon-NH$_2$ which retains phenylalanine at position 6 is a potent antagonist with a binding IC$_{50}$ value of 4 nM and a pA_2 value of 7.20.[10] Compounds **2**−**7** were designed to study the structural requirement and the effect of phenylalanine at position 6 on the transduction pathway and binding to the glucagon receptor. The substitutions of Phe^6 by Ala^6 (**2**), Tyr^6 and Trp^6 two aromatic residues analogues (**3**, **4**), and by a D-Phe^6 (**5**) resulted in antagonists with pA_2 values of 6.40, 6.20, 6.25, and 6.30, respectively. The binding potency IC$_{50}$ values for analogues **2**−**5** was determined as 126, 40, 19, and 100 nM, respectively. Surprisingly, [des-His^1,Nle^6,Glu^9]glucagon-NH$_2$ (**6**) produced by replacing Phe^6 with a hydrophobic residue Nle^6 was found to be a weak partial agonist with a maximum stimulation of 12% and a binding potency IC$_{50}$ value of

Table 3. Biological Activities of Glucagon Analogues

	receptor binding		adenylate cyclase		
peptide[a]	IC_{50} (nM)	rel binding potency (%)[b]	EC_{50} (nM)	max stimulation (%)	pA_2
glucagon	1.5 ± 0.20	100	8 ± 1.25	100	
1	48 ± 3.2	3.1	ia[c]	0	8.20
2	126 ± 17.5	1.2	ia	0	6.40
3	40 ± 3.7	3.8	ia	0	6.20
4	19 ± 2.4	7.9	ia	0	6.25
5	100 ± 13.6	1.5	ia	0	6.30
6	48 ± 4.2	3.1	−	12	ND[d]
7	2000 ± 215	<0.1	ia	0	6.30
8	52 ± 5.8	3.0	−	6	6.05
9	113 ± 15.	1.3	ia	0	6.20
10	512 ± 96	0.3	ia	0	6.30
11	128 ± 22.	1.2	ia	0	6.25
12	1000 ± 117	<0.1	ia	0	6.10
13	2000 ± 220	<0.1	ia	0	6.20
14	500 ± 106	0.3	ia	0	6.25
15	200 ± 22.5	1.0	ia	0	6.15

[a] See Table 1 for structures. [b] Relative binding potency = [(receptor binding IC_{50} for glucagon)/(receptor binding IC_{50} for glucagon analogue)] × 100. [c] ia, inactive at 10^{-5} M. [d] ND, not determined.

48 nM. When a hydrophilic amino acid residue was introduced as in [des-His^1,Asp^6,Glu^9]glucagon-NH_2 (**7**), the analogue had no agonist activity in the classical adenylate cyclase assay with a pA_2 value of 6.30 and an IC_{50} value of 2 μM indicative of a very weak receptor binding. Analogue (**8**) with the deletion of both His^1 and Gly^4 residues and the replacement of Asp^9 by Glu^9 was found to be a partial agonist with a maximum stimulation of 6% and a pA_2 value of 6.05. The glucagon analogues **9**−**15** were found to be weak antagonists in classical adenylate cyclase assay unable to activate the AC system up to a concentration of 10^{-5} M with pA_2 values of 6.10−6.30. The binding potency IC_{50} values for analogues **9**−**15** was determined to be 113, 512, 128, 1000, 2000, 500, and 200 nM, respectively.

Discussion

The use of solid-phase peptide synthesis[25] (SPPS) in classical structure−activity relationship studies of the polypeptide hormone glucagon has provided some insights into the essential structural elements of the hormone that are responsible for binding to the receptor and those that are crucial for the transduction process. These important ideas led to the conclusions that the amino-terminal region of glucagon was responsible for activity whereas the carboxyl-terminal sequence was essential for binding affinity.[17,26] It is well-accepted now that the entire sequence of glucagon is necessary for the hormonal binding and activity. Numerous glucagon analogues have been reported in the literature, including superagonists,[27] partial agonists,[28,29] and antagonists.[19,26,28,30]

It has been pointed out that histidine at position 1 contributes significantly to the glucagon activity and the removal of His^1 leads to a weak partial agonist.[16] The investigation of a series of substitutions at Asp^9 led to the discovery that this position was critical for transduction message, but not for receptor binding.[19] The results gave strong evidence of the essential roles of both His^1 and Asp^9 in glucagon's action.[20] The literature has provided evidence that other key amino acid residues are also involved in glucagon's activity. Briefly, our present investigation concentrates on the phenylalanine at position 6 by taking into consideration the idea that the polar histidine-1 and the negatively charged aspartic acid-9 may be electrostatically attracted.

Earlier studies of glucagon conformation by circular dichroism (CD)[31,32] and other biophysical techniques[13,14,33] revealed that this polypeptide chain has various conformations in different environments. According to the determination of the structure of the 29-amino acid hormone glucagon in perdeuterated dodecylphosphocholine (DPC) micelles,[13] it has been suggested that the glucagon chain in lipid−water interphase depends largely on the topology of the lipid support (DPC). Hence, the tertiary structure of glucagon in DPC has been described by the formation of two hydrophobic patches formed by the side chains of Phe^6, Tyr^{10}, and Leu^{14} and by the side chains of Ala^{19}, Phe^{22}, Val^{23}, Trp^{25}, and Leu^{26}, respectively. On the other hand, the X-ray crystal structure[14] indicated that glucagon adopts mainly a helical conformation which is stabilized by hydrophobic interactions. Two hydrophobic clusters formed by the nonpolar side chain residues of Phe^6, Tyr^{10}, and Tyr^{13} and the residues of Phe^{22}, Trp^{25}, and Leu^{26}, respectively, were found in the crystal structure. Taking into account these indications of the hydrophobic patches in NMR[12] and in X-ray structure studies,[13] which involve the Phe^6 and Tyr^{10} residues, we were interested in studying the role of Phe^6 in this hydrophobic patch and its influence on the binding affinity and transduction message on glucagon molecule. Note that the amino acid residue Tyr^{10} has been extensively studied[28,29] by substituting Phe^{10} and Ala^{10}, the D-amino acids of Tyr, Phe, and Ala, in addition to the incorporation of the four isomers of the β-MePhe.[28]

Several attempts have been made to clearly define glucagon antagonists. The development of a newly designed sensitive cAMP accumulation assay in our laboratory has proven to differentiate between antagonists and weak partial agonists in isolated hepatocytes.[9] The pure glucagon antagonists from this assay [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2 was further tested *in vivo* where it lowered blood glucose levels up to 65% of the originally elevated levels. It became critical to continue studying structure−activity relationship of phenylalanine at position 6 for its role in binding affinity and transduction by substituting aromatic, hydrophobic, hydrophilic, and D-configuration amino acid residues.

The removal of Phe^6 in the analogue [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2 (**1**) affected the hydrophobic side chain alignment between Phe^6, Tyr^{10}, and Tyr^{13},

which resulted in about a 10-fold decrease of binding potency compared to the glucagon antagonist [des-His^1,-Glu^9]glucagon-NH_2 that has a binding potency of 4 nM. However, this may cause an increase in the flexibility in the N-terminal region leading to an antagonist conformation which results in the increased antagonist potency of analogue **1**; this possibility will be discussed later in the section dealing with molecular modeling. Note that the removal of the phenyl ring by substituting Phe^6 with Ala^6 is not equivalent to the removal of the entire Phe^6 residue. Analogue **2** loses binding affinity as well as antagonist potency, which probably reflects both loss of a specific hydrophobic interaction with the receptor and lack of a specific antagonist conformation. Introducing Tyr^6 for Phe^6 in the analogue [des-His^1,-Tyr^6,Glu^9]glucagon-NH_2 (**3**) leads to a 10-fold loss in binding when compared with the reference analogue [des-His^1,Glu^9]glucagon-NH_2. This could be due to hydrogen bonding of the hydroxyl group of Tyr^6 not being favorable for receptor interaction. In analogue **4** where Trp^6 was substituted, the binding drop is one-fifth of the reference analogue. In this case, the bulky Trp side chain may become a barrier in its interaction with the binding pocket. When a D-Phe^6 residue was introduced in analogue **5**, where the orientation is reversed, the side chain interaction of aromatic residues in the N-terminal region is reduced and that caused a drop in binding to nearly 1/25th of the reference analogue. Altering the hydrophobicity by replacing Nle^6 for Phe^6 in analogue **6** also resulted in the loss of binding, and that is attributed to reduction in aromatic character. Interestingly, an IC_{50} of 48 nM of this analogue compares well with that of the potent antagonist analogue [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2. We believe that this is the result of the highly hydrophobic nature of Nle substitution which compensates for the reduced aromatic character. Placing a charged amino acid residue in position 6 in [des-His^1,Asp^6,Glu^9]-glucagon-NH_2 (**7**) led to almost complete loss of receptor binding. Presumably, hydrophilic substitution at this position is not at all tolerated. Once again, the removal of Gly at position 4 in analogue **8** resulted in a decrease in binding to one-tenth compared to [des-His^1,Glu^9]-glucagon-NH_2. The predicted model of the secondary structure of glucagon indicated that a β-turn conformation between residues 2 and 5 and a β-sheet structure between residues 5 and 10 existed.[24] Hence the deletion of Gly^4 presumably modified the secondary structure in the N-terminal region leading to a more flexible conformation with only a weak agonist activity with a maximum stimulation of 6% (Table 3).

Analogues **12**, **13**, **14**, and **15** were very similar to analogues **1**, **9**, **10**, and **11**, respectively, except that the NH_2-terminal was replaced by the COOH-terminal in each case. A big drop in binding potency for analogues **12** and **13** was observed when a comparison was made with their matched pairs, analogues **1** and **9**. Interestingly, the binding potencies of analogues **14** and **15** compared well with its matched pairs, analogues **10** and **11**. In the first instance, the only difference was the introduction of NH_2-terminal for COOH-terminal, and presumably that change explains its effect on ligand−receptor interactions. When Asp^9 was substituted with Glu^9, the loss in binding is more pronounced probably due to the interaction of the extra carbon of Glu^9 with the amide terminal. This is not so in the case of other matched pairs (analogues **10** and **11** and analogues **14** and **15**) where binding losses are attributed only to reduced hydrophobic interaction between the N-terminal aromatic amino acid side chains of the glucagon molecule and the glucagon receptor. Thus, we could suggest that when a glucagon molecule is made less hydrophobic, e.g., deletion of Phe^6, interaction between side chain of Glu^9 and amide terminal is very critical for the binding to the receptor.

One can overall summarize that hydrophobic side chain interaction in the aromatic residue of the N-terminal region is a critical component in the receptor-binding interaction of glucagon. The proper stacking of Phe^6, Tyr^{10}, and Tyr^{13} is very important for maximum binding and transduction. It is logical to speculate that when the secondary structure was altered, it resulted in a more flexible conformation, predominantly in the N-terminal region, leading to antagonistic activity. This was further demonstrated by molecular modeling studies.

Molecular Modeling

Although the three-dimensional structure of glucagon have been studied by X-ray crystallography[14] and NMR spectroscopy,[13] to our knowledge there has been no systematic conformational study of glucagon and its analogues by molecular modeling techniques. This is not surprising, because an extensive conformational search for the 29-amino acid peptide requires enormous amounts of computer time, and the results of the search may strongly depend on the force field and environment used in calculations. Nonetheless, we have initiated molecular studies of glucagon and two analogues, [des-His^1,Glu^9]glucagon-NH_2 and [des-His^1,des-Phe^6,Glu^9]-glucagon-NH_2, in order to get an insight into differences in the conformational properties of agonists and antagonists. For this purpose we have utilized the crystal structure[14] of glucagon as an initial conformation for mixed mode Molecular Dynamics/Monte Carlo (MD/MC)[34] simulations. Initial conformations for the two antagonists were aligned to the crystal structure of glucagon. The simulations were performed using the united atom AMBER force field[35] implemented into MacroModel program (Version 4.5),[36] with the generalized Born/surface area model[37] used for calculation of hydration energies. The initial conformations were equilibrated in a 300 K thermal bath. Then the MD/MC simulated annealing was performed by a slow decrease of temperature from 500 K to about 0 K during 500 ps, with random perturbations of torsional angles in the N-terminal parts of the molecules (residues 1−18). Conformations saved after each 50 ps of the simulated annealing procedure were energy minimized using the AMBER force field with conjugate gradient.[35]

The lowest-energy structure of glucagon obtained with the above simulated annealing protocol is shown in Figure 1. It is noteworthy that the backbone of glucagon retained mainly an α-helical conformation during the lengthy MD/MC trajectory, and the lowest-energy conformation is similar to the initial X-ray structure. The most interesting feature of this conformation is the cluster of aromatic rings of His^1, Phe^6, Tyr^{10}, and Tyr^{13}, which together with Leu^{14} side chain, form a continuous hydrophobic region in the N-terminal part of the mol-

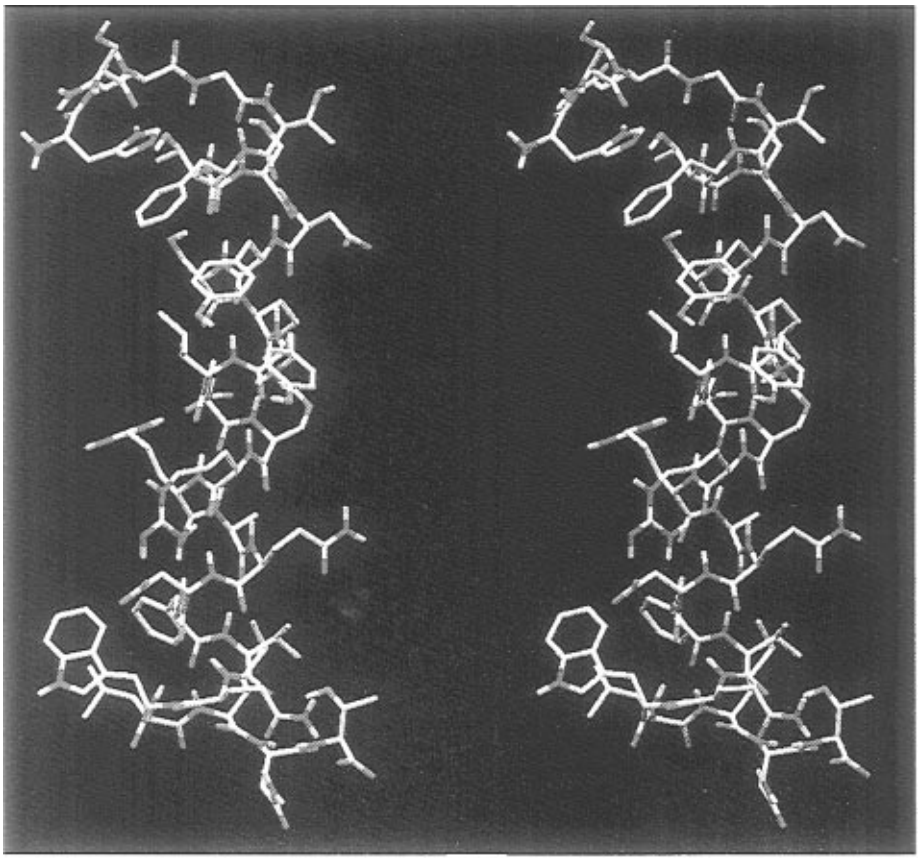

Figure 1. Stereoview of the lowest energy conformation of glucagon from simulated annealing.

ecule. Such a "hydrophobic patch" involving Phe[6], Tyr[10], Tyr[13], and/or Leu[14] was observed in the X-ray structure[14] and suggested for the micelle-bound conformation of glucagon.[13] This consensus feature of the 3D structure also may be conserved in the receptor environment and may play an important role in the receptor binding of glucagon. In contrast to the crystal structure, the hydrophobic cluster in the structure shown in Figure 1 also involves the N-terminal residue His[1]. Although His[1] seems to participate directly in hormone−receptor interactions which lead to signal transduction, it also may contribute in the stabilization of the hydrophobic patch that may be recognized by the receptor upon glucagon binding. Removal of His[1] generally increased the conformational mobility of the N-terminal part, although a stacking of the aromatic rings of Phe[6], Tyr[10], and Tyr[13] still was observed in low-energy conformations of [des-His[1],Glu[9]]glucagon-NH_2. Removal of the Phe[6] residue resulted in a further increase in the flexibility in the N-terminal part. The lowest-energy conformation found for [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2 (Figure 2) has a rather extended backbone in the N-terminal part which does not form a stable cluster of hydrophobic side chains.

Our preliminary molecular modeling was not aimed at a comprehensive conformation−activity study of glucagon and its antagonists, and the structures shown in Figures 1 and 2 should not be considered as models of biologically active conformations. Nevertheless, even the limited simulations based on the crystal structure of glucagon have revealed considerable differences in conformational properties of the N-terminal part of glucagon and its antagonists. Glucagon itself seems to have an ordered 3D structure stabilized by the cluster of aromatic rings which may be important for agonist recognition and binding by the glucagon receptor. Removal of His[1] and Phe[6] results in the loss of the hydrophobic patch which leads to a more disordered, flexible structure in the N-terminal region. The [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2 analogue has low binding affinity compared to glucagon, and yet it is one of the most potent antagonists of glucagon obtained so far. Our findings suggest that the flexible N-terminal part of this analogue may assume a specific conformation upon receptor binding, quite different from the conformation responsible for agonist binding. These results of our study support the suggestions of Tager and co-workers[38] that glucagon and its antagonists may have different binding modes by which they interact with the glucagon receptor. To elaborate this further, there is a marked difference in the interaction of glucagon receptors with glucagon when compared to the interactions of the glucagon receptor with many glucagon antagonist analogues and when compared to each other. For example, analogues **1** and **3** and **1** and **11** have similar binding affinities to the glucagon receptor, yet their pA_2 values are different by 2 orders of magnitude. It may be, as suggested by Tager and co-workers,[38] that when radiolabeled glucagon is used as a probe, the real affinity of glucagon receptor toward antagonist analogues such as [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2 (**1**) are not revealed. Alternatively, the antagonist analogues may have different kinetic characteristics than glucagon, and this is being examined by using radiolabeled antagonists. It is interesting to note that Unson et al.[19] have made similar observations for some of their antagonist analogues. The possibility of different kinds of desensitization also needs to be considered.

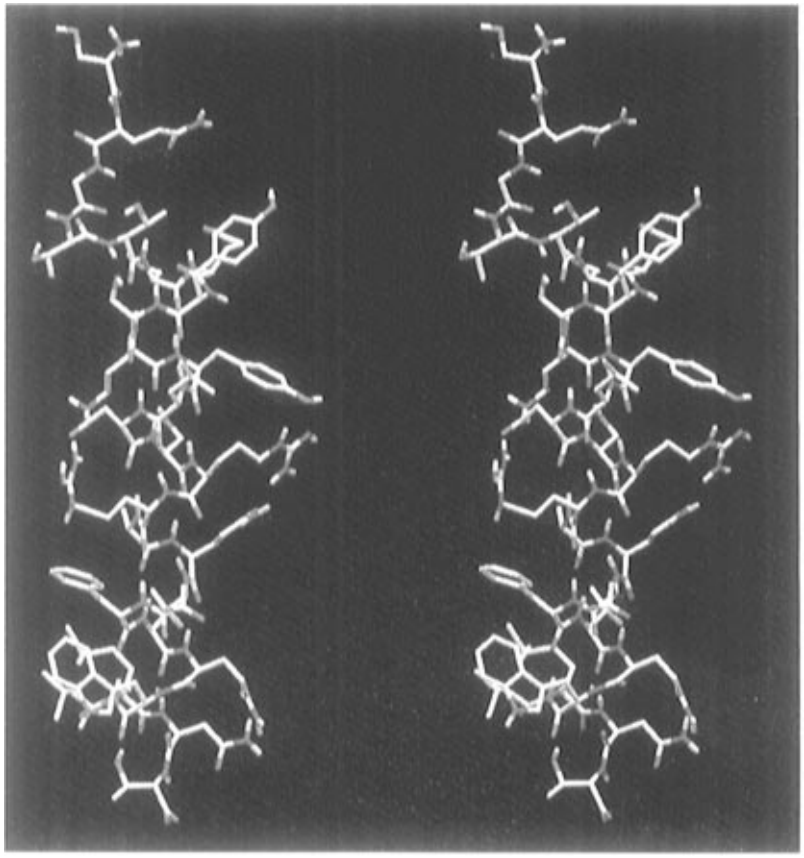

Figure 2. Stereoview of the lowest energy conformation of [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2 from simulated annealing.

Fifteen different glucagon analogues which have been reported here were not further tested in our newly developed highly sensitive cAMP accumulation assay[8] because pA_2 values of these were not potent enough to make any major difference in biological evaluations with the exception of our antagonist analogue [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2. Finally, the role of Glu[9] substitution for Asp[9], which considerably increased the antagonist potency (analogues **1** and **11** in Table 3), is not clear from the present study. One may speculate that the elongation of the negatively charged side chain in position 9 may enhance its electrostatic interaction with a polar or positively charged group, thus stabilizing an antagonist-binding conformation. The nearest positively charged groups in the glucagon sequence are the N-terminal α-amino group and the ϵ-amino group of Lys[12]. Covalent linking of side chains in positions 9 and 12 failed to produce analogues with potent antagonistic activity.[39] On the other hand, deletion of His[1] and Phe[6] brings the N-terminal amino group closer to position 9. Thus, the possibility of a salt-bridge formation between Glu[9] and the N-terminal amino group should be explored in the future.

Table 4. Physiochemical Properties of Glucagon Analogues Used in This Investigation

	MW		HPLC	R_f, TLC solvents[c]		
peptide	calcd	found[a]	k' [b]	I	II	III
1	3211.50	3212.00	4.53	0.47	0.46	0.53
2	3282.70	3283.00	4.67	0.39	0.37	0.41
3	3374.70	3375.10	5.06	0.34	0.30	0.39
4	3397.50	3397.50	5.15	0.37	0.33	0.41
5	3358.50	3358.90	4.98	0.31	0.36	0.44
6	3324.70	3324.90	4.85	0.34	0.34	0.46
7	3326.70	3326.80	4.62	0.33	0.33	0.39
8	3301.70	3302.10	4.72	0.37	0.30	0.40
9	3348.50	3348.00	3.54	0.45	0.42	0.48
10	3334.30	3334.50	3.39	0.42	0.40	0.47
11	3197.10	3197.30	3.70	0.40	0.43	0.49
12	3212.50	3212.90	4.05	0.43	0.41	0.47
13	3349.50	3349.80	4.24	0.42	0.40	0.45
14	3335.10	3335.40	3.97	0.44	0.39	0.42
15	3198.00	3198.50	3.85	0.41	0.38	0.42

[a] MW found by electrospray mass spectroscopy. [b] HPLC k' = [(peptide retention time − solvent retention time)/solvent retention time] under the following conditions: gradient 10−90% acetonitrile in 0.1% trifluoroacetic acid over 30 min, flow rate 1.5 mL/min. [c] TLC−silica gel 60F-245, 0.25 mm layer thickness; solvent systems: I, 1-butanol/acetic acid/pyridine/water (5:4:1:4), II, 1-butanol/acetic acid/pyridine/water (4:1:1:3), III, ethyl acetate/pyridine/acetic acid/water (12:4:4.2:2.2).

Experimental Section

Materials. N^{α}-Fmoc-protected amino acids, 4-alkoxybenzyl alcohol resin and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (0.44 mmol of NH_2/g of substitution), were purchased from Bachem (Torrence, CA). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA) (Halocarbon Products, NJ); anisole, 1,2-ethanedithiol, 1-hydroxybenzotriazole (HOBt), piperidine, and 1-methyl-2-pyrrolidinone (NMP) (Aldrich, Milwaukee, WI); dichloromethane (DCM) (Mallinckrodt Specialty Chemicals, Paris, KY); HPLC-quality acetonitrile (Burdick & Jackson, Muskegon, MI); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Richelieu Biotechnologies Inc., Saint Hyacinthe, Canada); bovine serum albumin, cAMP, GTP, ATP, chromatographic alumina (type WN3, neutral), and all enzymes (Sigma Chemicals, St. Louis, MO); [^{125}I]glucagon, [^{3}H]cAMP and α-^{32}P (New England Nuclear, Boston, MA); *N*,*N*-dimethylformamide (DMF) (Fischer Scientific, Pittsburgh, PA); and Dowex AG 50-W4 cation exchange resin (BioRad, San Diego, CA). TLC was performed using Merck silica gel 60 F-254 plates of 0.25 mm layer thickness, and the following solvents were used (Table 4): (A) 1-butanol/acetic acid/water/pyridine (5:4:4:1); (B) 1-butanol/acetic acid/

Table 5. Typical Protocol for N^{α}-Fmoc Chemistry SPPS

description	reagent/solvent	repetition	time (min)
(1) wash	DMF or NMP	1	15
(2) deprotection	piperidine−DMF or NMP (1:4)	1 1	3 17
(3) wash	DMF or NMP	3	1
(4) coupling	Fmoc amino acid (4 equiv), HBTU (3.8 equiv), HOBT (4 equiv) in DMF or NMP	1	40
(5) wash	DMF or NMP	3	1
(6) wash	DCM	2	1

water/pyridine (4:1:3:1); (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 was achieved on Perkin-Elmer Model 410-BIO instrument by preparative high-performance liquid chromatography (HPLC) C_{18}-bonded silica column (VYDAC 218 TBP-16, 16 × 250 mm). The peptides were eluted with a linear gradient of acetonitrile in 0.1% aqueous TFA (10−90%) over 30 min at a flow rate of 5.0 mL/min. The separations were monitored at 280 nm and integrated with a Perkin-Elmer LC-235 diode array detector. The amino acid analyses were done at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl and precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. The purity of the peptides were checked by analytical RP-HPLC using a VYDAC 218 TBP-16 column (4.6 × 250 mm) and by TLC in three different solvent systems (Table 4). The structures of the pure peptides were confirmed by electrospray mass spectroscopy (Table 4) and by amino acid analysis.

Peptide Synthesis. Using an N^{α}-Fmoc strategy, the glucagon analogues **1**−**11** (Table 2) were synthesized by solid-phase methodology using a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin[20] (0.44 mmol of NH_2/g of substitution) whereas analogues **12**−**15** used a 4-alkoxybenzyl alcohol resin. A typical protocol for N^{α}-Fmoc solid-phase peptide synthesis is illustrated in Table 5. The glucagon analogues [des-His[1],des-Phe[6],Glu[9]]glucagon-NH_2 (**1**), [des-His[1],Ala[6],Glu[9]]glucagon-NH_2 (**2**), [des-His[1],Tyr[6],Glu[9]]-glucagon-NH_2 (**3**), [des-His[1],Trp[6],Glu[9]]glucagon-NH_2 (**4**), [des-His[1],*D*-Phe[6],Glu[9]]glucagon-NH_2 (**5**), [des-His[1],Nle[6],Glu[9]]glucagon-NH_2 (**6**), [des-His[1],Asp[6],Glu[9]]glucagon-NH_2 (**7**), [des-His[1],des-Gly[4],-Glu[9]]glucagon-NH_2 (**8**), [desPhe[6],Glu[9]]glucagon-NH_2 (**9**), [desPhe[6]]glucagon-NH_2 (**10**), [des-His[1],des-Phe[6]]glucagon-NH_2 (**11**), [des-His[1],des-Phe[6],Glu[9]]glucagon (**12**), [des-Phe[6],Glu[9]]-glucagon (**13**), [des-Phe[6]]glucagon (**14**), and [des-His[1],desPhe[6]]-glucagon (**15**) were carried out on an Applied Biosystems (ABI) 431A automated synthesizer on a 0.25 mmol scale. The following side chain protecting groups were used: Arg(2,2,5,7,8-pentamethyl chroman-6-sulfonyl, Pmc, or 2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl, Pbf), Asn(N^{ϵ}-trityl), Asp(*O-tert*-butyl), Gln(N^{γ}-trityl), Glu(*O-tert*-butyl), Lys(N^{ϵ}-Boc), Ser(*O-tert*-butyl), Thr(*O-tert*-butyl), Trp (Boc), and Tyr(*O-tert*-butyl). The resin was placed in the reaction vessel while one cartridge with 1 mmol of the desired N^{α}-Fmoc amino acid was activated *in situ* as HOBt/HBTU ester followed by coupling to the growing peptide chain for 20 min. The N^{α}-protecting group was removed with 20% piperidine in NMP or DMF. After deprotection, the resin was washed with NMP or DMF, and the peptide resin was ready for next coupling. Four equivalents of the activated amino acid were used per equivalent of the growing peptide chain in the coupling. Finally, the N-terminal amino acid was deprotected with 20% piperidine in NMP or DMF, and the resin was washed thoroughly and dried over N_2. The protected peptide resin was cleaved to remove the resin from the desired peptide and to remove the side chain protecting groups.[40] The cleavage mixture consists of 90% trifluoroacetic acid, 5% anisole, 2.5% methyl sulfide, and 2.5% 1,2-ethanedithiol.

Isolation of Liver Plasma Membrane. The liver plasma membranes were obtained from Sprague−Dawley rats weighing 200−250 g as described by Neville[41] and modified by Pohl.[42] The protein concentration was determined by the method of Markwell et al.[43]

Receptor Binding Assay. The binding assay was performed according to Wright and Rodbell[44] 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, 15 × 10^4 CPM of [^{125}I]glucagon, and unlabeled glucagon or glucagon analogues at a desired concentration (range from 0 to 10 μM), all in 25 mM Tris-HCl with 0.5% BSA (pH 7.4 at 25 °C). The mixture was incubated for 10 min at 30 °C followed by immediate cooling in an ice bath, and filtered through 0.45 μm cellulose acetate filter previously soaked for 12 h in a Tris-BSA buffer. Four milliliters of ice cold Tris-BSA buffer was used for washing, and the amount of radioactivity remaining on the filter was quantitated using LKB1275 mini-gamma counter. Nonspecific binding, measured in the presence of excess unlabeled peptide (1024 nM), was 15−20% of the total binding and was substracted in each case to give the specific binding. Results were expressed as the percent inhibition of [^{125}I]glucagon specific binding. Assays were performed in triplicate and repeated twice.

Adenylate Cyclase Assay. The adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to 3′,5′-cyclic AMP as described by Lin et al.[16] Labeled cAMP was determined by the method of Solomon et al.[45] using Dowex 50 and alumina columns. Briefly, 0.1 mL of incubation medium consisting of 1 mM [α-^{32}P]ATP (∼50 cpm/pmol), 5 mM $MgCl_2$, 10 μM GTP, 1 mM EDTA, 1 mM cAMP containing 10^4 CPM of [^{3}H]cAMP, 25 mM Tris-HCl (pH 7.4 at 25 °C), 1% BSA, 35 μg of membrane protein, and an ATP regeneration system that had 20 mM phosphocreatine and 0.72 mg/mL (100 units/mL) creatine phosphokinase. Results were expressed as the percent of stimulation of cAMP production over basal. Assays were done twice in triplicate.

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 generated by using different concentrations ranging between 1 and 10 μM of antagonists. The dose/response curves exhibit shifts in the EC_{50} values which were used in calculating the pA_2 values as described by Schild.[46]

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References

(1) McKee, R. L.; Pelton, J. T.; Trivedi, D.; Johnson, D. G.; Coy, D. H.; Sueiras-Diaz, J.; Hruby, V. J. Receptor Binding and Adenylate Cyclase Activities of Glucagon Analogues Modified in the N-Terminal Region. *Biochemistry* **1986**, *25*, 1650−1656.
(2) Hruby, V. J. Strategies in the Development of Peptide Antagonists. In *Progress in Brain Research*; Joosse, J., Buijis, R. M., Tilders, F. J. H., Eds.; Elsevier Science Publishers: Amsterdam, 1992; Vol. 92, pp 215−224.
(3) Unger, R. H. Role of Glucagon in the Pathogenesis of Diabetes: The Status of the Controversy. *Metabolism* **1978**, *27*, 1691−1709.
(4) Unger, R. H.; Orci, L. The Essential Role of Glucagon in the Pathogenesis of Diabetes Mellitus. *Lancet* **1975**, No. 1, 14−26.
(5) Unger, R. H.; Orci, L. Role of Glucagon in Diabetes Mellitus. *Arch. Intern. Med.* **1977**, *137*, 482−491.
(6) Johnson, D. G.; Goebel, C. U.; Hruby, V. J.; Bregman, M. D.; Trivedi, D. Hyperglycemia of Diabetic Rats Decreased by a Glucagon Receptor Antagonist. *Science* **1982**, *215*, 1115−1116.
(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) Van Tine, B. A.; Azizeh, B. Y.; Trivedi, D.; Phelps, J. R.; 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.
(9) Azizeh, B. Y.; Van Tine, B. A.; Sturm, N. S.; Hutzler, A. M.; David, C.; Trivedi, D.; Hruby, V. J. [des His[1], des Phe[6], Glu[9]]-Glucagon Amide: A Newly Designed "Pure" Glucagon Antagonist. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1849−1852.
(10) 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.
(11) 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.
(12) Zechel, C.; Trivedi, D.; Hruby, V. J. Synthetic Glucagon Antagonists and Partial Agonists. *Int. J. Pept. Protein Res.* **1991**, *38*, 131−138.
(13) Braun, W.; Wider, G.; Lee, K. H.; Wüthrich, K. Conformation of Glucagon in a Lipid-Water Interface by 1H Nuclear Magnetic Resonanse. *J. Mol. Biol.* **1983**, *169*, 921−948.
(14) Sasaki, K.; Dockerill, S.; Adamiak, D. A.; Tickle, I. J.; Blundell, T. X-Ray Analysis of Glucagon and its Relationship to Receptor Binding. *Nature (London)* **1975**, *257*, 751−757.
(15) Campbell, R. M.; Scanes, C. G. Evolution of the Growth Hormone-Releasing Factor (GRF) Family of Peptides. *Growth Regul.* **1992**, *2*, 175−191.
(16) 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.
(17) Hruby, V. J.; Krystenansky, 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.
(18) Hruby, V. J. Structure-Conformation-Activity Studies of Glucagon and Semi-Synthetic Glucagon Analogs. *Mol. Cell. Biochem.* **1982**, *44*, 49−64.
(19) Unson, C. G.; MacDonald, D.; Ray, K.; Durrah, T. L.; Merrifield, R. B. Position 9 Replacement Analogs of Glucagon Uncouple Biological Activity and Receptor Binding. *J. Biol. Chem.* **1991**, *266*, 2763−2766.
(20) Unson, C. G.; MacDonald, D.; Merrifield, R. B. The Role of Histidine-1 in Glucagon Action. *Arch. Biochem. Biophys.* **1993**, *300*, 747−750.
(21) Unson, C. G.; Andreu, D.; Gurzenda, E. M.; Merrifield, R. B. Synthetic Peptide Antagonists of Glucagon. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4083−4087.
(22) Fields, G. B; Noble, R. L. Solid Phase Peptide Synthesis Utilizing 9-Fluorenylmethoxycarbonyl Amino Acids. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214.
(23) Dharanipragada, R.; Trivedi, D.; Bannister, A.; Siegel, M.; Toutwe, D.; Mollova, N.; Schram, K.; Hruby, V. J. Synthetic Linear and Cyclic Glucagon Antagonists. *Int. J. Pept. Protein Res.* **1993**, *42*, 68−77.
(24) Korn, A. P.; Ottensmeyer, F. P. A Model for the Three-Dimensional Structure of Glucagon. *J. Theor. Biol.* **1983**, *105*, 403−425.
(25) Merrifield, R. B. Solid Phase Peptide Synthesis. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
(26) Hruby, V. J.; Krystenanky, J. L.; McKee, R. L.; 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.
(27) Krystenansky, J. L.; Trivedi, D.; Johnson, D.; Hruby, V. J. Conformational Considerations in the Design of a Glucagon Analogue with Increased Receptor Binding and Adenylate Cyclase Potencies. *J. Am. Chem. Soc.* **1986**, *108*, 1696−1698.
(28) Azizeh, B. Y.; Shenderovich, M. D.; Trivedi, D.; Li, G.; Sturm, N. S.; Hruby, V. J. Topographical Amino Acid Substitution in Position 10 of Glucagon Leads to Antagonists/Partial Agonists with Greater Binding Differences. *J. Med. Chem.* **1996**, *39*, 2449−2455.
(29) Krystenansky, J. L.; Trivedi, D.; Hruby, V. J. Importance of the 10−13 Region of Glucagon for Its Receptor Interactions and Activation of Adenylate Cyclase. *Biochemistry* **1986**, *25*, 3833−3839.
(30) Unson, C. G.; Wu, C.-R.; Fitzpatrick, K. F.; Merrifield, R. B. Multiple-Site Replacement Analogs of Glucagon. *J. Biol. Chem.* **1994**, *269*, 12548−12551.
(31) Gratzer, W. B.; Creeth, J. M.; Beaven, G. H. Presence of Trimers in Glucagon Solution. *Eur. J. Biochem.* **1972**, *31*, 505−509.
(32) Formisano, S.; Johnson, M. L.; Edelhoch, H. Thermodynamics of the Self-Association Glucagon. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3340−3344.
(33) Bösch, C.; Brown, L. R.; Wüthrich, K. Physicochemical Characterization of Glucagon-Containing Lipid Micelles. *Biochim. Biophys. Acta* **1980**, *603*, 298−312.
(34) Guarnieri, F.; Still, W. C. A Rapidly Convergent Simulation Method: Mixed Monte Carlo/Stochastic Dynamics. *J. Comput. Chem.* **1994**, *15*, 1302−1310.
(35) Weiner, S. J.; Kollman, P. A.; Case, D. A. All-Atom Force Field for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* **1986**, *7*, 230−252.
(36) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C.; MacroModel-An Integrated Software System for Modeling Organic and Bioorganic Molecules Using Molecular Mechanics. *J. Comput. Chem.* **1990**, *11*, 440−467.
(37) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. Semianalytical Treatment of Solvation for Molecular Mechanics and Dynamics. *J. Am. Chem. Soc.* **1990**, *112*, 61127−6129.
(38) Post, S. R.; Rubinstein, P. G.; Tager, H. S. Mechanism of Action of des-His[1]-[Glu[9]]Glucagon amide, a Peptide Antagonist of the Glucagon Receptor System. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1662−1666.
(39) Hruby, V. J.; Gysin, B.; Trivedi, D. B.; Johnson, D. G. New Glucagon Analogues With Conformational Restrictions and Altered Amphiphilicity: Effects on Binding, Adenylate Cyclase and Glycogenolytic Activities. *Life Sci.* **1993**, *52*, 845−855.
(40) Lundt, B. F.; Johansen, N. L.; Volund, A.; Markussen, J. Removal of t-Butyl and t-Butoxycarbonyl Protecting Groups with Trifluoroacetic Acid. *Int. J. Pept. Protein Res.* **1978**, *12*, 258−268.
(41) Neville, D. M., Jr. Isolation of an Organ Specific Protein Antigen from Cell Surface Membrane of Rat Liver. *Biochim. Biophys. Acta* **1968**, *154*, 540−552.
(42) 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*, 1857−1860.
(43) Markwell, M. A. K.; Haas, S. M.; Bieleer, L. L.; Tolbert, N. E. A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Anal. Biochem.* **1978**, *87*, 206−210.
(44) Wright, D. E. and Rodbell, M. J. $Glucagon_{1-6}$ Binds to the Glucagon Receptor and Activates Hepatic Adenylate Cyclase. *J. Biol. Chem.* **1979**, *254*, 268−269.
(45) Salomon, Y.; Londos, G.; Rodbell, M. J. A Highly Sensitivity Adenylate Cyclase Assay. *Anal. Biochem.* **1974**, *58*, 541−548.
(46) Schild, H. O. A New Scale for the Measurement of Drug Antagonism. *Br. J. Pharmacol.* **1947**, *2*, 189−207.

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