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The Role of Salt Bridge Formation in Glucagon: An Experimental and Theoretical Study of Glucagon Analogs and Peptide Fragments of Glucagon

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Abstract

Background: Glucagon is a 29-residue peptide produced in the α cells of the pancreas that interacts with hepatic receptors to stimulate glucose production and release, via a cAMP-mediated pathway. Type 2 diabetes patients may have an excess of glucagon and, as such, glucagon antagonists might serve as diabetes drugs. The antagonists that bind to the glucagon receptor but do not exhibit activity could be analogs of glucagon. The presence of salt bridges between some residues of glucagons (such as aspartic acid) and others (such as lysine) might influence both the binding to the receptor and the activity.
Materials and Methods: *Experimental*—The solid phase method with 4-methylbenzilhydrilamine resin (p-MBHA resin) was used for the synthesis of glucagon analogs. Rat liver membranes were prepared from male Sprague-Dawley rats by the Neville procedure. The receptor binding essay was performed in 1% BSA, 1 mM dithiothreitol, 25 mM Tris-HCl buffer, pH 7.2. Adenyl cyclase activity was measured in an assay medium containing 1% serum albumin, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.025 mM GTP, 5 mM ATP, 0.9 mM theophylline , 17.2 mM creatine phosphate, and 1 mg/ml creatine phosphokinase. *Theoretical*—Quantum chemical calculations using the Titan program with the 6-31G* basis set were performed to calculate the binding energies of salt bridges between aspartic or glutamic acids and lysine. The relative stability of cyclic conformations of glucagon segments versus the extended segments was determined.
Results: It was found that the cyclic Glu9-Lys12 amide compound displayed a 20-fold decrease in binding affinity. DesHis1 cyclic compounds Glu20-Lys24 amide and DesHis1Glu9 Glu20-Lys24 amide behave as glucagon antagonists. The calculations show that cyclic conformations of tetrapeptidic and pentapeptidic segments of glucagon are more stable than the extended species.
Conclusions: The biological data and the theoretical calculations show that an intramolecular salt bridge might impart stability to some glucagon antagonists and, when situated at the C-terminus of glucagon, might facilitate induction of an α-helix upon initial hormone association with the membrane bilayer. These findings might be a useful tool for the design of new glucagon antagonists.

Introduction

Glucagon is a 29-residue peptide hormone secreted by pancreatic A cells responsible together with insulin for maintaining normal levels of glucose critical to the survival of an organism. The driving force for continued efforts to study glucagon lies primarily in the still unresolved problem of diabetes mellitus. There is persuasive evidence that the major metabolic complications of diabetes, hyperglycemia and ketoacidosis, are often accompanied by an abnormal increase in the level of plasma glucagon relative to insulin (1,2). Glucagon activity is mediated by the glucagon receptor, a member of family B receptors within the superfamily of G protein-coupled receptors that includes receptors for many important peptide hormones (3,4). The biological effects of glucagon are initiated by high-affinity binding to its membrane-bound receptor, the necessary first step in glucagon action. Upon glucagon binding, the extracellular signal is transduced across the cell membrane to activate adenylyl cyclase via the heterotrimeric G protein G_s resulting in an increase in cAMP, which mediates most of glucagon's cellular effects. Precise and efficient signaling that ultimately results in glucose production requires specific binding of glucagon to its cell surface receptors. Thus, structural information about the peptide ligand that influences receptor binding affinity is central to the design of antagonists of glucagon. An analog of glucagon that binds to the receptor but does not activate adenylyl cyclase is considered an antagonist. Thus, a good antagonist will compete for glucagon binding and inhibit adenylyl cyclase. A structural analog of glucagon that inhibits glucagon action by competing effectively for receptor binding sites is a reasonable target for drug design as an alternative or

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adjunct to insulin therapy in the management of diabetic complications.

Numerous structure–function studies of glucagon have singled out specific active site residues responsible for either high-affinity binding or activation and afforded some insight into its mechanism of action. We have demonstrated that the negatively charged side chain of aspartic acid residues at positions 9, 15, and 21 play important roles in either the binding or activity function of the hormone (5,6). The N-terminal histidine, which is strictly conserved within the family, furnishes determinants of both binding and activity of the hormone (7,8). The positively charged groups at positions 12, 17, and 18 contribute to receptor recognition and ensure maximum biological potency (9,10).

The molecular basis for the interaction of glucagon with the glucagon receptor remains to be elucidated. It is widely accepted that the glucagon binding site resides in the extracellular domain of the receptor, which consists of the long amino terminal tail and three extracellular loops that connect the transmembrane helices (11,12). In the presence of its receptor, glucagon changes its conformation to the activated form. It had been suggested that intramolecular salt bridges within the glucagon molecule stabilize the active conformation and allow formation of a more compact folded structure that facilitates hormone access to its binding pocket within the closely packed extracellular region of the receptor. There are amino acid residues in glucagon that could potentially form salt bridges. Indeed, the X-ray crystal structure of [$Lys^{17,18}$, Glu^{21}]glucagon amide revealed that the ability to form a salt bridge between Lys^{18} and Glu^{21} is likely responsible for the analog's enhanced binding and superagonist activity (10,13).

To examine the feasibility of salt bridge formation in glucagon and its significance to glucagon-induced receptor binding and activation, we synthesized four glucagon analogs (Table 1, **2–5**) with an internal lactam. Lactam cyclization resulting from a covalently bonded side chain salt bridge should stabilize secondary structures if they are present in the peptide. Amide bond formation was allowed to occur between Glu^2 and Lys^5, analog **2**, Glu^9 and Lys^{12}, analogs **3** and **4,** and Glu^{20} and Lys^{24}, analog **5**, in the corresponding glucagon analogs. The binding and activity parameters of the resulting lactam-containing glucagon analogs were compared to acyclic counterparts to determine whether formation of a cyclic structure was favorable at those positions.

In addition, the present work applied theoretical methods to study the energetics of salt bridge formation in glucagon by using the following as model structures. The complex formed by free aspartic acid and lysine residues was analyzed in two conformations. The first, featuring the formation of a salt bridge with the hydrogen of the aspartic acid carboxyl directed toward the lone pair of electrons on the lysine ϵ-amino nitrogen (Fig. 1A);

Table 1. Pharmacological parameters of cyclic analogues of glucagon

	Receptor Binding Activity		Adenylyl Cyclase		
Glucagon and Analogues	Relative Affinity[a], (%)	Fold Decrease	Relative Potency[b], (%)	Maximum Activity[c] (%)	pA_2[d] Value
1A Glucagon	100		100	100	—
1B Glucagon amide	100		15	100	—
1C DesHis[1]glucagon	8	12.5	0.1	36	—
1D DesHis[1]glucagon amide	63	1.6	0.16	44	—
2 Cyclic[Glu^2Lys^5]-amide	0.41	244	0.58	100	
3 Cyclic[Glu^9Lys^{12}]-amide	4.8	21	0.126	18	—
4 DesHis[1]cyclic[Glu^9 Lys^{12}]-amide	12.6	8	<0.00013	—	6.9
5 DesHis[1]Glu^9cyclic[Glu^{20} Lys^{24}]-amide	19.1	5.3	<0.003	—	6.8

[a]The ratio of unlabeled glucagon concentration required to displace 50% of receptor-bound [^{125}I]-glucagon, IC_{50}, to the concentration required for analogues of glucagon × 100.

[b]The ratio of glucagon concentration at 50% stimulation (EC_{50}) of natural glucagon to that of glucagon analogue × 100.

[c]The ratio of maximum response of glucagon analogue to that of natural glucagon × 100.

[d]pA_2 value is the negative logarithm of the concentration of inhibitor that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist.

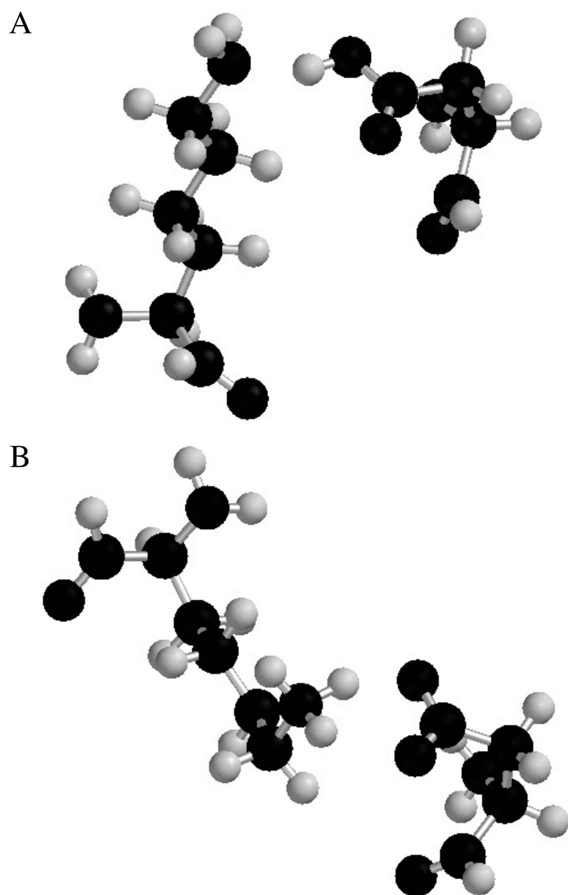


Fig. 1. (A) The complex formed by aspartic acid and lysine, featuring a hydrogen bond between the β-carboxyl hydrogen of aspartic acid and the lone pair of electrons on the ε-amino group of lysine. (B) The complex formed by the aspartate anion and the lysine cation, featuring hydrogen bonds between two hydrogens of the ε-amino group of lysine and the oxygens of the aspartate β-carboxylate group.

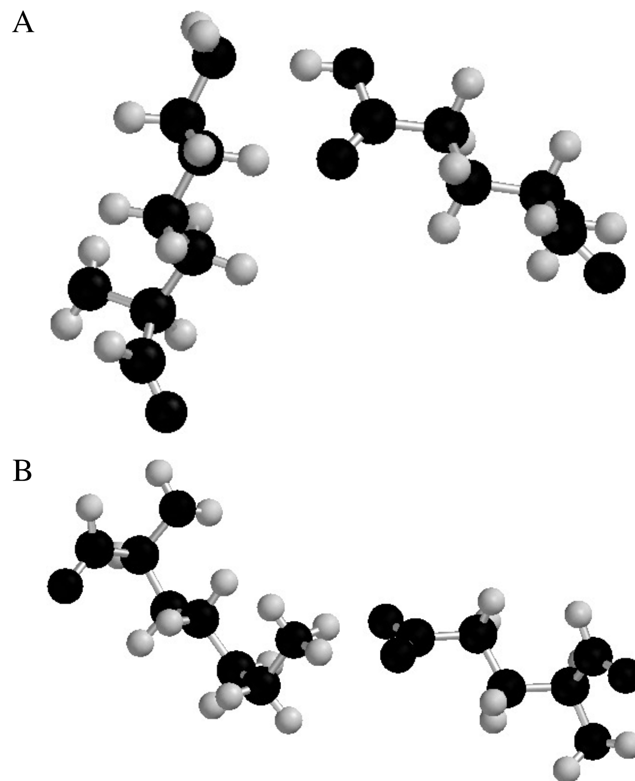


Fig. 2. (A) The complex formed by glutamic acid and lysine, featuring a hydrogen bond between the γ-carboxyl hydrogen of glutamic acid and the lone pair of electrons on the ε-amino group of lysine. (B) The complex formed by the glutamate anion and the lysine cation, featuring hydrogen bonds between two hydrogens of the ε-amino group of lysine and the oxygens of the glutamate γ-carboxylate group.

and the second, the formation of a salt bridge with the hydrogens of the nitrogen on lysine interacting with the carboxylate oxygens on aspartic acid. The latter model structure is a zwitterion (Fig. 1B). Identical calculations were performed on the same set of complexes formed by free lysine with a glutamic acid (Fig. 2A and B).

The energetics of intramolecular salt bridge formation were analyzed in three peptide models based on the glucagon sequence. First, a heptapeptide, Thr^{7}-Ser^{8}-Asp^{9}-Tyr^{10}-Ser^{11}-Lys^{12}-Tyr^{13}, represented residues 7–13 of glucagon. Several conformers of the peptide were studied with or without intramolecular salt bridge formation between Asp^{9} and Lys^{12} (Fig. 3). Second, a tetrapeptide, Asp^{9}-Tyr^{10}-Ser^{11}-Lys^{12}, represented residues 9–12 of glucagon. Several conformers with or without the salt bridge between Asp^{9} and Lys^{12} were analyzed (Fig. 4). A glucagon analog with an amide bond between an Asp^{9} and Lys^{12} has been synthesized (14). And third, a pentapeptide, Glu^{20}-Asp^{21}-Phe^{22}-Val^{23}-Lys^{24}, represented residues 20–24 of a glucagon analog synthesized with an amide bond between the γ-COOH of Glu^{20} and the ε-NH_2 of Lys^{24}.

Methods

Materials

tert-Butyloxycarbony (Boc)-protected amino acids were from Peptide Institute, Inc. (Osaka, Japan); 4-methylbenzlyhydrylamine resin (p-MBHA resin, 0.3 mmol/g) and benzotriazolyloxytris[dimethylamino]-phosphonium hexafluorophosphate (BOP) from Peptides International (Louisville, KY, USA); N^1-hydroxybenzotriazole (HOBt) and N,N′-dicyclohexylcarbodiimide, from Fluka (Milwaukee, WI, USA); diisopropylethylamine and trifluoroacetic acid (TFA) from Aldrich (Milwaukee, WI, USA); acetonitrile, dimethylformamide and dichloromethane from Fisher Scientific (Fair Lawn, NJ, USA); ATP, GTP, creatine phosphate, and creatine phosphokinase, from Sigma Chemical Co. (St. Louis, MO, USA); monoiodinated ^{125}I-glucagon from NEN Dupont (Boston, MA, USA); and the cAMP assay kit containing [8-^{3}H]cAMP was obtained from Amersham (Piscataway, NJ, USA).

Synthesis and Purification of Glucagon Analogs

Glucagon analogs were synthesized by the solid phase method with p-MBHA resin on an Applied Biosystems 430A peptide synthesizer using procedures developed for the synthesis of natural glucagons (15). All amino acids were coupled as their N^{α}-Boc derivatives. Standard protocol for double couplings with preformed symmetric anhydrides in dimethylformamide was used, except for arginine, asparagine, and glutamine, which were coupled as N^{1}-hydroxybenzotraizole esters. Aspartic acid and glutamic acids to be cyclized were coupled as N^{α}-Boc-Asp-(β-Ofm) and N^{α}-Boc-Glu-(γ-Ofm), respectively. Lysine to be cyclized was coupled as N^{α}-Boc-Lys (N^{ε}-Fmoc). After removing the side chain Fm and Fmoc protecting groups of aspartic and lysine derivatives with 20% piperidine for 40 min, the peptides were cyclized on the resin using three equivalents of

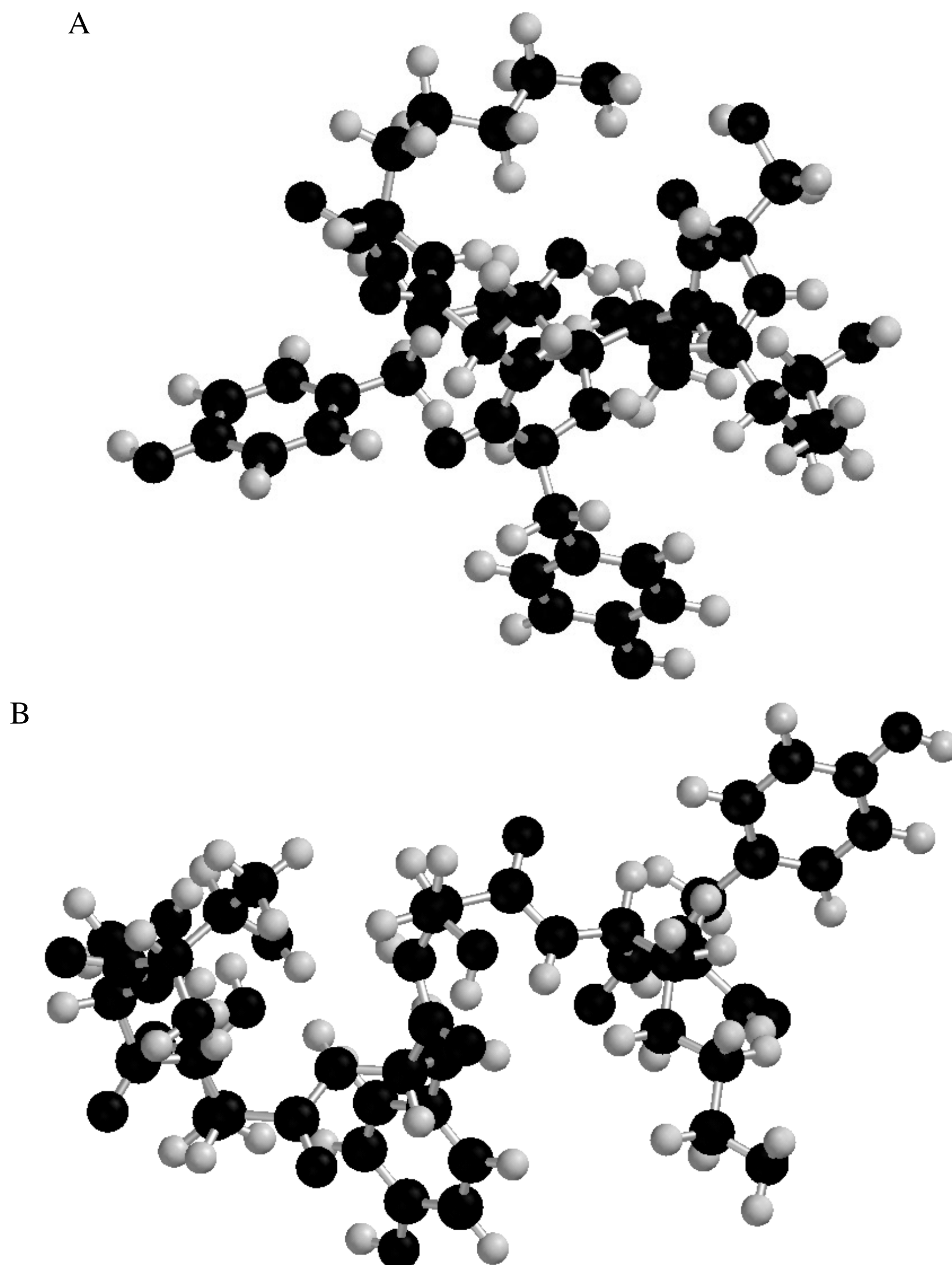


Fig. 3. (A) A conformation of a heptapeptide fragment derived from glucagon representing residues 7–13: Thr^{7}-Ser^{8}-Asp^{9}-Tyr^{10}-Ser^{11}-Lys^{12}-Tyr^{13}, featuring a hydrogen bond between the ε-amino group of lysine and the β-carboxyl group of Asp^{9}. (B) An extended conformation of the heptapeptide in Figure 3A. (C) A coiled conformation of the heptapeptide in Figure 3A. **(D)** Another extended conformation of the heptapeptide in Figure 3A, different from the conformation in Figure 3B.

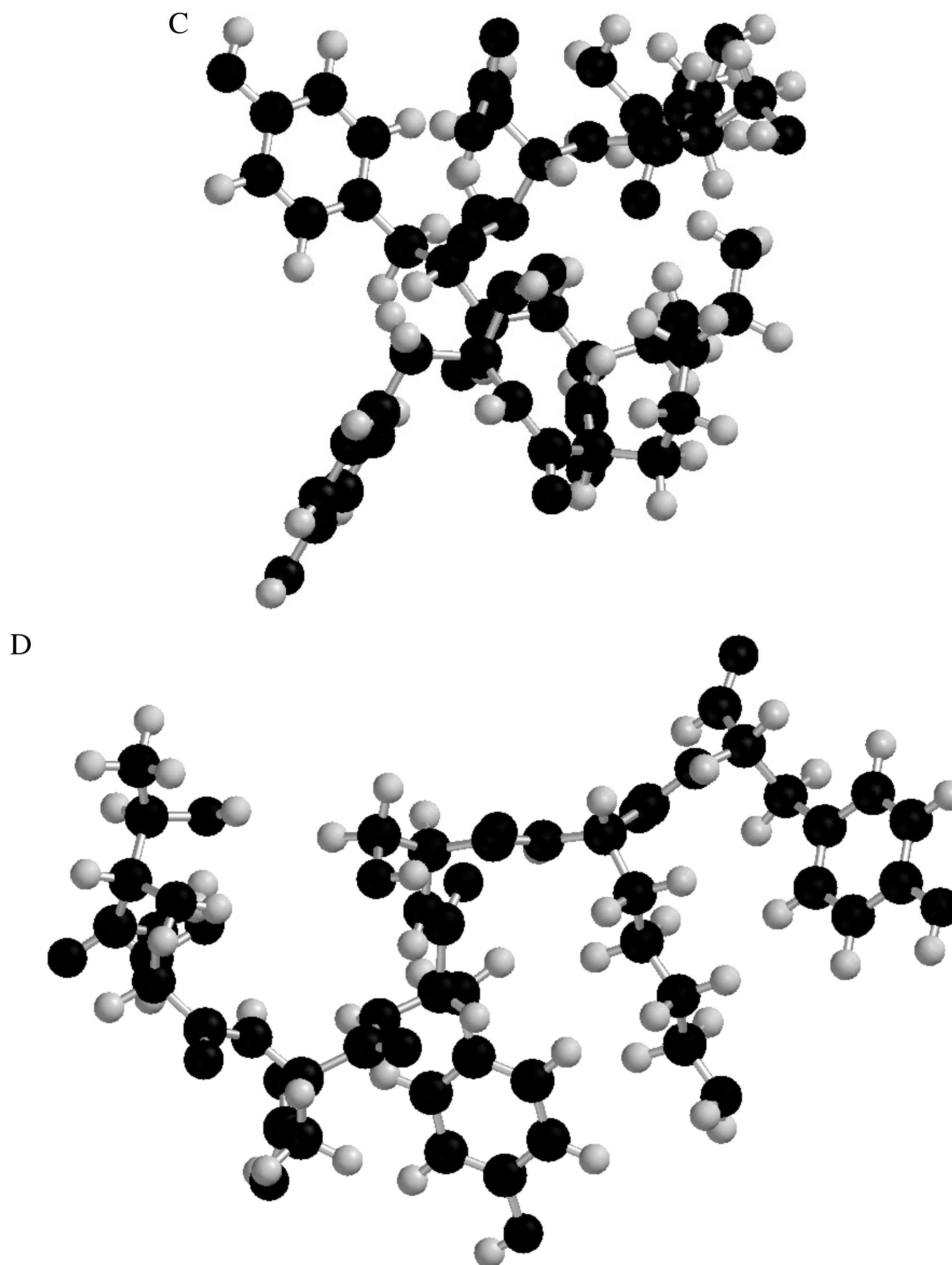


Fig. 3. (continued)

benzotriazolyloxytris[dimethylamino]-phosphonium hexafluorophosphate. The protected peptide resins were cleaved by the low–high HF procedure (16) and the crude peptides were extracted with 10% glacial acetic and purified as described previously (17). Purity of the peptide derivatives was verified to be greater than 95% by analytical HPLC on octadecylsilica (Vydac C18, Separations Group, Hesperia, CA, USA) applying a gradient of 25–45% acetonitrile in 0.05% aqueous TFA over 20 min. Amino acid analysis yielded amino acid compositions consistent with theory. Mass spectral analysis using the electrospray method determined the (M + H) + peak to be within ±0.3 da of theory.

Receptor Binding

Rat liver membranes were prepared from male Sprague-Dawley rats (Charles River Laboratories) by the Neville procedure, resuspended in $NaHCO_3$, and stored as aliquots under liquid nitrogen until use (18). Protein was determined by a modified Lowry method (19). The receptor binding assay

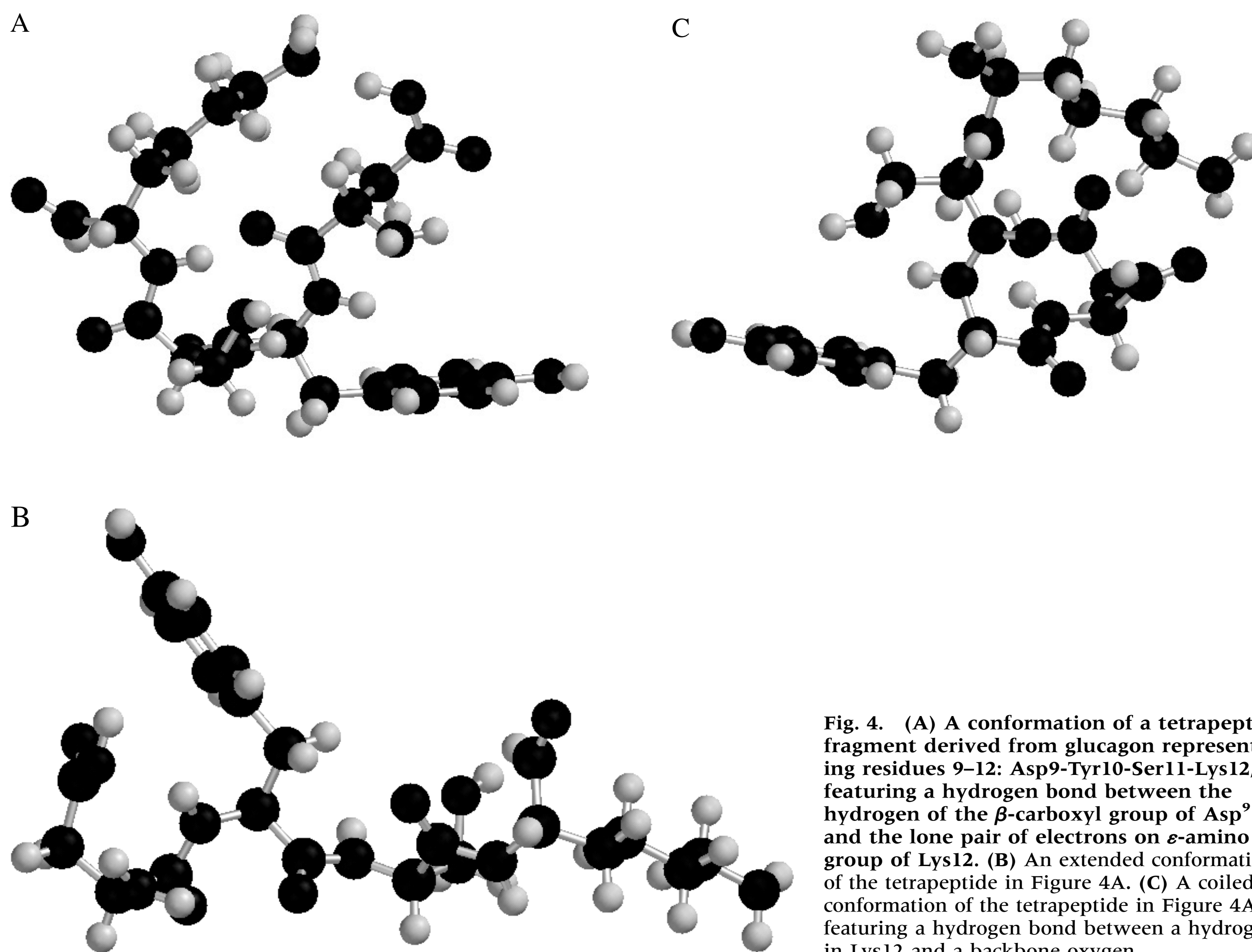


Fig. 4. (A) A conformation of a tetrapeptide fragment derived from glucagon representing residues 9–12: Asp9-Tyr10-Ser11-Lys12, featuring a hydrogen bond between the hydrogen of the β-carboxyl group of Asp[9] and the lone pair of electrons on ε-amino group of Lys12. (B) An extended conformation of the tetrapeptide in Figure 4A. **(C)** A coiled conformation of the tetrapeptide in Figure 4A, featuring a hydrogen bond between a hydrogen in Lys12 and a backbone oxygen.

was performed in 1% BSA, 1 mM dithiothreitol, and 25 mM Tris-HCl buffer, pH 7.2 (20). The amount of radioiodinated glucagon displaced from receptor sites by increasing concentrations of antagonist was measured. Binding affinity was expressed as the ratio of the concentration of natural glucagon to that of the antagonist required to displace 50% of receptor-bound labeled glucagon multiplied by 100.

Adenylyl Cyclase Activity

Adenylyl cyclase activity was measured in an assay medium containing 1% BSA, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.025 mM GTP, 5 mM ATP, 0.9 mM theophylline, 17.2 mM creatine phosphate, and 1 mg/ml creatine phosphokinase (21). The cAMP released was determined with a commercial kit from Amersham in which unlabeled cAMP was allowed to compete with [8-^{3}H] cAMP for a high-affinity cAMP-binding protein. Data for stimulation of adenylyl cyclase are expressed as picomoles of cAMP produced per milligram of protein per minute and plotted against the logarithm of peptide concentration. Relative activity is a measure of the potency of the analog in the adenylyl cyclase assay and is expressed as the ratio (×100) of the concentration of glucagon to that of the analog required to give half the maximum response of analog. This method of comparison was arbitrarily selected because the shapes of the activity response curves of natural glucagon and the analogs are not always parallel, especially in the case of partial agonists.

Inhibition of adenylyl cyclase was determined using the same procedure above except that increasing concentrations of peptide antagonist were allowed to compete with a constant concentration of natural glucagon. The ratio of the concentration of antagonist to agonist when the response is reduced to 50% of the response to glucagon in the absence of antagonist is the inhibition index (I/A_{50}). The pA2 value, calculated also from the dose–response curve, is the negative logarithm of the concentration of antagonist that reduces the response to 1 unit of agonist to the response obtained from 0.5 unit of agonist (22).

Theoretical Calculations

The Asp/Lys and Glu/Lys ion pairing complexes were investigated with ab initio calculations, using the

Titan computer program, version 1.05 (Wavefunction, Inc., Irvine, CA, USA). The basis set used is 6-31G*, at Hartree-Fock level. The 6-31G* basis set consists of one Slater orbital used for the description of the core electrons and expanded in a series of six gaussians, with the valence electrons described by two Slater orbitals each, one expanded in a series of three gaussians and the other approximated by one gaussian. In addition, d orbitals are set on nonhydrogen atoms. The geometries of the complexes are optimized and the results are shown in Table 2. Scheme 1 shows the numbering of the atoms. The optimized complexes are shown in Figures 1 and 2. The heptapeptide segment Thr-Ser-Asp-Tyr-Ser-Lys-Tyr (Fig. 3), which represents the 7–13 fragment of glucagon, has been investigated with the semi-empirical method AM1. Four different initial conformations were optimized and the resulting structures are shown in Figure 3. The Titan program was used. The tetrapeptide Asp-Tyr-Ser-Lys (Fig. 4), representing residues 9–12 of glucagon, was investigated with ab initio methods, using the 3-21G basis set, which uses one Slater orbital to the description of core electrons, expanded in a series of three gaussians. The valence electrons are described by two Slater orbitals, one expanded in a series of two gaussians, the other approximated by one gaussian. Geometry optimization was performed on three initial conformations and the results are shown in Figure 4. For all the structures considered, solvation energies were obtained using the SM5.4 model (23). In addition, the single-point energies of the tetrapeptides were also calculated with the 6-31G* basis set, using the 3-21G obtained geometries. Table 3 shows the total energies of the heptapeptide fragment in different conformers, in kcal/mol. Table 4 shows the energy values obtained for the tetrapeptide. The pentapeptide, Glu^{20}-Asp^{21}-Phe^{22}-Val^{23}-Lys^{24}, was optimized with the use of the 3-21G basis set in an extended conformation and in a cyclic conformation allowing hydrogen bonding between glutamic acid and lysine. The hydrogen bonds are between the carboxyl hydrogen and the lone pair of electrons on the ε-amino group of lysine. Single-point energies using the 3-21G obtained geometries were calculated using the 6-31G* basis set. The energy values are shown in Table 5. The optimized structures are shown in Figure 5.

Results and Discussion

We prepared glucagon analogs with intramolecular side chain lactams by solid-phase peptide synthesis using a Boc strategy (24). Internal lactams were designed at three different locations: at the N-terminal portion of the molecule between positions 2 and analog **2**, close to the central hinge region between positions 9 and 12, analogs **3** and **4**, and at the C-terminal end of the molecule between positions 20 and 24, analog **5**. Lactam formation occurred between a COOH side chain provided by a glutamic

Table 2. Geometry of complexes 1 and 2 (X and degree)

Bond Lengths

	1a	1b	2a	2b
N1-H1′	1.874	—	1.905	—
H1-O1′	—	1.601	—	1.579
H2-O2′	—	2.000	—	1.924
N1C1	1.467	1.488	1.465	1.481
C1C2	1.525	1.524	1.524	1.524
C2C3	1.531	1.533	1.530	1.531
C3C4	1.533	1.532	1.533	1.529
C4C5	1.537	1.533	1.537	1.530
C5C6	1.516	1.520	1.516	1.506
C5N2	1.455	1.455	1.455	1.454
C6O1	1.186	1.187	1.191	1.158
C5H5	1.088	1.094	1.088	1.091
H1′O1′	0.975	—	0.973	—
O1′C1′	1.314	1.235	1.315	1.252
O2′C1′	1.198	1.255	1.195	1.252
C1′C2′	1.512	1.525	1.510	1.523
C2′C3′	1.529	1.543	1.525	1.529
C3′C4′	1.525	1.520	1.531	1.564
C4′O3′	1.182	1.192	—	—
C3′N1′	1.447	1.446	—	—
C3′H3′	1.091	1.092	—	—
C4′N1′	—	—	1.448	1.453
C4′C5′	—	—	1.523	1.511
C5′O3′	—	—	1.188	1.188
C4′H4′	—	—	1.094	1.090
C5′H5′	—	—	1.092	1.095

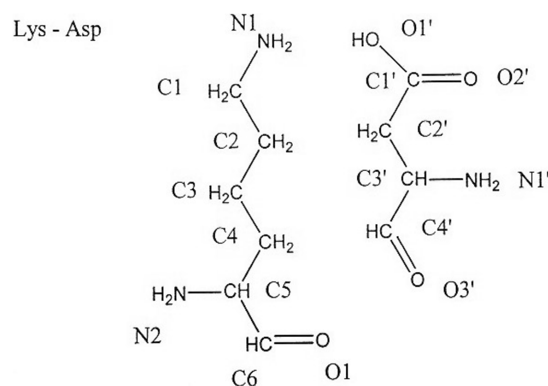
Bond Angles

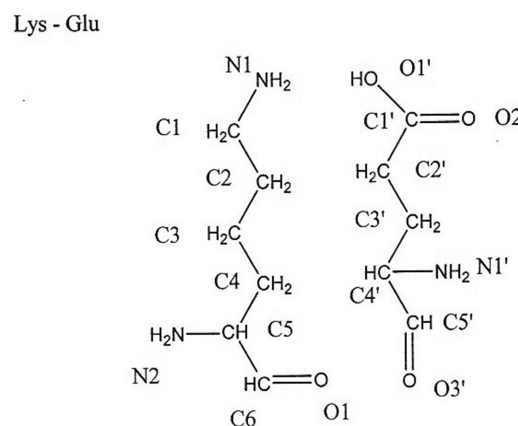
	1a	1b	2a	2b
O1′H1′N1	174.56	—	173.23	—
O1′ H1N1	—	155.48	—	157.07
O2′H2N1	—	123.12	—	128.23
N1C1C2	110.60	111.47	110.52	110.19
C1C2C3	112.93	114.42	112.69	113.84
C2C3C4	111.79	111.48	111.60	112.27
C3C4C5	115.85	114.92	116.06	114.70
C4C5C6	111.09	112.05	111.60	112.13
C5C6O1	123.54	123.91	123.20	123.58
C4C5N2	110.07	111.43	110.53	111.91
H1′O1′C1′	110.67	—	110.35	—
O1′C1′C2′	112.19	117.23	112.70	116.9
H1O1′C1	—	102.90	—	102.74
H2O2′C1′	—	108.11	—	107.79
C1′C2′C3′	114.03	109.44	112.17	112.83

C2′C3′C4′	111.89	110.08	112.82	112.85
C3′C4′C5′	—	—	110.70	111.88
C2′C3′N1′	111.89	110.99	—	—
C3′C4′O3′	123.45	123.47	—	—
C3′C4′N1′	—	—	111.36	107.17
C4′C5′O3′	—	—	123.38	124.77

Dihedral Angles

	1a	1b	2a	2b
C1′O1′H1′N1′	−125.13	—	−174.78	—
H1′N1C1C2	− 57.80	—	64.07	—
N1C1C2C3	−171.44	− 57.19	−175.88	− 57.68
C1C2C3C4	−171.86	−171.05	−171.02	−175.22
C2C3C4C5	−164.96	−170.46	−168.36	−170.69
C3C4C5N2	64.46	60.71	64.04	60.50
C3C4C5C6	− 57.05	− 60.80	− 55.40	− 61.14
O1C6C5C4	−105.96	− 36.14	−110.99	− 36.14
O1′C1′C2′C3′	173.50	88.73	168.60	−124.74
C1′C2′C3′C4′	− 62.28	− 55.99	178.51	−174.62
C1′C2′C3′N1′	66.80	70.98	—	—
C2′C3′C4′O3′	−105.96	125.21	—	—
C2′C3′C4′N1′	—	—	− 67.04	−151.19
C2′C3′C4′C5′	—	—	165.86	82.78
C3′C4′C5′O3′	—	—	141.56	119.78





acid residue and an NH_2 side chain supplied by a lysine residue at those positions. With this strategy, we aimed to probe for that portion of glucagon that can be stabilized by lactam cyclization formed by an amide bond between the γ-carboxyl group of a glutamic acid and the ε-amino group of a lysine. In the case of cyclic[Glu^2 Lys^5], cyclic[Glu^9 Lys^{12}], and desHis[1]cyclic[Glu^9 Lys^{12}]glucagon amide, analogs **2, 3,** and **4** (Table 1), the oppositely charged side chains were positioned at i and i + 3 relative to each other. In the cyclic[$Glu^{20}Lys^{24}$] derivative, analog **5** (Table 1) the charged residues were situated i and i + 4 relative to each other. Substitution of glutamic acid for aspartic acid at position 9 of glucagon had been shown previously to result in an analog that behaved as a glucagon antagonist (17). The effect of intramolecular lactam formation on receptor binding affinity and subsequent stimulation of adenylyl cyclase was measured. Binding affinity and adenylyl cyclase activity were assayed on rat liver membranes that are

Table 3. Energies (au), binding energy (kcal/mol) of complexes 1 and 2

Complex	
Aspartic-Lysine complex (1a)	−853.69864
Aspartate ion-lysine ion (1b)	−853.68014
Glutamic-lysine complex (2a)	−892.73339
Glutamate ion-lysine ion (2b)	−892.70054

Complex	Binding energy	Binding energy + Solvation energy
1a	−13.6	−25.4
1b : vs. ions	−121.2	−7.0
1b : vs. neutral species	−2.0	−13.7
2a	−12.9	−24.4
2b : vs. ions	−119.9	−4.6
2b : vs. neutral species	−4.0	−13.4

Table 4. Energies of some conformations of the heptapeptidic fragment of glucagon: Thr7-Ser8-asp9-tyr10-ser11-lys12-tyr13 (kcal/mol)

Structure	Energy	Energy + Solvation Energy
3a	−538.62	−572.25
3b	−573.49	−611.02
3c	−539.49	−571.42
3d	−576.35	−611.30

Table 5. Energies (au) of the tetrapeptidic fragment of glucagon Asp-Tyr-Ser-Lys as calculated with ab initio calculations, using 3-21G and 6-31G*//3-21G basis sets

	3-21G	6-31G*//3-21G
4a (containing Asp-Lys bridge)	−1713.92181	−1723.48502
4b (extended)	−1713.88362	−1723.44827
4c (containing a Lys-backbone H bond)	−1713.93222	−1723.47322

	Energies (kcal/mol)			
	Solvation:	*Relative:* ΔE (3-21G)	ΔE(3-21G) + Solvation energy	ΔE(6-31G*//3-21G)
4a	−60.48	6.53	−53.95	0.0
4b	−29.97	30.49	.52	23.06
4c	−44.10	0.0	−44.10	7.41

rich in glucagon receptors. The binding and activity parameters of the glucagon lactams were compared to those of their acyclic counterparts. The cyclic analogs were designed to indirectly provide insight on the importance of internal ion pair formation to glucagon binding and activity. Information from these studies might point to new directions in the design of novel glucagon antagonists.

In addition to synthetic analogs, theoretical calculations were performed on model cyclic peptides based on sequences from glucagon. The energetics of intramolecular hydrogen bond formation within a tetrapeptide, and a heptapeptide, containing an aspartic/glutamic acid at position i and a lysine at the i + 3 position, and a peptapeptide with a glutamic acid at the i position and a lysine at the i + 4 position, were calculated using the AM1 semi-empirical method for the heptapeptide and the ab initio method using the 3-21G basis set and the 6-31G* basis set for the tetrapeptide and pentapeptide. The tetrapeptide Asp-Tyr-Ser-Lys represented residues 9–12 of glucagon, the heptapeptide Thr-Ser-Asp-Tyr-Ser-Lys-Tyr represented residues 7–14, and the pentapeptide Glu-Asp-Phe-Val-Lys represented residues 20–24. Internal hydrogen bonding between the carboxylic group and amino functional groups of the peptides resulted in a 17-membered cyclic peptide in the case of the i and i + 3 cyclization (Figs. 3A and 4A) and a 21-membered cyclic peptide in the i and i + 4 cyclization (Fig. 5A). The peptide models mimicked the cyclic glucagon analogs listed in Table 1. The minimum energies obtained were contrasted with the biological data.

Analog **3**, cyclic[Glu^9Lys^{12}]amide, displayed a 20-fold decrease in binding affinity to 4.8% and retained weak agonist activity. The acyclic counterpart

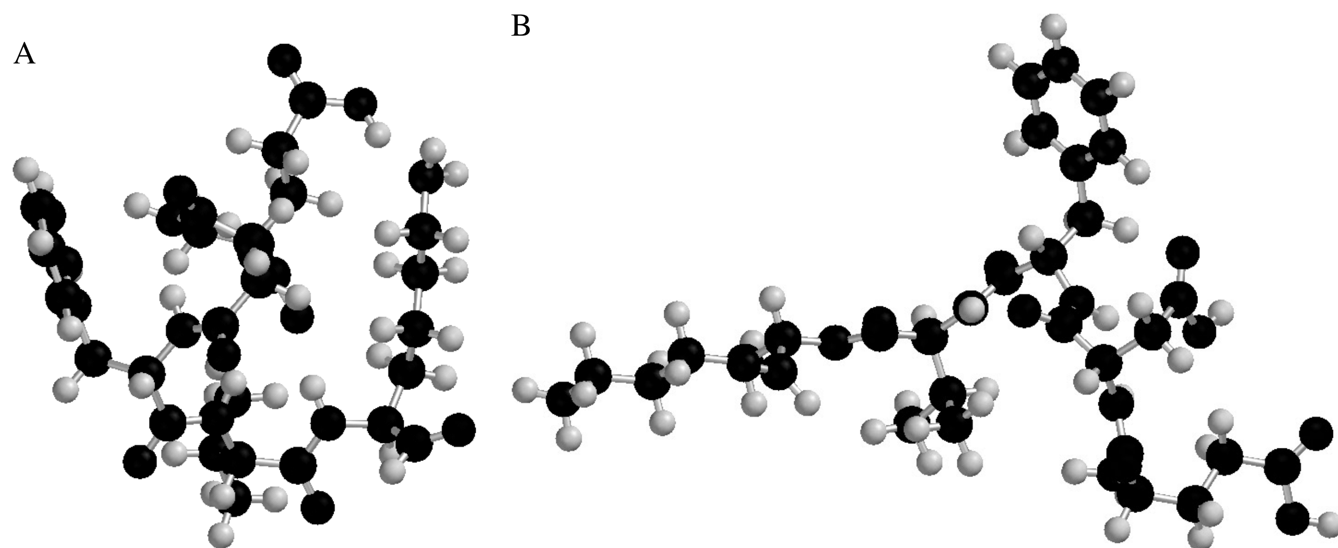


Fig. 5. (A) A cyclic conformation of Glu-Asp-Phe-Val-Lys that features a hydrogen bond between the glutamic and lysine residues. (B) An extended conformation of Glu-Asp-Phe-Val-Lys.

had a binding affinity of 14% (17). Deleting histidine 1 in desHis^{1}cyclic[Glu^{9}Lys^{12}]amide, analog **4**, abolished the ability to activate adenylyl cyclase even at the highest concentration tested, but the binding affinity was enhanced to 12.6%. Lactam formation between Glu^{20} and Lys^{24} in desHis^{1}Glu^9-cyclic[Glu^{20}Lys^{24}]amide, analog **5**, preserved 20% binding affinity for glucagon receptors and the derivative did not activate adenylyl cyclase. Not surprisingly, both analogs **4** and **5** were able to inhibit glucagon-stimulated adenylyl cyclase activation and behaved as glucagon antagonists with a comparable pA_2 value of 6.8. In contrast, cyclic peptide formation between Glu^2 and Lys^5 in cyclic[Glu^{2}Lys^5]-amide, analog **2**, was not well-tolerated and resulted in a 99% loss in binding affinity (Table 1). However, cyclic[Glu^{2}Lys^5]amide was still a full agonist, although with significantly reduced potency.

We have established the critical importance of Asp^9 glucagon for the activation of the glucagon receptor (5). Moreover, we and others have shown that the positive charge of Lys^{12} contributes both to the binding affinity and to the potency of activation (9,25). The data cannot specify, however, whether these charged residues interact with each other in an intramolecular salt bridge or whether these observations support ion pairing with oppositely charged residues in the receptor binding pocket. Interestingly, because the aspartic acid at position 9 and lysine at position 12 were not altered in cyclic[Glu^{2}Lys^5]amide, the analog retained full agonist activity.

What is evident from the bioassay data is that the ligand receptor interface can accommodate a cyclic structure at the C-terminal half but less so at both the midsection of the molecule and close to the N-terminus. The C-terminal half has been shown to be largely helical, while the N-terminus has a more flexible conformation (26,28). The lactam between Glu^2 and Lys^5 in cyclic[Glu^{2}Lys^5]amide, analog **2**, restricts chain flexibility and prevents efficient contact with the receptor. However, Asp^9 in cyclic-[Glu^{2}Lys^5]amide maintained its ability to bring about full activation. Despite the notion that the N-terminal half is mostly responsible for activation and the C-terminal half for binding to the receptor, our results suggest that the first five residues of glucagon are critical for binding.

Lactam cyclization at the midsection of the peptide also impaired receptor binding. Cyclic [Glu^{9}Lys^{12}]amide, analog **3**, showed only 5% binding affinity but deletion of histidine in desHis^1-cyclic[Glu^{9}Lys^{12}]amide, analog **4**, restored binding affinity to 13%. The acyclic derivatives [Glu^9]glucagon amide and desHis^1[Glu^9] glucagon amide had binding affinities of 14% and 41%, respectively. These results are consistent with a report that cyclic[Asp^9 Lys^{12}]glucagon amide displayed only 1% binding affinity and was inactive (14) and reinforce the notion that Asp^9 and Lys^{12} are not likely to be involved in an intramolecular salt bridge.

A lactam between Glu^{20} and Lys^{24} at the C-terminal region of DesHis^{1}Glu^{9}cyclic[Glu^{20}Lys^{24}]amide, analog **5**, was better tolerated and the peptide retained 20% binding affinity. The C-terminal end of the glucagon molecule is largely helical and studies have suggested that helical content is enhanced by conformational constraints such as a lactam bridge. In this analog the connecting side chains are at the i and i + 4 positions, which has a stabilizing effect on a helix. The lactam in analog **5** is a 21-membered ring. Formation of a lactam bridge between a Lys^{17} i and a Glu^{21} i + 4 was recently shown to be favorable and further supports our finding (29). The lactams in cyclic[Glu^{2}Lys^5]amide and cyclic[Glu^{9}Lys^{12}]amide, analogs **2** and **3**, are more constricted because the connecting side chains are at the i and i + 3 positions. The lactam in these analogs is an 18-membered ring that might distort the backbone rather than stabilize it.

Binding energies of the model peptides were calculated as the difference between the energy of the complex and the sum of the energies of the subsystems. As shown in Table 6, the binding energies of aspartic acid and lysine (complex 1) and of glutamic acid and lysine (complex 2) are substantial. Complex 1a, which features a hydrogen bond between the two neutral species, is slightly more bound than the complex 2a showing that replacing the aspartic acid residue by a glutamic acid does not influence the binding energy significantly. The same is true of complexes 1b and 2b, which feature the bond between two opposite charges, the aspartate or glutamate anions, respectively, with the lysine cation. In these complexes, two of the lysine NH_3^+ group are bound to the two oxygens of the carboxylate anion. This bond, compared to the separate ions, is very strong due to the electrostatic attraction upon its formation. However, when compared to the

Table 6. Energies (au) of the pentapeptide Glu-Asp-Phe-Val-Lys, relative and solvation energy

	Energy (3-21G)	ΔE(3-21G)	(kcal/mol) Energy(6-31G*)	ΔE(6-31G*)	Solvation Energy
Cyclic	−2112.56024	0.0	−2124.34392	0.0	−51.89
Extended	−2112.54067	12.28	−2124.33707	4.30	−21.66

independent neutral species, it is weaker than the bond in the 1a and 2a complexes. Taking into consideration the solvation energies did not influence the trend.

In previous studies, we investigated possible reasons for the loss of activity of the glucagon analog with Asp^9 to Glu^9 substitution. It was reasoned that shifting the carboxyl position affects the topographic interaction of Asp^9 with a critical contact point in the receptor that switches on the activation response (30). According to the results in Table 2, the stabilization energy of salt bridge formation between aspartic acid and lysine would not be measurably different if aspartic acid were replaced with a glutamic acid. Thus, the addition of one methylene group in glutamic acid would not be a contributing factor to the loss in activity if aspartic acid were involved in an intramolecular salt bridge.

Table 3 shows that at AM1 calculational level, the extended species of the heptapeptide Thr-Ser-Asp-Tyr-Ser-Lys-Tyr, which represents the 7–13 fragment of glucagon, is more stable than the cyclic model, with or without the inclusion of the solvation energy. However, as shown in Table 4, salt bridge formation between Asp and Lys of the (i + 3) tetrapeptide Asp-Tyr-Ser-Lys, resulted in a cyclic conformation that had a lower energy than the extended tetrapeptide Asp-Tyr-Ser-Lys, at both 3-21G and 6-31G*//3-21G levels. This peptide fragment represents residues 9–12 of glucagon. The same system investigated at AM1 level shows the extended conformation as more stable, indicating that AM1 calculations are not appropriate for the description of these systems. This is probably due to a poor description of hydrogen bonds.

As seen in Table 4, at 3-21G calculational level, the energy of the structure 4c, which features a hydrogen bond between a backbone oxygen and one of the hydrogens on the ε-amino nitrogen, is lower than the one of the structure 4a, which features the Asp^9-Lys^{12} hydrogen bond in which the hydrogen is positioned on the carboxyl as in Figure 1A or 2A. However, when the solvation energy is taken into consideration, the order is reversed, which suggests that the $Asp^{9-}Lys^{12}$ salt bridge is less likely to form in a hydrophobic pocket. This observation is consistent with the idea that the peptide ligand binds very close to the lipid bilayer and is most likely stabilized by hydrophobic interactions with the membrane-bound receptor protein. At 6-31G*//3-21G calculational level, 4a is the lowest in energy, even without solvation energy added.

A peptide lactam $desHis^1cyclic[Glu^9Lys^{12}]$-amide produced from an i to i + 3 cyclization of a γ-COOH and ε-amino of Glu^9 and Lys^{12}, respectively, was shown to have a lower binding affinity than its acyclic counterpart, which was reported to have a binding affinity of 41% (17). This observation supports the notion that the Glu^9 carboxyl and the Lys^{12} amino groups are not likely to interact intramolecularly, but may instead engage in ionic interactions with receptor protein residues (Table 1). The Glu^9 derivative is uncoupled from the receptor, and thus, the decreased binding may be due to the loss of the Lys^{12} interaction. These results are consistent with the finding that cyclic[Asp^9 Lys^{12}]glucagon amide retained only 1% binding affinity and was inactive (14).

An amide bond between γ-COOH and ε-amino of Glu^{20} and Lys^{24}, respectively, was tolerated in the analog $DesHis^1Glu^9cyclic[Glu^{20}Lys^{24}]$amide and 20% binding affinity was retained. As seen in Table 5, a pentapeptide Glu-Asp-Phe-Val-Lys, which represents the 20–24 segment of a glucagon analog, exhibited a lower energy for the cyclic conformation involving an Asp/Lys salt bridge than for the extended one using ab initio calculations, Hartree-Fock, with the 3-21G basis set. The pentapeptide is more stable in the cyclic form than the extended form by 12.28 kcal and also exhibits a stronger solvation energy (Table 5). In the tetrapeptide the cyclic conformation containing the salt bridge in Figure 4A is more stable than the extended form in Figure 4B by about 23 kcal/mol, but its solvation energy is much higher, making it much more stable. As seen in Tables 4 and 5, both with and without taking into account the solvation energy, the cyclic tetrapeptide shows a greater stabilization compared to the extended form than does the cyclic pentapeptide.

Our results are consistent with tentative models that have been proposed for the binding of peptide ligands to members of family B-type GPCRs (31,32). In this model, binding of the peptide ligand to its receptor occurs in two steps. First, a helical conformation is induced as the peptide associates nonspecifically with the membrane bilayer, followed by a second conformational change when the N-terminus of the peptide interacts with the membrane-bound receptor and activates it. The C-terminal end of the glucagon molecule has been shown by x-ray structure analysis to be largely helical (26). The current model indicates that this structure may closely represent the physiologic conformation of glucagon in the presence of its membrane-bound receptor, contrary to earlier assumptions.

In summary, data obtained from our cyclic glucagon analogs support a current model of the active conformation of glucagon. The peptide ligand in solution can assume many conformations. A small population of these conformations will predominate when the ligand interacts with its receptor on the cell surface. We tested the idea that an internal salt bridge might contribute to the stabilization of this putative conformation. Biological data bolstered by ab initio calculations indeed show good probability that an intramolecular salt bridge at the C-terminus of glucagon might facilitate provide local stabilization within the peptide as it interacts with its receptor. In contrast, an intramolecular salt bridge has a destabilizing effect at the N-terminal 1–13 sequence

that contains residues required for interaction with the receptor. Thus, an internal i to i + 4 intramolecular salt bridge at the C-terminal half of glucagon may be a useful feature in antagonist design and may lead to novel glucagon antagonists.

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