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Water Proton NMR Detection of Amide Hydrolysis and Diglycine Dimerization

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ELECTRONIC SUPPLEMENTARY INFORMATION

Experimental

Sample preparation

Dry powders of glycine (Gly), N-acetylglycine (Ac-Gly), glycinamide (Gly-am), diglycine (Gly-Gly), *L*-glutamic acid (Glu), *L*-glutamine (Gln) and *L*-pyroglutamic acid (pGlu) were purchased from Sigma-Aldrich (St. Louis, MO). Each compound was dissolved in 1x phosphate buffered saline (PBS) from a 10x stock (1.0 M NaCl, 0.5 M NaH_2PO_4, adjusted to pH 7.4 with concentrated NaOH solution). The pH of the stock solutions of compounds was adjusted to pH 7.4 and then each solution was 1:1 (v:v) serially diluted with 1x PBS buffer for a total of 6 concentrations per compound. Concentrations for the Gly-derivatives, including Gly, Ac-Gly, Gly-am and Gly-Gly are 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/mL (see corresponding molar concentrations in Table S1). Concentrations the Glu-derivatives, including Glu, Gln and pGlu, are 8, 4, 2, 1, 0.5 and 0.25 mg/mL (Table S1). The Glu-series have lower concentrations than the Gly-series because they have lower solubility in water. NMR samples were pipetted into 3-mm NMR inner tubes (Norell®; Morgantown, NC), which were inserted into 5-mm NMR outer tubes (Wilmad-LabGlass; Vineland, NJ) containing cyclohexane-d_{12} (Sigma-Aldrich; St. Louis, MO) for the lock signal. This sample-in-inner-tube/standard-in-outer-tube setup avoids mixing the sample with the NMR standard and avoids expusing the sample to deuterated solvents.

Nuclear magnetic resonance experiments

Water proton transverse relaxation rate measurements

Measurements were made on a Varian 400 MR NMR spectrometer (Agilent, Inc. Santa Clara, CA) (9.4 T; 399.75 MHz for ^1H) with a 2-channel broadband multi-nuclei probe. A Carr-Purcell-Meiboom-Gill (CPMG)[1] pulse sequence was used following previous methods[2], with the exception of the flip angle, typically 90°, was instead set to ~8.5° so as to prevent radiation damping due to high intensity water proton signal. The NMR sample temperature was regulated at 22°C. Signal intensities were processed using SpinWorks 4.2 freeware from University of Manitoba (Winnipeg, MB, Canada), and final data fitting was performed with Origin 8.1 Software from OriginLab Corporation (Northampton, MA) to a single exponential[3] to obtain the T_2 and error of the fit, using the following equation:

$$I(t) = I_0 \cdot \exp(-t/T_2) \quad (1)$$

The transverse relaxation rate R_2 in s^{-1} is then calculated from the transverse relaxation time T_2 in seconds as $R_2 = \frac{1}{T_2}$.

Pulsed Field Gradient Diffusion Measurement

A bipolar pulse longitudinal eddy current delay method was used for pulsed-field gradient (PFG) diffusion measurements.[4] The ^1H_2O signal intensity in three samples (Gly in 1x PBS, Gly-Gly in 1x PBS, and 1x PBS) was measured as a function of gradient strength. In addition, the $-C^1H_2-$ signal intensity in Gly and Gly-Gly solutions was measured as a function of gradient strength as a measure of the solute (Gly or Gly-Gly) diffusion.

The diffusion coefficient was extrapolated from the fit of intensity integrals of either ^1H_2O or $-C^1H_2-$ in Gly and Gly-Gly:[5]

$$I(G_z) = I_0 \cdot \exp[(-\gamma\delta G_z)^2 \cdot (\Delta - \delta/3) \cdot D] \quad (2)$$

where γ is the gyromagnetic ratio of ^1H (42.576 MHz/Tesla for ^1H); Δ corresponds to the diffusion interval (100 ms for ^1H_2O and 200 ms for CH_2 ^1H) and δ to the length of the PFG pulse (4 ms for both ^1H_2O and CH_2 ^1H); G_z is the gradient strength and I_0 is the initial intensity of the signal, and D is the diffusion coefficient. These measurements were made on a Varian MR Direct Drive Console equipped with a two-channel, 5 mm broadband probe. Data were processed using the software programs VNMRJ 3.0 and SpinWorks 4.2, and were plotted and fit in the software program Origin 8.1.

Table S1. Solute Concentrations of NMR Samples

Solute	Sample #	[mg/mL]	[mM]	Solute	Sample #	[mg/mL]	[mM]
Gly	1	25.00	333.02	**Glu**	1	8.00	54.38
	2	12.50	166.51		2	4.00	27.19
	3	6.25	83.26		3	2.00	13.60
	4	3.13	41.63		4	1.00	6.80
	5	1.56	20.81		5	0.50	3.40
	6	0.78	10.41		6	0.25	1.70
Gly-am	1	25.00	226.16	**Gln**	1	8.00	54.75
	2	12.50	113.08		2	4.00	27.38
	3	6.25	56.54		3	2.00	13.69
	4	3.13	28.27		4	1.00	6.84
	5	1.56	14.14		5	0.50	3.42
	6	0.78	7.07		6	0.25	1.71
Ac-Gly	1	25.00	213.49	**pGlu**	1	8.00	61.96
	2	12.50	106.75		2	4.00	30.98
	3	6.25	53.37		3	2.00	15.49
	4	3.13	26.69		4	1.00	7.75
	5	1.56	13.34		5	0.50	3.87
	6	0.78	6.67		6	0.25	1.94
Gly-Gly	1	25.00	189.25	**NH_4^+**	1	2.89	54.38
	2	12.50	94.63		2	1.44	27.19
	3	6.25	47.31		3	0.72	13.60
	4	3.13	23.66		4	0.36	6.80
	5	1.56	11.83		5	0.18	3.40
	6	0.78	5.91		6	0.09	1.70
Acetate	1	25.00	416.32	**Glu + NH_4^+***	1	8.00	54.38
	2	12.50	208.16		2	4.00	27.19
	3	6.25	104.08		3	2.00	13.60
	4	3.13	52.04		4	1.00	6.80
	5	1.56	25.98		5	0.50	3.40
	6	0.78	12.99		6	0.25	1.70
Gly + Acetate*	1	25.00	333.02				
	2	12.50	166.51				
	3	6.25	83.26				
	4	3.13	41.63				
	5	1.56	20.81				
	6	0.78	10.41				

*Mixed at 1:1 molar ratios.

Table S2. Transverse relaxivities of reactants and products of amide hydrolysis (in descending order).

Compound	r_2 $(mM)^{-1} \cdot s^{-1}$ (x 10^{-3})	R^2
Glu	41.37	0.99919
Gly	16.57	0.98133
Gln	20.63	0.99838
Gly-Gly	2.70	0.99886
Gly-am	2.09	0.98949
Ac-Gly	0.25	0.92097
pGlu*	—	—

The measurements of $R_2(^1H_2O)$ for each compound at six different concentrations was fit to a straight line where the slopes, r_2, are tabulated with the fit quality of linear regression, R^2.

*The $R_2(^1H_2O)$ data for pGlu are essentially flat, no quality linear fit was possible.

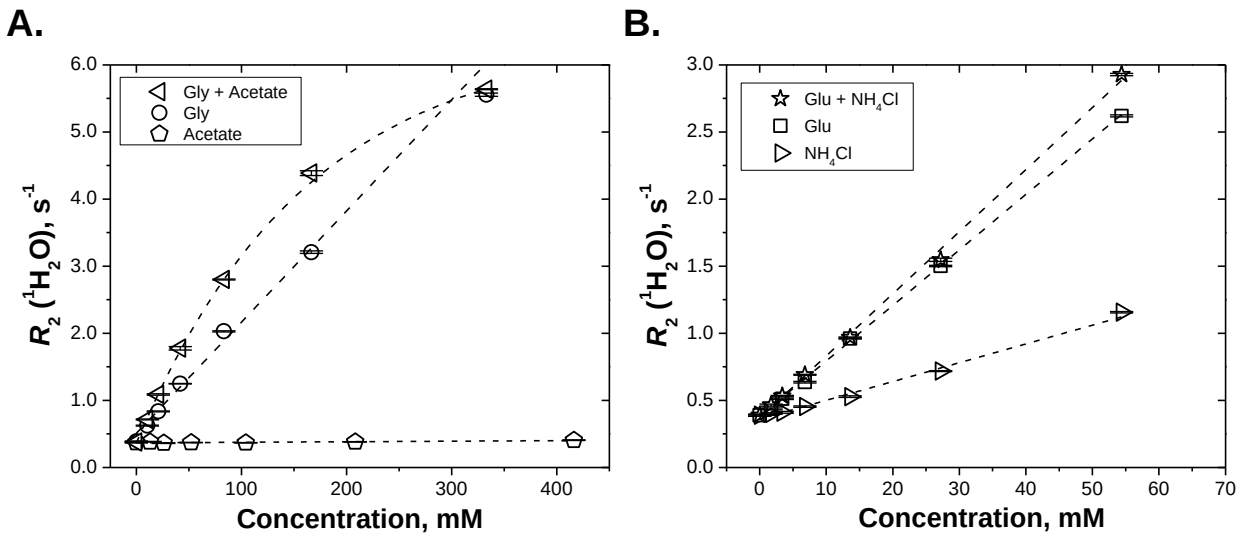

Figure S1. R_2 (^1H_2O) Contribution of Ammonium and Acetate
R_2 (^1H_2O) data are shown as a function of concentration of compounds. **A.** Gly (circle), acetate (pentagon), Gly + acetate (left triangle). The non-linearity of R_2(^1H_2O) of the Gly + acetate sample suggests a concentration-dependent interaction between Gly and acetate. **B.** Glu (square), NH_4Cl (right triangle), Glu + NH_4Cl (star).

Methods: Calculation of self-association of Gly-Gly

The self-diffusion coefficient, *D*, of a diffusant relates to its radius, *r*, through the Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta r} \tag{3}$$

where *k* is the Boltzmann constant, *T* is the absolute temperature, and η is the viscosity coefficient of the solution. Here, the diffusant shape is treated as spherical, which is a reasonable approximation for diffusants that are not fibers or sheets.[6] To eliminate η, we normalize the diffusant diffusion coefficient *D* by the water diffusion coefficient $D(^1H_2O)$, i.e.,

$$D^{\text{norm}} = \frac{D}{D(^1\text{H}_2\text{O})} = \frac{r(^1\text{H}_2\text{O})}{r} \tag{4}$$

For this reason, both *D* and $D(^1H_2O)$ are measured by PFG NMR for each solution. Note that D^{norm} is a dimensionless parameter of the ratio of *D* to $D(^1H_2O)$. When a molecule self-associates in solution, its diffusion will slow down, leading to smaller *D* and D^{norm}. To compare the aggregation status of two solutes, we take the ratio of D^{norm} of the two solutes:

$$\frac{D_2^{\text{norm}}}{D_1^{\text{norm}}} = \frac{r_1}{r_2} \tag{5}$$

Denote the monomeric molecular weight of solutes 1 and 2 respectively as m_1 and m_2, and the aggregation number of solutes 1 and 2 respectively as n_1 and n_2. The molecular weight of the two possibly associated diffusants are then n_1m_1 and n_2m_2, respectively. The radius of a diffusant r_1 or r_2 is proportional to the cubic root of its molecular weight, therefore,

$$\frac{D_2^{\text{norm}}}{D_1^{\text{norm}}} = \sqrt[3]{\frac{n_1m_1}{n_2m_2}} \tag{6}$$

Rearrange eqn. (4), the ratio of the two aggregation numbers is then given by:

$$\frac{n_2}{n_1} = \frac{m_1}{m_2} \times \left(\frac{D_1^{\text{norm}}}{D_2^{\text{norm}}} \right)^3 \tag{7}$$

Table S3 lists the values of D_1, $D_1(^1H_2O)$, D_1^{norm} for the Gly solution and m_1 for Gly, and D_2, $D_2(^1H_2O)$, D_2^{norm} for the Gly-Gly solution and m_2 for Gly-Gly.

Table S3. Diffusion Coefficients of Gly, Gly-Gly and ^1H_2O at 22°C

Diffusant	D, $m^2 \cdot s^{-1}$ (x 10^{-10})	$D(^1H_2O)$, $m^2 \cdot s^{-1}$ (x 10^{-10})	D^{norm}	m, g/mol
1.) Gly	9.83	20.80	0.473	75.07
2.) Gly-Gly	6.91	20.50	0.337	132.12

$D(^1H_2O)$ of pure water at is 17.77 x 10^{-10} $m^2 \cdot s^{-1}$ at 15°C and 22.99 x 10^{-10} $m^2 \cdot s^{-1}$ at 25°C.[7] Assuming $D(^1H_2O)$ grows linearly with the temperature between 15°C and 25°C, this gives a pure water $D(^1H_2O)$ value of ~21.42 x 10^{-10} $m^2 \cdot s^{-1}$ at 22°C. $D(^1H_2O)$ values that we measured at 22°C were from solutions containing 25 mg/mL solute (Gly or Gly-Gly) and therefore should be slightly lower than that of pure water. This is indeed the case. The values of the self-diffusion coefficient, D, of each diffusant, 25 mg/mL Gly or Gly-Gly, the diffusion coefficients of each solution, $D(^1H_2O)$, and the ratio of D to $D(^1H_2O)$, D^{norm}, as well as each diffusant's monomeric molecular weight value, m, in g/mol are tabulated in Table S3. All PFG Diffusion experiments were measured on a 400 MHz NMR at 22 °C.

From these, we can calculate

$$\frac{n_2}{n_1} = \frac{m_1}{m_2} \times \left(\frac{D_1^{norm}}{D_2^{norm}} \right)^3 = \frac{75.07}{132.12} \times \left(\frac{4.73}{3.37} \right)^3 = 0.568 \times 2.765 = 1.57 \quad (8)$$

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