Unveiling the neutral forms of glutamine

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Abstract: Neutral glutamine has been evaporated by laser ablation of its solid sample to seed a rare gas carrier prior to a supersonic expansion and proved by Fourier transform microwave techniques. We report on three distinct neutral conformers that show a singular non-interacting and flexible amide sidechain in contrast with the other proteinogenic aliphatic amino acids. It could explain the essential biological role of glutamine as a nitrogen source, and its unique ability to form a variety of hydrogen bonds with peptide backbones. Common computational methods fail to predict the delicate balance of intramolecular interactions controlling the geometry of the most stable conformer. The spectroscopic data here reported can be used to benchmark novel computational methods in quantum chemistry.

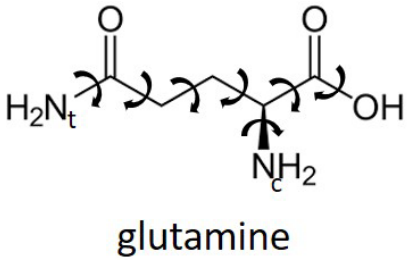
Glutamine ($C_5H_{10}N_2O_3$, Scheme 1) is one of the twenty proteinogenic amino acids present in many essential proteins and used in their biosynthesis.[1] It is the most abundant amino acid in the human blood and comprises approximately half of the whole-body pool of free amino acids. Furthermore, unlike some of the other amino acids, is conditionally essential amino acid that plays a significant role in the synthesis of lipids, regulation of kidneys functionality, purines synthesis and safe circulation of ammonia in a human circulatory system.[1–3] It is the most essential amino acid in muscle growth and is involved in the synthesis of a variety of enzymes. Additionally, it is the primary source of nitrogen in human bodies,[4] donating nitrogen atoms during the synthesis of purines, pyrimidines, and amino acids. The biological functions of glutamine are intimately connected to their three-dimensional structure;[1,5] knowledge of its structure is therefore essential.

In traditional studies in the condensed phase, the strong intermolecular interactions fix glutamine as a doubly charged zwitterion, wiping out its conformational variability. So far, the few experiments to obtain any structural information are electron-density projections,[6] infrared and Raman studies, either in aqueous solutions or in crystal.[7–12] In these condensed media, glutamine is in its zwitterionic form, i.e. not the neutral form present in polypeptides chains. The intrinsic conformational choices of amino acids can be revealed only when they are studied in isolation, where the neutral species present in polypeptide chains are the most stable in detriment of ionic or zwitterionic forms. A supersonic expansion with a carrier gas is the preferred experimental approach as it provides the isolation conditions to determine the intrinsic conformational preferences[13–16]. Electronic spectroscopy,[17] can be used to investigate the conformational behaviour of α-amino acids and a variety of relevant biological molecules provided that they contain aromatic chromophores. Phenylalanine, tyrosine,[18] and tryptophan[19] meet this criterion, and they have been extensively studied by laser spectroscopic techniques. The only work in gas phase related to glutamine so far is a recent study of capped glutamine-containing peptides.[20] To date, there is no structural information of free glutamine, despite it is one of the most essential proteinogenic amino acids.

Pure rotational spectroscopy requires no chromophore and leads to accurate rotational parameters that can distinguish unambiguously between different neutral forms. Glutamine is solid with a high melting point (185 ºC), and its thermal instability prevents the vaporization by classical heating methods. Laser ablation techniques combined with Fourier transform microwave spectroscopy have boosted the investigation of solid proteinogenic[21] and non-proteinogenic amino acids.[22] However, the numerous attempts to acquire the rotational spectrum of glutamine so far proved unsuccessful due to the photofragmentation effects originated by the laser ablation process. Only the latest improvements of our laser ablation system[23,24] have finally led to the first rotational study of glutamine therein presented.

We have produced neutral glutamine using picosecond laser pulses in combination with two different chirped-excitation Fourier transform microwave spectrometers (LA-CP-FTMW)[25] to generate the broadband spectrum between 3 to 14 GHz, shown in Figure 1a. Most of the lines disappeared when the laser was turned off indicating the coexistence of various conformers of glutamine with enough population in the supersonic jet. The remarkable flexibility offered by glutamine due to its seven torsional degrees of freedom (see Scheme 1) results in a large manifold of plausible conformers close in energy. The polar amide group NH_2-CO-CH_2- side chain can originate intramolecular hydrogen bonds to the backbone amino and carboxylic groups affecting the conformational panorama.

Prior to the analysis of the rotational spectrum, we have performed an exhaustive conformational search on glutamine to assist in the identification of the conformational species present in the supersonic expansion. Hence, multiple molecular structures were tentatively screened using molecular mechanics methods. More than 100 structures were obtained within an energetic window of 2500 cm^{-1}. We focused our attention on the 18 lower-energy conformers within 1500 cm^{-1} which were optimized by *ab*



glutamine

Scheme 1. The sketch of the proteinogenic amino acid glutamine and its seven torsional degrees of freedom. N_t and N_c make reference to the terminal and central nitrogen atoms, respectively.

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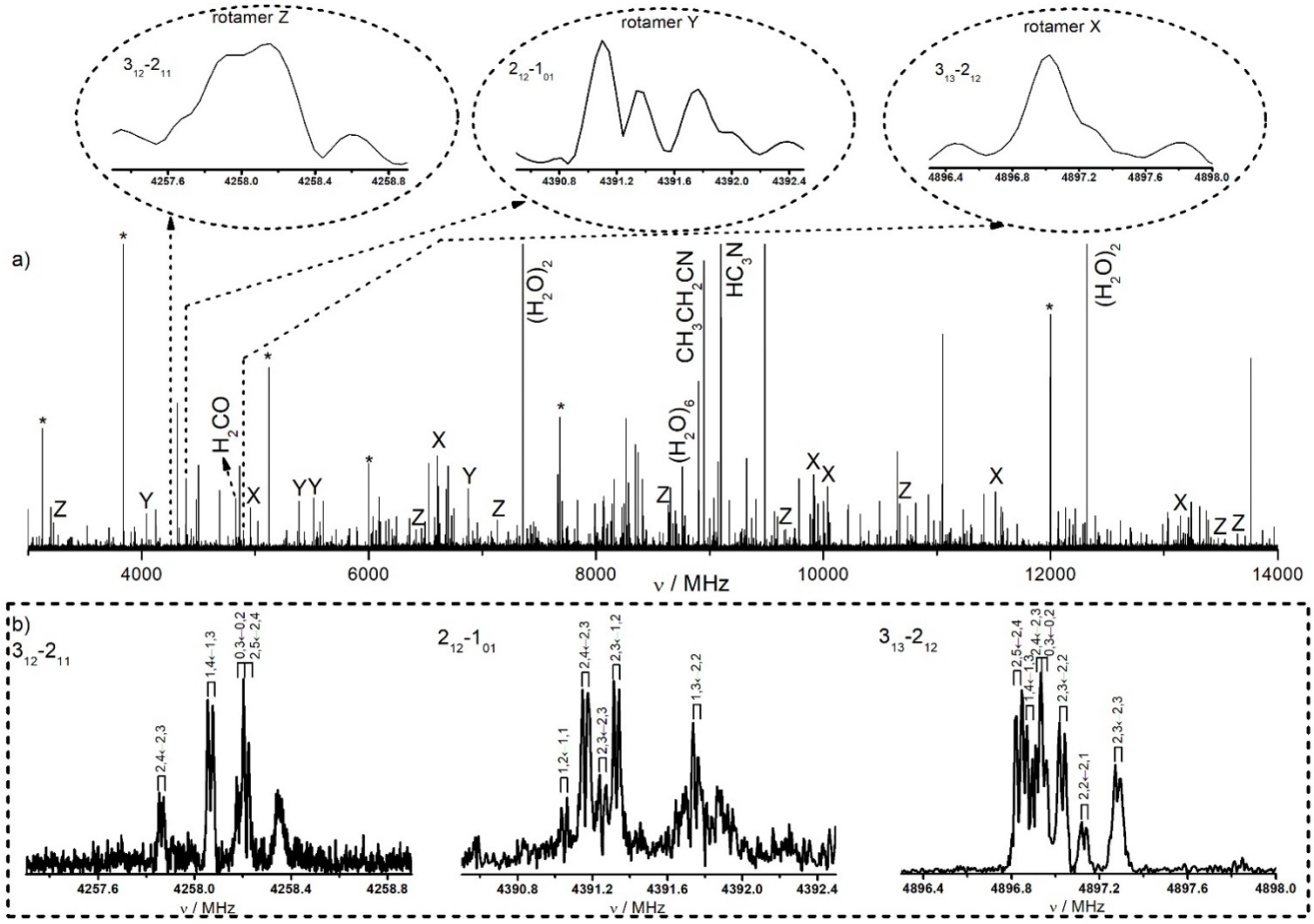

Figure 1. (a) The broadband LA-CP-FTMW rotational spectrum of glutamine indicating some of the transitions of the observed X, Y, and Z rotamers. Some photofragmentation products such as formaldehyde (CH_2O), cyanopolyynes, and water clusters are also marked. A star is used to indicate the electronic instrumental lines. The upper insets show some examples of individual transitions of the three detected conformers of glutamine not well resolved in the broadband spectrum. (b) The narrowband LA-MB-FTMW spectrum of the three above transitions highlighting the fully-resolved hyperfine structure. The nuclear quadrupole hyperfine components were analyzed using a semirigid rotor Hamiltonian complemented with a quadrupole coupling interaction term $H = H_R + H_Q$.[26] The H_Q Hamiltonian was set up in the coupled basis set (I_1I_2IJF), $I_1+I_2 = I$, $I+J = F$. The energy levels involved in each transition were thus labeled with the quantum numbers J, K_{-1}, K_{+1}, I, F.

initio MP2 methods using the 6-311++G(d,p) basis set. They are depicted in Fig. S01 of Electronic Supplementary information (ESI), and the essential spectroscopic ingredients to guide spectral assignments, namely rotational constants, nuclear quadrupole coupling constants, and electric dipole moment components are collected in Table S01. The predicted conformers have been labeled according to the intramolecular hydrogen bonds established between the amino acid and carboxylic moieties as I (N-H···O=C), II (N···H-O), and III (N-H···O-H), following the previous convention used for amino acids.[27] Suffixes a,b and c have been added attending to their increasing energy within each type.

All the plausible structures of glutamine are near-prolate asymmetric rotor with characteristic patterns of μ_a-type R-branch transitions. On this basis, the spectral analysis rendered the identification of three rotamers labeled as X, Y, and Z. μ_b-type transitions were also observed and measured for the last two species. As shown in the upper inset of Fig. 1a, all the assigned lines show a not well-resolved hyperfine structure rotational transitions due to the presence of two ^{14}N nuclei with electric quadrupole moment (I=1). It helped us to confirm that the assigned lines belonged to glutamine conformers. No attempt was initially made to analyze the quadrupole structure but rather the center of the lines was measured and fitted to a rigid rotor Hamiltonian[28] to give a preliminary set (A/B/C) of rotational constants (in MHz) for rotamer X (2100/848/806) Y (2190/884/734) and Z (2468/728/656). The unassigned lines remained in the spectrum could not be attributed to other glutamine conformers.

To ascertain which glutamine conformers are the carriers of the spectrum, we first compared the above experimental values with those computed using the MP2 method in Table S01 of the ESI. The conformational assignment is unequivocal for rotamers Y and Z; the experimental values correlate well with those predicted for conformers II_a and I_a, respectively. The observed line intensities are also consistent with the selection rules. Surprisingly, the rotational constants of rotamer X are not in good agreement with any of the calculated conformers. It should correspond, however, to conformer II_b, predicted as the second

Table 1 Experimental spectroscopic parameters obtained for the detected rotamers X, Y and Z of glutamine compared with those calculated by *ab initio* MP2/6-311++G(d,p) for the lowest energy conformers IIb, IIa and Ia (see Table S01 in the ESI for the rest of the calculated conformers).

	Rotamer X			Rotamer Y		Rotamer Z	
	II_b (MP2)	Experimental	II_b (B3LYP)	Experimental	II_a	Experimental	I_a
A[a]	2024	2099.82(18) [g]	2088	2190.4159(16)	2171	2467.7117(17)	2456
B	947	847.87013(35)	825	884.45682(36)	899	727.9450(25)	739
C	904	805.74201(28)	751	733.62565(53)	742	655.81021(18)	665
Δ_J	0.68	1.0650(63)	0.84	0.086(12)	0.10	0.1184(36)	0.09
Δ_{JK}	1.93	3.083(76)	4.91	---[h]	0.20	---[h]	0.17
$\lvert\mu_a\rvert$	1.5	observed	4.3	observed	2.2	observed	2.6
$\lvert\mu_b\rvert$	1.7	not observed	0.3	observed	2.4	observed	1.9
$\lvert\mu_c\rvert$	0.1	not observed	1.8	not observed	0.5	not observed	0.0
N_c/χ_{aa}	1.71	1.1000(34)	-0.52	-1.9242(67)	-1.93	-4.0823(89)	-4.25
N_c/χ_{bb}	-3.76	1.4471(64)	3.07	1.8699(90)	1.99	2.378(11)	2.48
N_c/χ_{cc}	2.04	-2.5469(64)	-2.55	0.0543(90)	-0.06	1.704(11)	1.73
N_t/χ_{aa}	-0.60	-0.1719(46)	0.37	-0.3546(60)	-0.39	-1.551(17)	-1.64
N_t/χ_{bb}	1.96	1.6357(85)	2.18	0.6527(69)	0.66	0.613(17)	0.66
N_t/χ_{cc}	-1.36	-1.4639(85)	-2.56	-0.2981(69)	-0.27	0.938(17)	0.99
σ[b]		3.4		2.8		2.9	
N[c]		93		66		42	
ΔE[d]	180		0		0		381
ΔE_{ZPE}[e]	49		0		0		244
ΔG[f]	0		0		104		139

[a]*A*, *B*, and *C* represent the rotational constants (in MHz); Δ_J and Δ_{JK} are the quartic centrifugal distortion constants (in kHz); μ_a, μ_b, and μ_c are the electric dipole moment components (in D); χ_{aa}, χ_{bb}, and χ_{cc}, are the diagonal elements of the ^{14}N nuclear quadrupole coupling tensor (in MHz); N_c and N_t correspond to the central and terminal ^{14}N nuclei, respectively. [b]RMS deviation of the fit (in kHz). [c]Number of measured transitions. [d]Relative energies (in cm^{-1}) with respect to the global minimum. [e]Relative energies (in cm^{-1}) with respect to the global minimum, taking into account the zero-point energy (ZPE). [f]Gibbs energies (in cm^{-1}) calculated at 298 K. [g]Standard error in parentheses in units of the last digit. [h]Not enough data for their determination. The values obtained using B3LYP/6-311++G(d,p) for structure II_b are also indicated.

most stable structure by MP2. This fact prompted us to extend our theoretical calculations to DFT methods. Interestingly, the experimental values of rotamer X are in between those calculated by MP2 and DFT methods (see Table 1) for the II_b conformer. The most pronounced structural difference is the C-C-C=O dihedral angle (see Figure 2, and all details in text and Figure S02 in the ESI), which varies from -39.5º predicted by MP2, to 10º obtained by B3LYP. Such a difference is connected with the N-H•••O=C interaction that seems to be not correctly described theoretically and affects the rotational constants and dipole moment components dramatically. We tried several common theoretical methods and basis sets, but none of the methods gives accurate rotational constants for structure IIb (see all details in the ESI and in Tables S02 to S05).

The $^{14}N_c$ and $^{14}N_t$ nuclei of the amine groups of glutamine have quadrupole moment (I=1), which interacts with the electric field gradient at the site of these nuclei resulting in a hyperfine structure for all the rotational transitions.[26] The nuclear quadrupole coupling constants $(\chi_{aa}, \chi_{bb}, \chi_{cc})$ extracted from its analysis provide information on the electronic environment of the nitrogen nuclei, as well as the orientations of the corresponding NH_2 groups. This information could be decisive in identifying the observed rotamers. We took advantage of the subdoppler resolution of the LA-MB-FTMW technique[29,30] to resolve the hyperfine structure completely (see Fig. 1c). A total of 66 and 42 hyperfine components were measured for rotamers Y and Z, respectively (see Tables S07 to S08 of the ESI). The fitted values of the quadrupole coupling constants are collected in Table I. A final assessment of the conformational assignments to the Ia and IIa conformers comes from the excellent agreement between the experimental and theoretical values of nuclear quadrupole coupling constants.

The analysis of the quadrupole hyperfine structure for rotamer X, on the other hand, resulted in an impossible task due to the structural uncertainties mentioned above, which significantly affects the predicted quadrupole coupling constants. We have addressed this complicated problem with a custom script that explores all the possible combinations of the quadrupole coupling constants. The predicted hyperfine patterns are compared with the experimental transitions until a proper matching is achieved (see all details in the ESI). A total of 93 hyperfine components (see Table S06 of the ESI) were assigned and fitted to give the accurate values of quadrupole coupling constants listed in Table 1. These values are between those predicted by MP2 and B3LYP, as stated above for the rotational constants. This further supports the initial assignment of rotamer X to conformer IIb. Its abnormal non-rigid behavior reflected in the high values of its centrifugal distortion constants, Δ_J *and* Δ_{JK}, can be attributed to a large amplitude motion involving the C-C-C=O dihedral angle (see Figure S02 of the ESI).

A first glance at the conformers shown in Figure 2 reveals that glutamine does not follow the conformational behavior demonstrated by the other proteinogenic amino acids studied so far.[27] In aspartic acid,[31] serine,[32] cysteine,[33] glutamic acid[34] or asparagine,[21] the side chain functional groups participate in stabilizing networks of strong intramolecular hydrogen bonding with the backbone. In contrast, no strong side-chain backbone interactions are apparent in glutamine conformers. Only conformer IIb presents a possible weak N_c-H···O=C interaction not well described theoretically. Contrary, only one conformer has been observed for asparagine, which is stabilized due to the cooperative effects of the intramolecular network formed by four hydrogen bonds.[21] The additional CH_2 unit in the carbon chain in going from asparagine to glutamine increases the flexibility of the amino acid drastically. This enhanced flexibility exhibited by

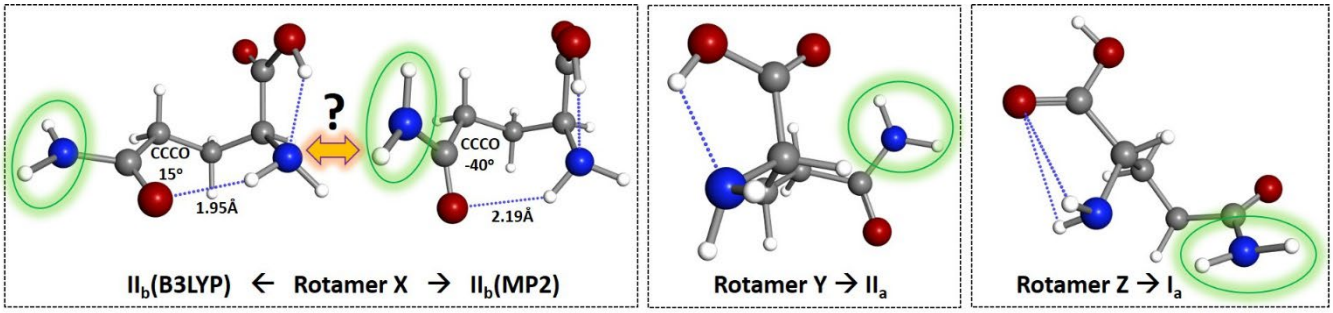

Figure 2. Comparison of the assigned structures using MP2. For structure II$_b$, the exact structure cannot be reproduced by any of the employed theoretical methods: while MP2 underestimates the N-H•••O=C interaction, B3LYP overestimates it (see text). Encircled in green is the terminal NH_2 group not involved in any strong intramolecular interaction.

glutamine could have important biological implications. For example, the flexible amide-containing side chain of glutamine repeats is believed to be responsible of joining specific transcription factors bound to separate DNA segments, by acting as polar-zippers.[35]

Another significant structural feature is that glutamine, unlike the rest of the aliphatic amino acids, always has an NH_2 group of the side chain free of any intramolecular interaction. Surprisingly, one of the crucial roles of glutamine is the nitrogen donation for many anabolic processes,[4,36] the regulation of acid-base balance in the kidney by producing ammonium,[36] or as a precursor to the neurotransmitter glutamate,[36] which is in fact provided by glutamine donating an NH_2 group.[37–39] The results in this work seem to indicate that this fact could be correlated with the unique structural property of glutamine due to having an "unprotected" NH_2 group. Additionally, the observation of the non-interacting side chain of glutamine is also in good agreement with recent results on the β-sheets in amyloid fibrils, in which it is thought that the sheets stack by zippering and hydrogen bonding the glutamine side chains.[40]

In summary, we have extracted the neutral proteinogenic glutamine from the solid by laser ablation to interrogate it by Fourier transform microwave techniques. The three unveiled neutral forms that are intimately connected to its biological functions, provide the first experimental information on the shape of this relevant amino acid. Noticeably, the amide side chain is not mainly involved in intramolecular interactions in right contrast with that observed for the other natural α-amino acids with polar side-chain. This singular structural behavior is intimately connected to its biological functions. Hence, it could explain the implications of glutamine as a nitrogen source and its unique ability to form a variety of hydrogen bonds with a peptide backbone in the surrounding. It is not surprising why evolution has selected certain amino acids for specific functions.

Noticeably, the accuracy of the conventional computational methods in predicting the structure and properties for individual stable conformers has been tested in glutamine. Due to the delicate balance of intramolecular interactions, neither *ab initio* MP2 nor DFT theoretical treatments are capable of predicting the structure of the most stable conformer of glutamine. The accurate spectroscopic parameters reported in this work will help to validate novel computational methods.

Acknowledgments

The financial fundings from Ministerio de Ciencia e Innovación (CTQ2013-40717-P and CTQ2016-76393-P), Junta de Castilla y León (Grant VA077U16) and the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013) / ERC-2013-SyG, Grant Agreement n. 610256 NANOCOSMOS, are gratefully acknowledged. E. R. A. thanks Ministerio de Ciencia e Innovación for FPI grant (BES-2014-067776) and I.L.O. thanks Junta de Castilla y León and Universidad de Valladolid for a postdoctoral contract.

Keywords: glutamine • FTMW spectroscopy • non-covalent interactions • amino acid • benchmark

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