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Ab initio study of aspartic and glutamic acid: supplementary evidence for structural requirements at position 9 for glucagon activity

Anne-Marie Sapse*[a,d], Mihaly Mezei[b], Duli C. Jain[c], Cecille Unson[d]

[a]*John Jay College and the Graduate School, City University of New York, 445 West 59th St., New York, NY 10019, USA*
[b]*Mount Sinai School of Medicine, 1 Gustav Levy Plaza, New York, NY 10029 USA*
[c]*York College, City University of New York, 94-20 Guy R. Brewer Boulevard, Jamaica, NY 11451, USA*
[d]*Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA*

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Abstract

Our previous work established that position 9 aspartic acid in glucagon was a critical residue for transduction of the hormone response. An uncoupling of the binding interaction from adenylate cyclase activation was demonstrated by the observation that amino acid replacements at position 9 resulted in peptides that had no measurable adenylate cyclase activity yet were still recognized by the glucagon receptor. It was also later shown that His^1 played a major role in activation, and it was suggested that an electrostatic interaction between the aspartic acid carboxylate and the histidine imidazole occurred as part of the activation mechanism. This did not preclude intermolecular interactions of this aspartic acid with other residues within the receptor binding site. The observation that a conservative substitution of glutamic acid for aspartic acid at position 9 was sufficient to result in the potent antagonist, des-His^1d[Glu^9]glucagon amide, implied that even glutamic acid possessed the minimum properties necessary for inhibition, and that the precise position of the carboxyl group at position 9 in glucagon was an absolute requirement for full agonist activity. The present investigation was conducted with ab initio calculations and molecular modeling to shed some light on the source of this phenomenon.

Introduction

Glucagon is a peptide hormone of the pancreas that, together with insulin, is primarily responsible for the maintenance of the plasma glucose concentration that is critical to survival in man and animals. Most of the effects of glucagon are mediated by the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) produced by activation of adenylate cyclase when the hormone binds to its receptor in the liver membrane. The elevation of cAMP levels triggers a cascade of enzymatic reactions that leads to the breakdown of glycogen and a consequent production of hepatic glucose within minutes.

Glucagon is a well-studied member of a peptide family that includes vasoactive intestinal peptide, gastrin releasing factor and secretin. These peptide hormones may have evolved from a common precursor, and in spite of startling similarities in sequence each one couples to a specific receptor protein that triggers distinct physiological events. Receptors of this family are linked to their effector enzymes by GTP-binding proteins (G proteins), and also share conserved sequences, especially in their membrane-spanning α-helices. This suggests

* Corresponding author.

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that topologically equivalent domains of receptor protein interact with the peptide ligand, and highly specific variations in the amino acid sequences at the binding site enable receptors to discriminate between members of a family of hormones. The structural basis by which these receptors "recognize" their ligands as well as the subsequent generation of the transmembrane signal is not understood.

Structure activity studies have been directed towards sorting out the functional groups and conformational features of glucagon that are responsible for recognition and binding from those that transduce the biological response. The ability to segregate these properties will allow us to design glucagon antagonists that may be clinically relevant to the management of diabetes mellitus. Indeed, previous work has established that aspartic acid at position 9 [1,2] and histidine at position 1 [3] are critical for transduction of the hormone response and not for binding to the glucagon receptor. When combined with the omission of histidine at the amino terminus, all position 9 replacement analogs have proven to be glucagon antagonists [2]. One antagonist, des-His^{1}d[Glu^9]glucagon amide, has also proven to be an effective inhibitor of endogenous glucagon in vivo [4]. In addition, we have also shown that while the analog is unable to activate adenylate cyclase, it remains coupled to the phospholipase C signaling system and stimulates the breakdown of inositol lipids [4]. More recent studies demonstrate that while des-His^{1}d[Glu^9]glucagon amide binds well to the glucagon receptor, its affinity for the receptor is insensitive to GTP concentration [5]. This non-responsive behavior towards the presence of exogenous GTP in contrast to the GTP-sensitive glucagon, might explain its inability to transduce the hormone effect. That the conservative replacement of an Asp^9 residue with a Glu^9 is sufficient to transform glucagon into a pure antagonist with remarkably altered pharmacology, raises the possibility that intrinsic differences in the structure and preferential conformation of the two amino acids are large enough to perturb an interaction necessary for activation. This work is an attempt to obtain information about these differences at the molecular level using computational ab initio methods and modeling.

Methods and results

The GAUSSIAN 90 computer program [6] has been used to perform ab initio calculations at the Hartree–Fock level of theory. This method solves the Hartree–Fock equations without using any approximations to the integrals or to the Hamiltonian operator, but is completely specified by the choice of the nuclear coordinates and the basis set used. In this case, the basis set is the 6-31G basis set, which uses one Slater orbital for each core electron and expands it in a series of six gaussians, and two Slater orbitals for each valence electron, one of them being expanded in a series of three gaussians, and one being approximated by one gaussian. This approach to amino acid structure elucidation has proven to predict results which are in agreement with the experimentally obtained parameters, as in the case of the amino acid arginine [7].

The theoretical values of equilibrium bond lengths and angles were obtained by the Berny optimization method [8]. In order to distinguish local minima from the global minimum, different conformations of L-aspartic and L-glutamic acids were investigated. These are structures 1–5 shown

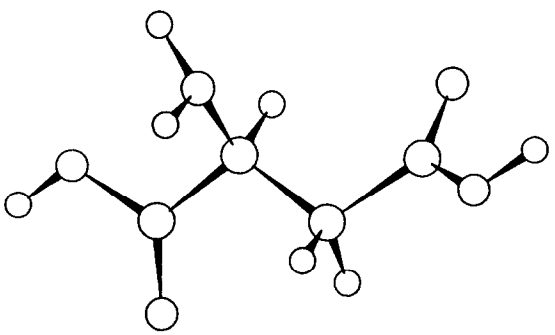


Fig. 1. The most stable conformation of aspartic acid (structure 1).

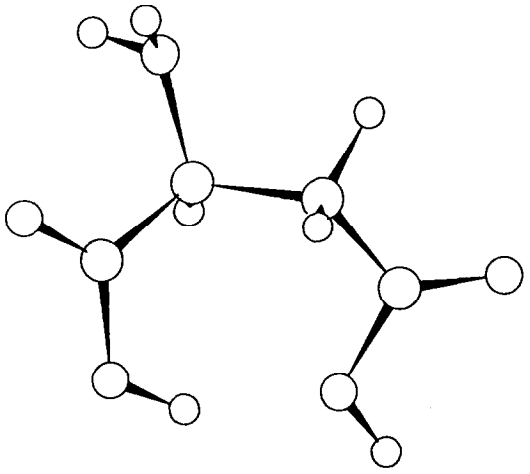


Fig. 2. Minimized cyclic structure of L-aspartic acid (structure 2).

in Figs. 1–5. Figures 1 and 4 show extended structures of aspartic acid and glutamic acid respectively, in their most stable conformations. Figure 2 shows a structure of aspartic acid in a cyclic conformation which features a hydrogen bond between the two carbonyl groups. Structures of aspartic acid and glutamic acid in a cyclic conformation brought about by a hydrogen bond between the side chain carboxyl group and the amino group are shown in Figs. 3 and 5. Complete geometry optimizations were performed for the five structures.

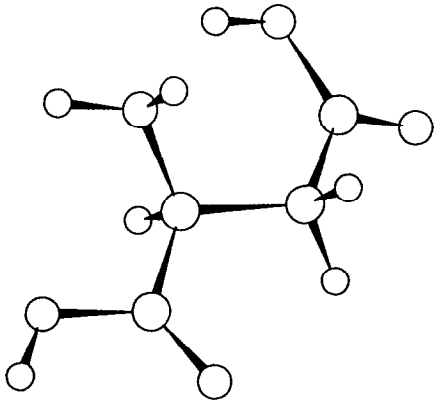


Fig. 3. Minimized cyclic structure of L-aspartic acid (structure 3).

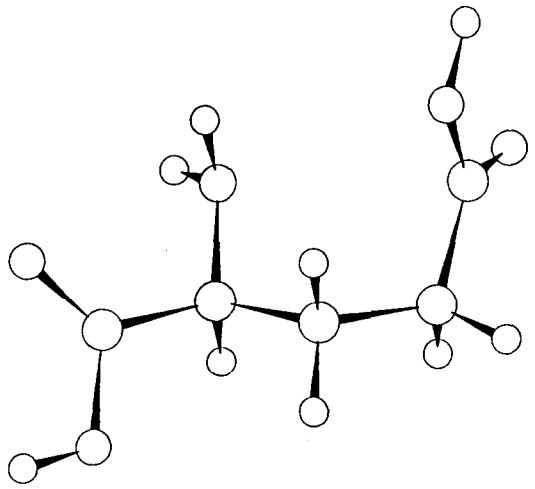


Fig. 4. The most stable conformation of L-glutamic acid (structure 4).

The computer graphics program INSIGHT II from Biosym, San Diego, CA, was then used to superimpose the most stable structures (structures 1 and 4) of aspartic and glutamic acids. In addition, they were superimposed in turn onto Asp^9 in the X-ray structure of glucagon [9,10] obtained from the Brookhaven Protein Data Bank. These results are displayed on Figs. 6–8, respectively. Table 1 shows the optimized geometrical parameters of L-aspartic acid in the investigated conformations, Table 2 shows the optimized parameters of L-glutamic

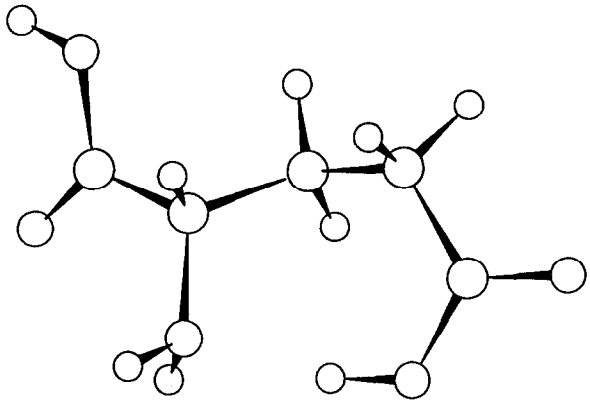


Fig. 5. Minimized cyclic structure of L-glutamic acid (structure 5).

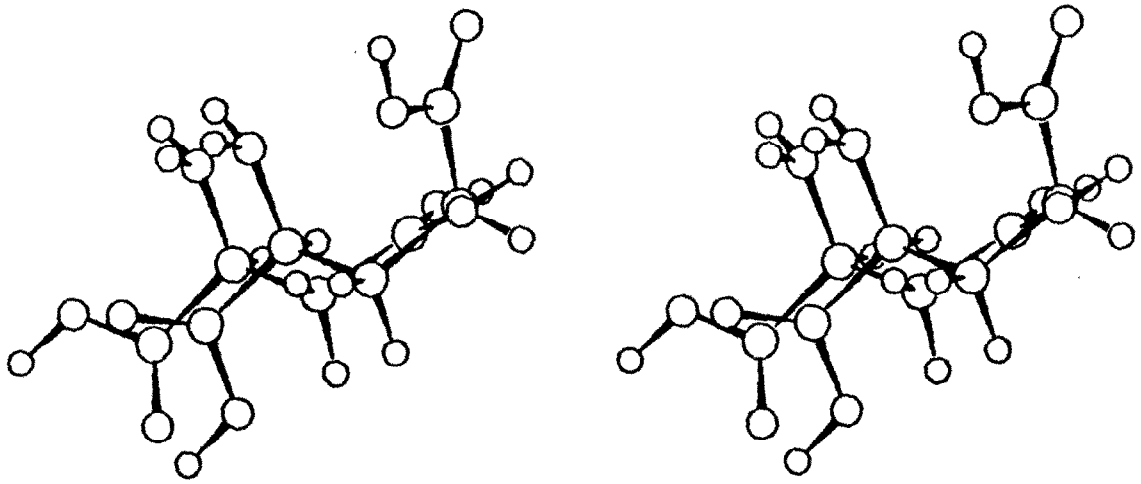


Fig. 6. Stereo picture of the superposition of L-aspartic and L-glutamic acid in their most stable conformations. A final shift was added for clarity.

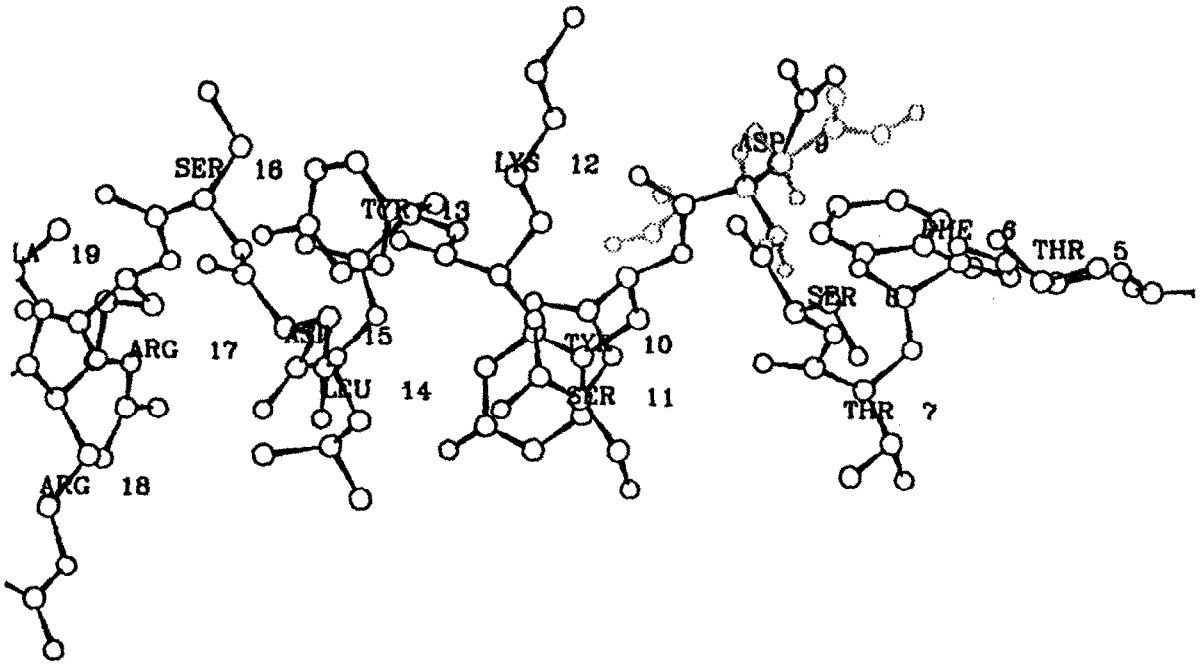


Fig. 7. Superposition of L-aspartic acid in its most stable conformation on the Asp^9 residue of the X-ray structure of glucagon.

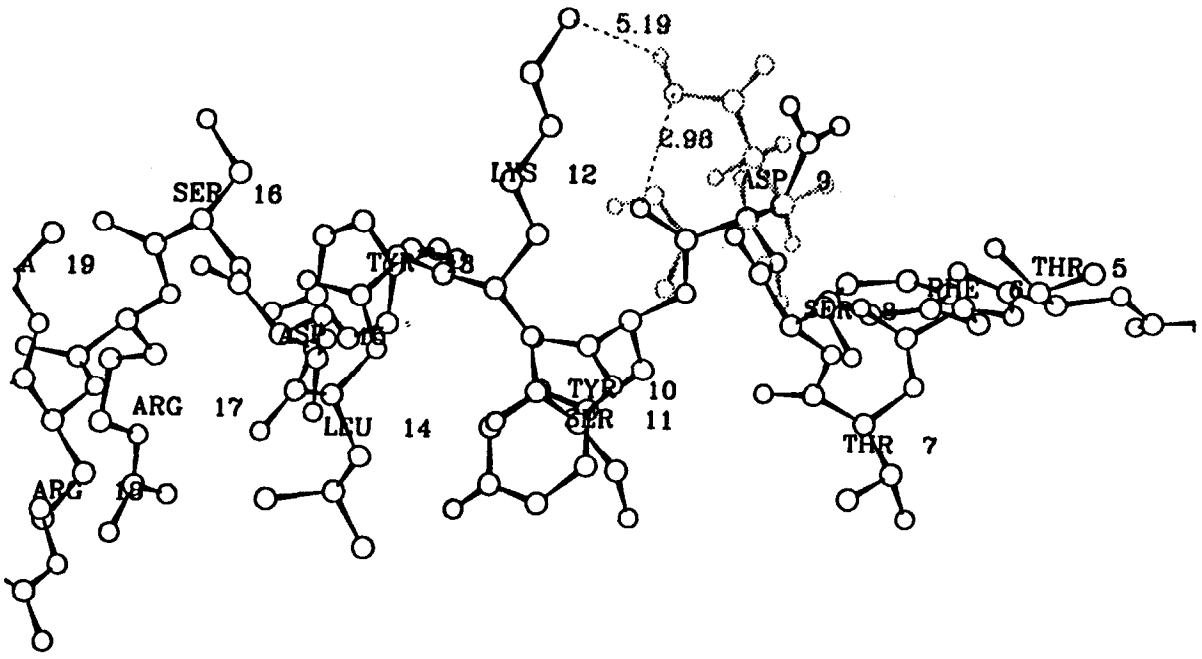


Fig. 8. Superposition of L-glutamic acid on the Asp[9] residue of the X-ray structure of glucagon. Backbone torsion angles were modified to avoid unfavorable contact with Phe[6].

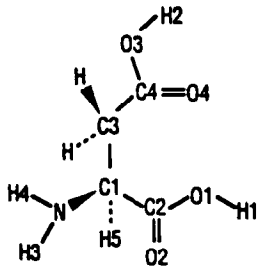
acid, Table 3 shows the total energies and the energy differences between different conformations of structures 1–5, and Table 4 displays the net atomic charges on the heavy atoms in each of the investigated conformations as obtained by Mulliken population analysis.

Discussion

One approach to the study of glucagon action is to attempt to dissociate structural and conformational properties of the hormone that are important for binding (recognition message) from those that are necessary for transduction (biological activity message). Structure–function studies have resulted in the development of the analog, des-His[1][Glu[9]]glucagon amide [11], which has proven to be a potent antagonist of glucagon in in vivo systems and in vitro studies [4]. Further investigation has shown that any replacement at position 9 of glucagon leads to analogs that do not have adenylate cyclase activity, but are still recognized by the glucagon receptor [1]. All of the resulting analogs are moderate to good antagonists of glucagon, and we have established that Asp[9] is critical for activation of the hormone response [2]. In addition, we have confirmed that the protonatable imidazole group of His[1] is required for interaction with Asp[9] [3]. To complete the triad, we have subsequently identified Ser[16] also to be an important residue for the expression of a full agonist response [12,13].

The involvement of histidine, aspartic acid, and serine residues in an active intermediate is reminiscent of serine proteases. We have hypothesized that Asp[9] is part of a putative catalytic triad together with His[1] and Ser[16]. Upon binding, the glucagon–receptor complex acquires enzyme activity, and the His, Asp, Ser residues of the hormone are aligned in a charge relay network for the catalyzed hydrolysis of an amide bond in the receptor. The role of the carboxylate group of Asp[9] would be to stabilize the positive imidazole ring of His[1] in the oxyanion hole, wherein Ser[16] is involved in a

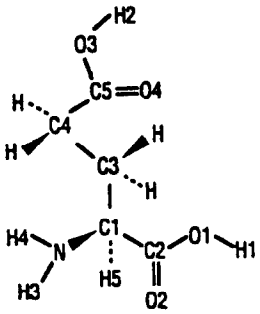
Table 1
Geometrical parameters of the optimized structures of L-aspartic acid



L-Aspartic Acid

Parameter	Structure 1	Structure 2	Structure 3
Bond length (Å)			
C1C2	*1.516*	*1.532*	*1.514*
C1C3	*1.525*	*1.554*	*1.530*
C3C4	*1.496*	*1.496*	*1.516*
C1N	*1.445*	*1.443*	*1.463*
C2O1	*1.350*	*1.344*	*1.351*
C2O2	*1.212*	*1.206*	*1.208*
C4O3	*1.350*	*1.369*	*1.343*
C4O4	*1.212*	*1.205*	*1.207*
O1H1	*0.955*	*0.958*	*0.955*
O3H2	*0.955*	*0.956*	*0.965*
C1H5	*1.083*	*1.084*	*1.085*
C3H	*1.082*	*1.082*	*1.082*
NH	*0.995*	*0.997*	*0.999*
Bond angle (deg)			
C2C1C3	110.7	111.7	111.0
C1C3C4	110.4	115.0	114.3
NC1C2	113.4	112.1	112.1
O1C2C1	112.0	116.5	111.3
O2C2C1	126.3	122.6	126.4
O3C4C3	112.6	112.5	117.0
O4C4C3	125.6	126.6	121.8
Torsion angle (deg)			
C4C3C1C2	162.7	97.8	170.0
O2C2C1C3	−7.6	120.8	−4.9
NC1C2C3	−124.2	−123.7	−124.0
O3C4C3C1	63.0	−45.1	35.3

Table 2
Optimized geometrical parameters of L-glutamic acid



L-Glutamic Acid

Parameters	Structure 4	Structure 5
Bond length (Å)		
C1C2	*1.515*	*1.513*
C1C3	*1.540*	*1.546*
C3C4	*1.531*	*1.542*
C4C5	*1.498*	*1.517*
NC1	*1.439*	*1.456*
O1C2	*1.353*	*1.347*
O2C2	*1.210*	*1.210*
O3C5	*1.356*	*1.336*
O4C5	*1.211*	*1.210*
O1H1	*0.955*	*0.955*
O3H2	*0.954*	*0.971*
NH	*0.996*	*1.000*
C1H5	*1.083*	*1.083*
C3H	*1.084*	*1.083*
Bond angle (deg)		
C2C1C3	109.6	110.4
C1C3C4	112.2	114.7
C3C4C5	115.7	117.6
O1C2C1	112.1	112.3
O2C2C1	126.0	125.2
NC1C2	112.8	110.8
O3C5C4	113.1	117.5
O4C5C4	125.5	121.3
Torsion angle (deg)		
C4C3C1C2	175.1	162.2
O2C2C1C3	114.1	114.8
NC1C2C3	−124.0	−124.7
C5C4C3C1	71.2	87.6
O3C5C4C3	44.0	−1.9

Table 3
Total energies (a.u.) of the L-aspartic acid and L-glutamic acid conformations and energy differences (kcal mol^{-1})

	E	ΔE
Structure 1	−509.2384	0.0
Structure 2	−509.2283	6.4
Structure 3	−509.2337	2.9
Structure 4	−548.2599	0.0
Structure 5	−548.2552	2.9

nucleophilic attack on the receptor. That the hormone–receptor complex acquires serine protease-like activity and that this proteolytic event might be the triggering step in the transduction of glucagon action, is supported by the observation that the serine protease inhibitor 4-aminophenylmethanesulfonyl fluoride completely suppressed cAMP production by glucagon-stimulated liver membranes [13].

The hypothesis may also explain antagonism in the case of des-His^1[Glu^9]glucagon amide and position 9 replacement analogs. An agonist bound to the receptor may lead to a conformation that forms an active center, while an antagonist stabilizes a conformation that either does not bring His, Asp, Ser into proper orientation, or has the correct conformation but is missing the required residue. The conservative substitution of glutamic acid for aspartic acid, if our hypothesis is correct, would not be expected to result in complete abolition of cyclase activity. Indeed, catalytic triads comprising His–Glu–Ser are well documented [14]. Nevertheless, the analog des-His^1[Glu^9]glucagon amide behaves as a pure antagonist that, in more recent work, has been shown to be insensitive to GTP concentration [5], which suggests that the receptor moiety bound to this analog is probably unable to interact with the G protein. Thus, Glu^9 of glucagon possesses subtle properties that are sufficient to lead to antagonism. In terms of our hypothesis, we can reason that the glutamic acid replacement perturbs the charge relay scheme, and receptor autolysis does not occur. The "modified" receptor resulting from this proteolysis is needed to interact with the G protein which in turn activates the effector, adenylate cyclase.

To obtain a molecular-level understanding of this phenomenon, ab initio calculations were performed to study the conformational surface of both amino acids and to determine the minimum energy conformations, and the results were studied in the context of the known glucagon crystal structure.

The total energies of the possible structures of aspartic and glutamic acids with the lowest minima are listed in Table 3. These structures are displayed on Figs. 1–5. The energy differences between various conformations, ΔE, range from 2.9 to 6.4 kcal mol^{-1}.

As seen from Table 3, the most stable conformation of aspartic acid is structure 1, followed by structure 3 and with structure 2 the least stable.

Table 4
Partial charges for the five conformations studied

Atom	Structure 1	Structure 2	Structure 3	Structure 4	Structure 5
C1	−0.074	−0.079	−0.108	−0.050	−0.038
C2	0.768	0.778	0.778	0.750	0.750
C3	−0.377	−0.401	−0.404	−0.313	−0.337
C4	0.785	0.790	0.777	−0.366	−0.423
C5				0.728	0.767
O1	−0.735	−0.763	−0.741	−0.742	−0.743
O2	−0.553	−0.528	−0.530	−0.546	−0.533
O3	−0.730	−0.808	−0.755	−0.729	−0.775
O4	−0.561	−0.516	−0.535	−0.552	−0.544
N	−0.830	−0.861	−0.877	−0.849	−0.918

Structures 2 and 3 are local minima on the energy hypersurface. These structures are depicted in Figs. 1–3. All the structures feature only positive eigenvalues of the Hessian matrix, showing them to be real minima. Table 1 lists the bond lengths and angles of the optimized structures of aspartic acid. Structure 2 exhibits longer bond lengths for the C1C2 and C1C3 bonds than the other structures. Structure 3 shows, as expected, a longer O3H2 bond length since H2 is involved in hydrogen bonding with the nitrogen. It also shows a larger O3C4C3 angle and a smaller O4C4C3 angle.

In Table 3, glutamic acid features as its most stable conformation, structure 4 (Fig. 4), with the cyclic structure 5 (Fig. 5) higher in energy only by 2.9 kcal mol^{-1}. As listed in Table 2, structure 5 shows a longer O4H2 bond, since H2 is hydrogen bonded to N. The C1C3C4 angle is also somewhat larger in structure 5. A rotation of the C4C5 bond around the C4C3 bond shows that, with a few exceptions which entail steric hindrance, the energies are within 3 kcal mol^{-1}, showing the molecule to be quite flexible, as found through a series of single point calculations.

As our hypothesis concerning the reason for the loss of activity is based on changes in the electrostatic interactions, we also examined the partial charges (obtained from Mulliken population analysis) for each conformation studied, shown in Table 4 for the heavy atoms. There are indeed significant (although not too large) changes both upon conformational change and on comparison of the two molecules. However the position of the carboxyl carbon in aspartic acid (C4) is replaced by an aliphatic carbon in glutamic acid (C4), resulting in a large charge shift, from 0.8 to 0.4 (see Table 4).

When glutamic acid is superimposed onto aspartic acid, both being in their most stable conformations as seen in Fig. 7, it can be seen that the position of the carboxyl group in aspartic acid is occupied by a methylene group in glutamic acid. This is evident also in the superposition of glutamic acid on the Asp^9 residue in the X-ray structure of glucagon as shown in Fig. 8.

Superimposing structure 1 onto the Asp^9 of the X-ray structure of glucagon, as seen in Fig. 6, it is realized that the aspartic acid residue in the peptide hormone is quite similar in geometry to the most stable conformation of the amino acid itself, indicating that the minimum energy conformation of the isolated amino acid adequately predicts the sidechain conformation in the peptide.

When, however, we have attempted to superimpose the glutamic acid in its lowest energy conformation onto the Asp^9 of the X-ray structure, the carboxyl group overlaps the sidechain of Phe^6. This shows that the substitution of aspartic by glutamic acid requires additional energy "investment". Accordingly, the superimposition of the glutamic acid shown in Fig. 8 does not correspond to the optimized structure but to one where the C4C5 bond is rotated around the C3C4 bond. Such a rotation involves a cost in energy of about 3 kcal mol^{-1}.

These results allow the following possible events: (1) the glutamic acid is substituted in a conformation similar to the one shown in Fig. 8; (2) there is a conformational change around the Phe^6 residue to accommodate the glutamic acid sidechain in its minimum energy conformation. In the case of (1), the carboxyl group will get closer to the lysine and is likely to change the lysine conformation, displacing its positive charge. In the case of (2) the effects are less easy to predict, since the change is to a hydrophobic group. In this case it is likely that other nearby hydrophobic groups are affected through hydrophobic interactions.

In any event, comparison of our calculations with the glucagon structure shows that the substitution of aspartic acid with glutamic acid destabilizes the protein and is guaranteed to induce conformational changes. This would indeed change the position of the charges on the sidechain and product an alteration of the charge relay mechanism. The reliable prediction of the actual sidechain conformational change, however, is beyond the scope of this work since it would require simulations in the presence of a large

number of water molecules. This challenge may be taken up in future work, however.

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