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Conformation of the Neurotransmitter γ-Aminobutyric Acid in Liquid Water

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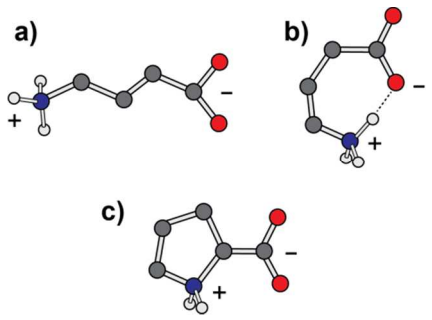
We study the conformation and reorientation dynamics of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) at neutral and acidic conditions using a combination of broadband dielectric relaxation spectroscopy and polarization-resolved femtosecond mid-infrared pump-probe spectroscopy. We find that both zwitterionic and cationic GABA adopt nearly linear conformations in aqueous solution, meaning that the two charged functional groups of the GABA zwitterion are hydrated separately.

γ-Aminobutyric acid ($COOH(CH_2)_3NH_2$, abbreviated GABA) is considered to be the major inhibitory neurotransmitter in the mammalian central nervous system.[1, 2] A deficiency of GABA therefore leads to a number of neurological disorders such as anxiety, insomnia, and depression as well as diseases like Parkinsonism and epilepsy.[3, 4] The molecule performs its biological function by binding to three classes of neuroreceptors; $GABA_A$, $GABA_B$ and $GABA_C$, distributed throughout the mammalian brain. Unfortunately, GABA does not penetrate the blood-brain barrier and thus cannot be administrated as a tranquilizing drug.[5] Novel compounds which are able to bind to GABAoid receptors and mimic functions of GABA are therefore continuously being developed.[6]

To design a suitable GABA analogue, a thorough understanding of the conformation of the molecule in physiological solution is needed. While neutral in the gas phase[7] the molecule predominantly forms zwitterions in both the crystalline phase[8] and in neutral aqueous solutions (see Scheme 1a).[9, 10] H^1 NMR and Raman spectroscopy studies have shown that a large number of possible GABA rotamers are equally populated in water;[9] a consequence of the molecule's flexible aliphatic backbone. Curiously though, no consensus has been reached concerning the nature of the molecule's dominant hydration patterns. More specifically, there is still substantial disagreement in the literature as to the importance of folded GABA conformations with an internal hydrogen bond between the NH_3^+ and COO^- group (see Scheme 1b).[11-13] From Hartree-Fock calculations, where the solvent was implicitly described using a continuum Onsager reaction field approach, Odai et al. concluded that extended forms of zwitterionic GABA are the only stable in aqueous solution.[11] Crittenden *et al.* further studied the hydration of GABA within the density functional theory (DFT) framework, using either polarizable continuum models or by an explicit description of the hydration in small clusters.[12] They found that folded conformers are dominantly present at low hydration levels, while unfolded conformations become more prominent with increasing number of added water molecules. Hence, they concluded that unfolded species dominate in the aqueous phase. In stark contrast, Song and Kang found in another DFT study that as much as 94% of the GABA molecules adopts a folded conformation in water forming intramolecular H-bonds between the COO^- and NH_3^+ functional groups.[13]



Scheme 1) *Molecular structures of a) extended zwitterionic GABA, b) folded and internally bonded zwitterionic GABA and c) zwitterionic L-proline.*

In this work we use two complementary experimental techniques, namely broadband dielectric relaxation spectroscopy (DRS) and polarization resolved femtosecond infrared spectroscopy (fs-IR) to probe the conformation and hydration dynamics of GABA in both neutral and acidic solutions. Aqueous zwitterionic L-proline is studied as a reference case, due to its structural similarity with folded GABA conformers (compare Scheme 1b and c). DRS probes the dipolar reorientation of all polar species (given that a sufficient frequency range is covered) in terms of the sample's complex permittivity $\hat{\varepsilon}(\nu) =$

$\varepsilon'(\nu) - i\varepsilon''(\nu)$.[14] Our broadband measurement (10 MHz – 90 GHz) allows us to single out the dielectric relaxation modes of both the solutes and the water solvent. Fs-IR is an excellent tool to study the reorientation dynamics of water molecules.[15-18] With this technique we measure the reorientation dynamics of an anisotropically excited subset of OD groups of HDO molecules in isotopically diluted water.

By simultaneously fitting the permittivity spectra and the fs-IR anisotropy dynamics, we can separate the contributions of water molecules showing bulk-like reorientation behavior and of water molecules that are slowed down in their reorientation due to interactions with the solute. With this information we can accurately determine the dipole moment and reorientation time of the solute from the DR data. Since the dipole moment of a zwitterionic molecule strongly depends on the distance between the charged groups, we can relate the dipole moment directly to the conformation of the solute in aqueous solution.

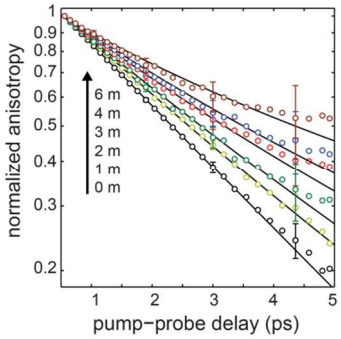


Figure 1) *Anisotropy decay (logarithmic vertical scale) of the bulk OD vibrational band of HDO in H_2O solutions of zwitterionic GABA at 1,2,3,4 and 6 m concentrations. For visual clarity error bars are only showed for selected pump-probe delays.*

Figure 1 shows measured anisotropy decays (circular markers) of the OD stretch vibration (centered at 2500 cm^{-1}) of HDO molecules for solutions of zwitterionic GABA in HDO:H_2O, at concentrations ranging from 1 to 6 molal (m). For comparison, we also include the anisotropy decay of the OD stretch vibration of HDO molecules in neat HDO:H_2O, showing a characteristic mono-exponential decay with a time constant of 2.5±0.1 ps.[15] These curves were obtained according to the experimental and data analysis procedures described in detail in the Electronic Supplementary Information (ESI). The anisotropy decay curves are proportional to the second Legendre polynomial of the microscopic orientational correlation function,[19] thus providing direct information on the reorientation of the water molecules in the sample. As has been previously observed, the solvation of amphiphilic molecules causes a distinct slowdown of the reorientational dynamics of a sub-ensemble of water molecules.[20, 21] Accordingly, the solid lines represents least-square fits to a bi-exponential function, fitted in conjunction with the dielectric permittivity spectra shown in Fig. 2. DRS, in contrast to fs-IR, measures a collective relaxation time that is similar to the first Legendre polynomial of the orientational correlation function.[22] Based on previous DR and fs-IR experiments, we multiply the relaxation time constants obtained by fs-IR by a factor of ~3.4 to enable a direct comparison of the water reorientation dynamics measured with the two techniques[23,19, 22, 24] The details of the combined fit model are given in the ESI (section ESI.4). From the combined fits to the fs-IR and DRS data we find that both the number of slowed-down water molecules and the associated reorientational time constants increase with solute concentrations (see Fig. ESI.5). The reorientation time increases from ~20 to 35 ps when the GABA concentration is increased from 1 to 6 m.

The leftmost panels in Fig. 2 show complex permittivity spectra of aqueous solutions of 1-6 m zwitterionic GABA. Panel a) gives $\varepsilon'(\nu)$, the in-phase contribution to the polarization while the middle panel b) gives the corresponding out-of-phase contribution $\varepsilon''(\nu)$. The colored markers give experimental data points while the solid black lines represent least-

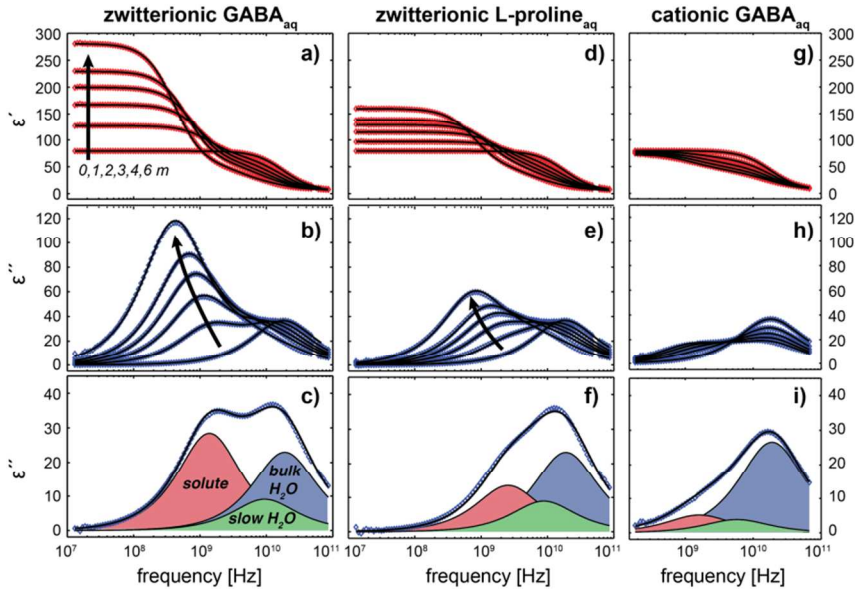


Figure 2) *Complex permittivity spectra of zwitterionic GABA (left panel), zwitterionic L-proline (middle panel), both at 1-6 m concentrations, as well as cationic GABA (right panel) at 1-4 m concentrations. For the latter solutions a term $i\sigma/\varepsilon_0 2\pi\nu$, arising due to the ionic conductivity σ, has been subtracted for visual clarity. For comparison, permittivity spectra of pure water are shown together with all samples. The solid lines represent fits to the relaxation model described in the ESI. The decomposition of $\varepsilon''(\nu)$ into a solute mode, a slow water mode and a bulk water mode is shown in the bottom row panels for 1m solutions.*

square fits to the fit model consisting of a sum of three Debye relaxation modes pertaining to the solute, slow water and bulk-like water, respectively. In the bottom panel c) we show the decomposition of the fit to $\varepsilon''(\nu)$ at 1 m GABA concentration, explicitly separating the contributions from the three separate relaxation modes, together with the total resulting fit.

As is directly evident from the raw data, and explicitly shown in the decomposition in panel c), the dissolution of GABA results in the appearance of a strong low-frequency mode (τ_{GABA}= 110 ps at 1 m) that red-shifts with increasing concentration. This is indicative of a general slowdown of the solution dynamics. The addition of GABA to the solution leads to a strong increase of the dielectric response, e.g. 6 m GABA has a dielectric constant ε_S (i.e. $\hat{\varepsilon}(\nu \to 0)$) of ~280, which is more than three times that of pure water (~80).[25] Using the Cavell equation the dielectric molecule's dipole moment, μ_{GABA}, can be computed from the dielectric strength S_{GABA};[26] the results are given as filled red circular markers in Fig. 3 (see ESI for details). The dipole moment show only a minor (negative) concentration dependence, in agreement with the findings of a recent systematic DRS study of aqueous zwitterionic amino acids.[27] From a linear fit (solid line) the dipole moment at infinite dilution can be estimated to $\mu_{GABA}^{c\to 0} = 25.8 \pm 0.3$ Debye, a high value for such a small molecule.

In order to relate these results to the conformation of zwitterionic GABA we make an experimental comparison with a molecule whose shape is similar to GABA in its folded state, namely L-proline (Scheme 1c). Being one of the 21 protein encoding amino acids L-proline is also predominately a zwitterion in neutral aqueous solutions. It is therefore expected that the dipole moment of folded GABA conformers and L-proline are similar (compare Schemes 1b and c). The dielectric data measured for zwitterionic L-proline at 1-6 m concentration are given in the center panels d-f) of Fig. 2. Our combined fit approach of the fs-IR and DR data again yield excellent results. The most striking difference between the data for zwitterionic GABA and L-proline is the far weaker dielectric response of the solute in the latter case, reflecting a pronounced difference in dipole moment between the two molecules in aqueous solution. From a fit of the extracted dipoles, given in Fig. 3, we find that $\mu_{L-proline}^{c\to 0} = 17.9 \pm 0.4$ Debye, in good agreement with the value reported in Ref[27]. This value is significantly smaller than $\mu_{GABA}^{c\to 0}$, which indicates of that the NH_3^+ and COO^- functional groups are much more separated in aqueous zwitterionic GABA than in L-proline.

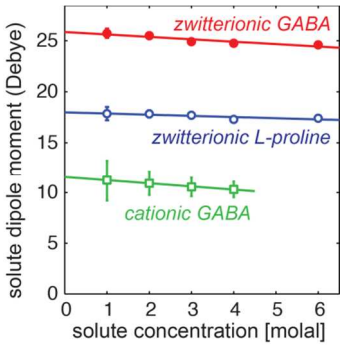


Figure 3) *Extracted dipole moments for zwitterionic (red) and cationic (green) GABA, as well as zwitterionic L-proline (blue) from the fits to the data shown in Fig. 2.*

Crittenden *et al.* calculated dipole moments of a number of low-energy conformations of aqueous zwitterionic GABA on the B3LYP/6-31+G* level of theory.[12] The calculated values fall in two distinct classes; for conformations with intramolecular hydrogen-bonds between the NH_3^+ and COO^- groups they report values around 16.5 Debye. This value is close to what we observe for L-proline, and supports the conjecture that the dipole moment of GABA in a folded conformation is similar to that of L-proline. In contrast, for extended structures of GABA where the ionic groups are individually hydrated, the calculated values are around 25 Debye. This is in excellent agreement with the here determined experimental value $\mu_{GABA}^{c\to 0} = 25.8 \pm 0.3$ Debye. We thus find strong evidence that GABA in aqueous solution is predominantly present in the form of an unfolded zwitterionic conformer.

In order to further investigate the charge state dependence of the GABA conformation we have performed analogous experiments on the cationic form ($GABA^+$): Fig. 2 g-i) shows fitted permittivity spectra of solutions at 1-4 m concentration, acidified to pH = 1.8 using perchloric acid (details described in ESI). The net positive charge of $GABA^+$ is localized on the ammonium group, which causes the ion to possess an appreciable dipole moment, reflected in the concentration dependent ingrowth of a low-frequency mode around 1 GHz. As seen in Fig. 3, fits to the experimentally obtained dipole moment yields $\mu_{GABA+}^{c\to 0} = 12 \pm 1$ Debye. Interestingly, this value is about half of that of the zwitterion, which indicates that $GABA^+$ and zwitterionic GABA possess a similar unfolded conformation. Since the charges of zwitterionic GABA is located on the terminal groups, nearly symmetrically with the respect to the rotational center of mass, the mere neutralization of the carboxylate group (without any conformational change) will reduce the dipole moment by a factor of 2, as is indeed observed.

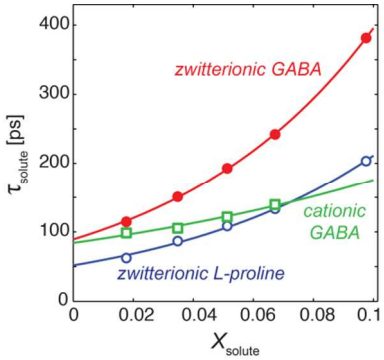


Figure 4) *Measured solute reorientation times of zwitterionic and cationic GABA, as well as zwitterionic L-proline as a function of solute molar fraction, X_{solute}.*

Finally, we will analyze the solute dipolar relaxation time pertaining to the three species here investigated; the data are given in Fig 4. Following the approach in Ref.[27] we find that the data can be excellently fitted to the functional form $\log(\tau_{solute}) = A \times X_{solute} + B$, where X_{solute} is the solute molar fraction (fit parameters given in ESI table 1). We can thus extract the solute reorientation time at infinite dilution, $\tau_{solute}^0 = \exp(B)$. This parameter can be related to the microscopic properties of the rotating solutes using the modified Stokes-Einstein-Debye theory:[22, 28] $\tau_{solute}^0 = 3V_{eff}\eta/(k_BT)$, where V_{eff} is the effective volume occupied by the solute and η is the solution viscosity. At infinite dilution η is equal to that of

the solvent. Hence, an observed difference in τ^0_{solute} between two solutes must therefore, within this theoretical framework, exclusively be due to a corresponding difference in the effective volumes of the two.

The reorientation times of GABA in its two charge state are nearly identical ($\tau^0_{GABA} = 89 \pm 3$ ps and $\tau^0_{GABA+} = 84 \pm 3$ ps) while the value for L-proline is substantially smaller; $\tau^0_{L-Proline} = 52 \pm 3$ ps. The solute's effective volume of rotation can be related to the actual molecular volume V via $V_{eff} = V \cdot f \cdot C$.[28] Here C gives the hydrodynamic boundary conditions ($C_{stick} = 1$ and $C_{slip} = 1 - f^{-2/3}$ representing the extreme values) whereas f is a shape parameter. For spheres $f = 1$, while the value increases nearly linearly with the aspect ratio of prolate spheroids.[29] Since the molecular volume of GABA and L-proline are similar, the difference in τ^0 must primarily originate from a change in the product $f \cdot C$. The value of $f \cdot C$ for L-proline has previously been determined to be ~0.5.[27] The value for GABA here obtained is ~0.9, which is significantly larger than what was found for most small amino acids (~0.6) whose geometry can be reasonably well estimated as spherical. Larger $f \cdot C$ values, observed e.g. for lysine (~1) and argenine (~1.1), were interpreted by Rodríguez-Arteche *et al.*[27] as originating from the increasing aspect ratio of these larger amino acids. The solute reorientation time constants are thus consistent with the observed solute dipole moments: zwitterionic GABA is extended in solution (lacking intramolecular bonding between the charged functional groups), and thus give rise to a considerably larger aspect ratio than zwitterionic L-proline, which is folded.

Conclusions

We have studied the dielectric response and molecular reorientation dynamics of aqueous solutions of zwitterionic and cationic GABA, and of zwitterionic L-proline. Both the measured dipole moment and the solute reorientation time constant show that both zwitterionic and cationic GABA adopt nearly linear, unfolded conformation in aqueous solution, meaning that the COO^- and NH_3^+ groups of the zwitterion are separately hydrated. This result is in excellent agreement with the theoretical study by Crittenden *et al.*[12] This work addresses a longstanding problem related to neural biochemistry, and demonstrates the advantages of combining fs-IR and DRS to detect the molecular conformation and reorientation dynamics of (bio)molecules and (bio)molecular ions in aqueous solution.

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Notes and references

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Experimental details

Aqueous solutions of GABA and L-proline were prepared at concentrations between 1 and 6 molal (m) by mixing demineralized water with appropriate amounts of high-purity chemicals (Sigma-Aldrich, >99.9%), which were used without further purification. In the DRS measurements pure water (Milli-Q, ≥18.2 MΩ cm) was used as a solvent whereas a mixture of 5% (w/w) D_2O (Aldrich, >99.99% D-atoms) and 95% (w/w) H_2O was used in the fs-IR experiments. Solutions of cationic $GABA^+$ were additionally prepared by acidifying the solution to pH = 1.8 with perchloric acid, $HClO_4$. With $pK_a = 4.23$ and of GABA,[30] this means that the cationic form was the overwhelmingly dominant species (>99.6%).

We measured permittivity spectra of all samples at a temperature of 22.5 ± 0.5 °C over the frequency range 10 MHz – 90 GHz by means of a vector network analyzer (VNA, Rhode-Schwartz model ZVA67).[31] To cover such a broad frequency range three different types of sample cells were employed, based on either coaxial line reflectrometry or free-space microwave propagation in a waveguide (see ESI for details).

The fs-IR experiments were performed using mid-infrared femtosecond laser pulses which were obtained in a number of conversion steps using the output of a Ti:Sapphire laser (Hurricane, Spectra-Physics). The majority of the resulting 2500 cm^{-1} pulses (4 μJ, 150 fs) was used to excite the OD stretch vibration in a fraction of the HDO molecules in isotopically dilute water to their first excited state. The transient absorption changes in the polarization plane both parallel and perpendicular to the polarization of the pump pulses were alternatingly probed with a weak (200 nJ) pulse, which was delayed relative to the pump pulse by an optical delay stage (see ESI for details).

Electronic Supplementary Information (ESI) available: Additional information on experimental details, analysis and modeling. See DOI: 10.1039/c000000x/

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23. If dipolar correlations between neighboring molecules are negligible it can be shown that the Debye relaxation τ_D time measured with DRS is identical to the first order microscopic reorientation time $\tau_r^{(1)}$. Furthermore, assuming diffusive reorientational dynamics it can be shown that the relaxation time of the l:th dipolar correlation function is $\tau_r^{(l)} = (D_{rot} l(l+1))^{-1}$, where D_{rot} is the rotational diffusion constant. Under these conditions the following identity thus holds: $\tau_D = \tau_r^{(1)} = 3\tau_r^{(2)}$. In liquid water dipole-dipole correlations are however non-negligible and reorientation is rather facilitated by large-angular jumps than by rotational diffusion [D. Laage & J. T. Hynes, Science, 2006, 311, 832-835]. As a result of these effects the relation between the time constants determined in our DRS and fs-IR experiments are instead $\tau_D \approx 3.4\ \tau_r^{(2)}$.
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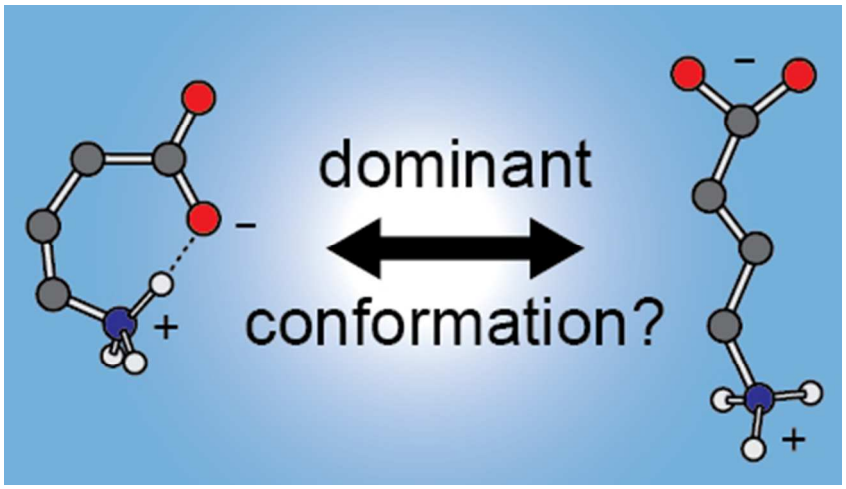

A combined dielectric and mid-IR pump-probe spectroscopic study reveals zwitterionic GABA to exist in predominately extended conformations in liquid water.