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Electronic Supporting Information (ESI)

A diffusion NMR method for the prediction of weight-average molecular weight of globular proteins in aqueous media of different viscosity

Francisco M. Arrabal-Campos, Luis M. Aguilera-Sáez, Ignacio Fernández*

Department of Chemistry and Physics, Research Centre CIAIMBITAL, University of Almería, Ctra. Sacramento, s/n, 04120, Almería (Spain).

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Samples preparation and reagents.

A series of globular proteins (ubiquitin, lysozyme, equine myoglobin, β-chymotrypsinogen, α-lactoglobulin, ovalbumin and albumin) were analyzed. One mg of protein was dissolved in 0.5 mL of D_2O, with HDO as a diffusion reference. Protein concentrations were determined by weight and were not controlled spectrophotometrically. All globular proteins were purchased from Sigma-Aldrich and used after dialysis in D_2O (16 hours) for contaminant removal.

NMR spectroscopy.

All ^1H NMR measurements were performed on a Bruker Avance III 500 spectrometer equipped with a microprocessor-controlled gradient unit and a third radiofrequency channel using an indirect 5 mm TBI $^1H/^{31}P$/BB triple probe with an actively shielded Z-gradient coil. Tetramethylsilane was used as spectral reference. The shape of the gradient pulse was sinusoidal, and its strength varied automatically during the experiments. The calibration of the gradients was carried out via a diffusion measurement of HDO in D_2O at room temperature, where we get the slope value m_{HDO}. The D value of each protein sample was calculated according to the equation 1, where $D_{HDO} = 1.9\ 10^{-9}\ m^2\ s^{-1}$.[1]

$$D_{P1-P6} = \frac{m_t}{m_{HDO}} D_{HDO} \quad (1)$$

The m_t parameter has been calculated for each measurement with the help of the equation 2, which takes into account the settings for the diffusion experiment (delay Δ, gradient length δ, as well as the nature of the observed nucleus $\gamma_{P1\text{-}P6}$) with respect to those used in the calibration with HDO.

$$m_t = \frac{P1-P6\ slope}{\left(\frac{\gamma_{P1-P6}}{\gamma_{HDO}}\right)^2 \left(\frac{\delta_{P1-P6}}{\delta_{HDO}}\right)^2 \frac{\left(\Delta - \frac{\delta}{4}\right)_{P1-P6}}{\left(\Delta - \frac{\delta}{4}\right)_{HDO}}} \quad (2)$$

The Δ values varied from 100 to 200 ms and the δ values from 5.0 to 7.5 ms. The δ was incremented in steps of 4%, so that 23 points were used for the regression analysis. The recovery delay was set to 10 s. The number of scans per increment were set to 256, giving rise to experimental times of 12 h. All experiments were run without spinning to avoid convection. The standard Bruker pulse program stegp1s (which uses a stimulated echo sequence with monopolar pulses and 1 spoil gradient) was employed. The gradient recovery delay was set to 0.1 ms. To check the reproducibility of the method and the lack of convection, three different measurements with different Δ parameters were always carried out. LMS fittings were processed using the DiffAtOnce® software package available at www.diffatonce.com.[2] *D* values reported herein were extracted from linear-squared fittings-whose correlation coefficients were above 0.99. ITAMeD solutions were obtained by the use of the algorithm provided by Urbanczyk *et al*.[3] The number of iterations was set to 10,000 and the sparsity-promoting λ to 10^{-5}. TRAIn solutions were obtained by the use of the algorithm provided by Xu and Zhang,[4] the number of iterations was set to 250

and the sparsity-promoting alpha to 1.02. NNLS calculations were performed using the built-in MATLAB function *nnls*.[5]

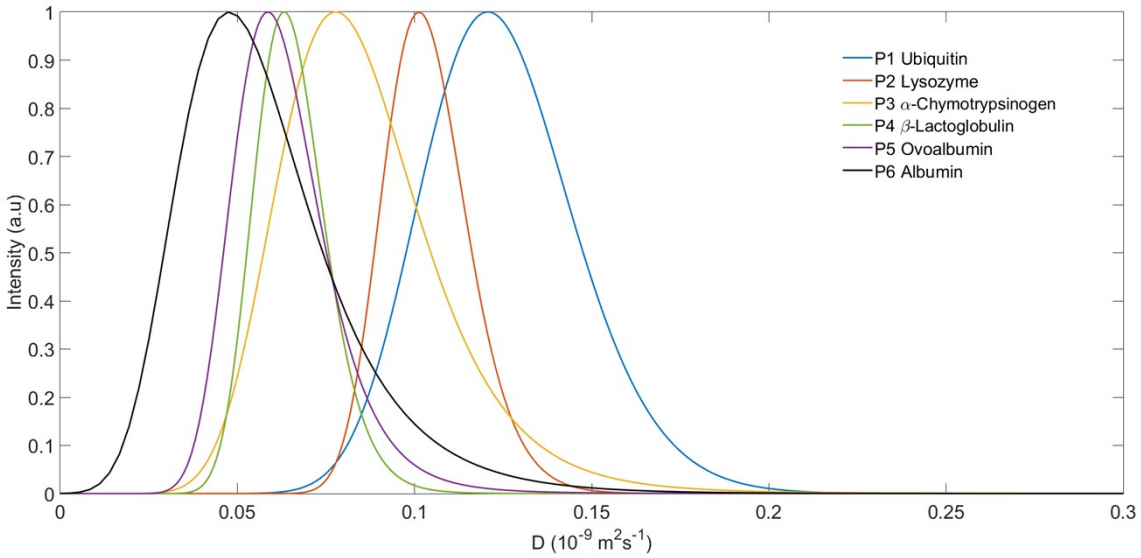

Figure S1. TRAIn solutions for proteins P1 to P6 in D_2O (viscosity of 0.9740 cP).

$$r_H = \frac{kT}{6\pi\eta D}$$

$$\eta = \frac{kT}{6\pi\, r_H TSP\, D}$$

$$D = \frac{m_t}{\left(\frac{\gamma_{TSP}}{\gamma_{HDO}}\right)^2 \left(\frac{\delta_{TSP}}{\delta_{HDO}}\right)^2 \frac{\left(\Delta - \frac{\delta}{4}\right)_{TSP}}{\left(\Delta - \frac{\delta}{4}\right)_{HDO}}} * \frac{1}{m_{HDO}} * D_{HDO} = \frac{2.913}{5.528} * \frac{1}{1.8875} * 1.902$$

$$D = \ 0.5582\ 10^{-9} m^2 s^{-1}$$

$$r_H TSP = \frac{kT}{6\pi\eta D} = \frac{1.38 * 295}{6 * \pi * 0.9740 * 0.5582} = 3.97\ \text{Å}$$

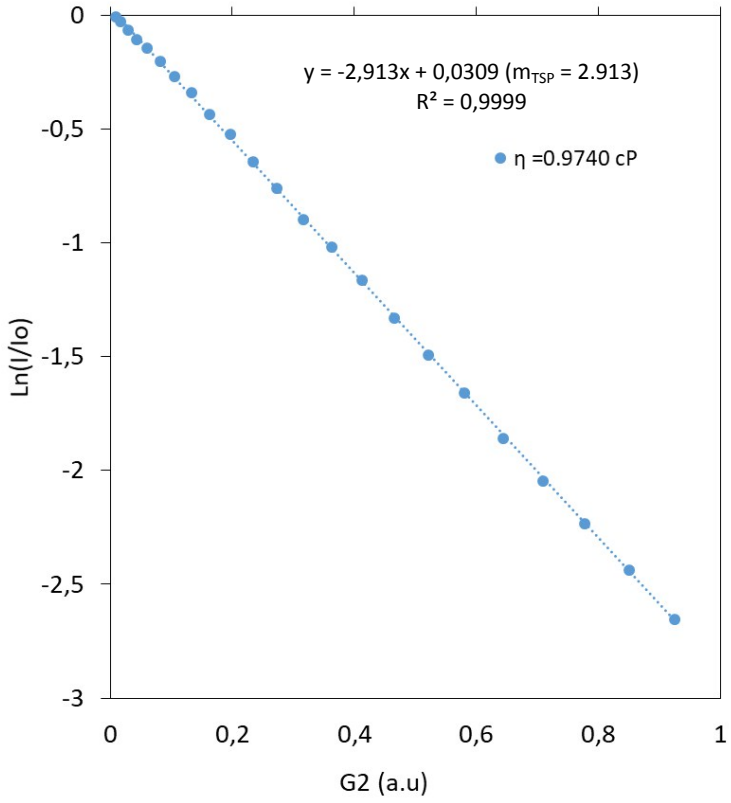

Figure S2. Stejskal-Tanner plot obtained from ^{1}H PGSE diffusion experiments applied to TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) in D_2O at 295 K. The solid line represents the linear least-squares fitting of experimental data. We have used the viscosity value of 0.9740 cP by applying the equation $\log_{10}\eta = A + B/T + CT + DT^2$ at 295 K given in reference 6.

Table S1. Regression analysis of Figure S2.

Ln(I/Io)	**G2**
0	0,0064
-0,018963219	0,0144
-0,056053621	0,0256
-0,096956233	0,04
-0,136716166	0,0576
-0,193877837	0,0784
-0,261354486	0,1024
-0,330734034	0,1296
-0,427498573	0,16
-0,514446968	0,1936
-0,635554282	0,2304
-0,751261766	0,2704
-0,888439522	0,3136
-1,008759287	0,36
-1,154107707	0,4096
-1,323189204	0,4624
-1,486264875	0,5184
-1,649694948	0,5776
-1,851416584	0,64
-2,039160193	0,7056
-2,223984466	0,7744
-2,429193886	0,8464
-2,644928217	0,9216

$y = -2,913x + 0,0309$ ($R^2 = 0,9999$)

$$r_H = \frac{kT}{6\pi\eta D}$$

$$\eta = \frac{kT}{6\pi\, r_H TSP\, D} \qquad \eta = \frac{1.38 * 295}{6 * \pi * 3.97 * D}$$

Fructose added (mg)	D-value (10^{-9} m^2 s^{-1})	Viscosity (cP)
2.0	0.5570	0.9763
4.0	0.5415	1.0042
6.0	0.5283	1.0294
18	0.5087	1.0690

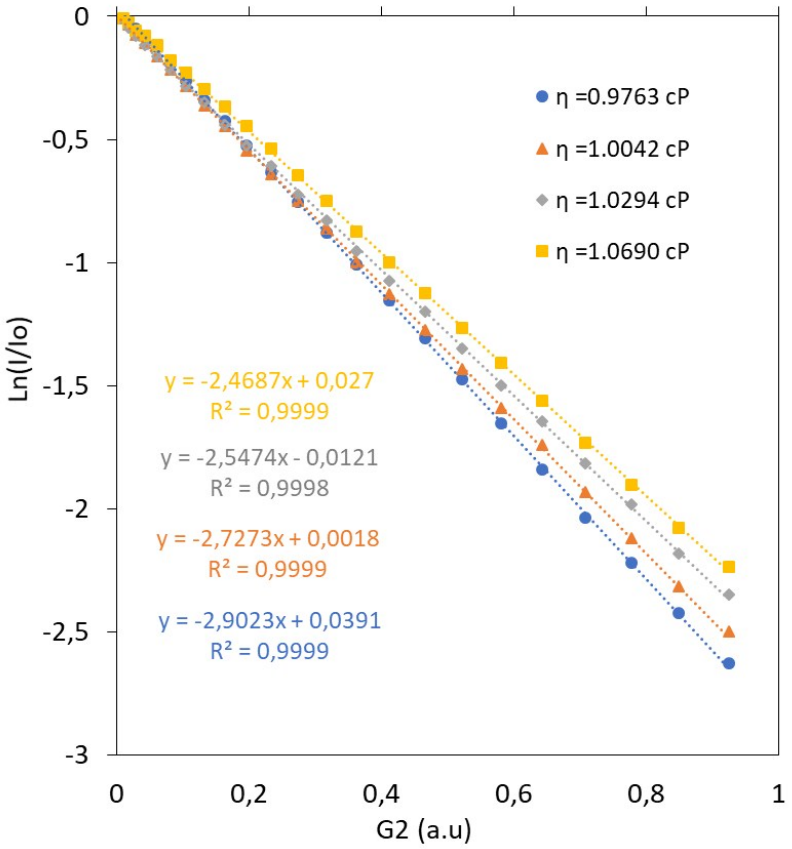

Figure S3. Stejskal-Tanner plots obtained from ^{1}H PGSE diffusion experiments applied to TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) in D_2O at four different fructose concentrations and measured at 295 K. The solid lines represent the linear least-squares fitting of the experimental data.

Table S2. TRAIn diffusion values (x 10^{-10} m^2 s^{-1}), hydrodynamic radii (Å) and M_W UCC-predicted (Da) for P1-P6 and Myoglobin at 294 K. The viscosity η used in the Stokes-Einstein equation was 0.9976 cP, obtained by applying the equation $\log_{10}\eta = A + B/T + CT + DT^2$ at 294 K given in reference 6.

	M_W[a]	D[b]	r_H	Predicted M_W	diff (%)
Ubiquitin (P1)	8938	1.212	18.2	9248	3.5
Lysozyme (P2)	14889	0.992	22.3	14609	1.9
α-chymotrypsinogen (P3)	26051	0.779	28.4	25365	2.6
β-lactoglobulin (P4)	37226	0.667	33.2	36088	3.1
Ovalbumin (P5)	45049	0.600	36.8	45995	2.1
Albumin (P6)	67474	0.501	44.1	69344	2.8
Myoglobin	17900	0.940	23.5	17449	2.5

[a] Considering masses that arise from the exchange by deuterium of labile protons in the backbone and side chains. [b] The experimental error is about ±0.9 %.

Table S3. LMS diffusion values (x 10^{-10} m^2 s^{-1}), hydrodynamic radii (Å) and M_W UCC-predicted (Da) for P1-P6 and Myoglobin at 294 K. The viscosity η used in the Stokes-Einstein equation was 0.9976 cP, obtained by applying the equation $\log_{10}\eta = A + B/T + CT + DT^2$ at 294 K given in reference 6.

	M_W[a]	D[b]	r_H	Predicted M_W	diff (%)
Ubiquitin (P1)	8938	1.227	18.0	9201	2.9
Lysozyme (P2)	14889	1.015	21.8	14308	3.9
α-chymotrypsinogen (P3)	26051	0.797	27.7	25772	1.1
β-lactoglobulin (P4)	37226	0.695	31.8	35999	3.3
Ovalbumin (P5)	45049	0.611	36.2	49198	9.2
Albumin (P6)	67474	0.543	40.7	65585	2.8
Myoglobin	17900	0.951	23.2	16764	6.3

[a] Considering masses that arise from the exchange by deuterium of labile protons in the backbone and side chains. [b] The experimental error is about ±2.4 %.

Table S4. NNLS diffusion values (x 10^{-10} m^2 s^{-1}), hydrodynamic radii (Å) and M_W UCC-predicted (Da) for P1-P6 and Myoglobin at 294 K. The viscosity η used in the Stokes-Einstein equation was 0.9976 cP, obtained by applying the equation $\log_{10}\eta = A + B/T + CT + DT^2$ at 294 K given in reference 6.

	M_W[a]	D[b]	r_H	Predicted M_W	diff (%)
Ubiquitin (P1)	8938	1.228	18.0	9432	5.2
Lysozyme (P2)	14889	1.031	21.4	13892	6.7
α-chymotrypsinogen (P3)	26051	0.801	27.6	24268	6.84
β-lactoglobulin (P4)	37226	0.667	33.1	36379	2.3
Ovalbumin (P5)	45049	0.600	36.8	46031	2.2
Albumin (P6)	67474	0.501	44.1	68552	1.6
Myoglobin	17900	0.949	23.3	16850	5.9

[a] Considering masses that arise from the exchange by deuterium of labile protons in the backbone and side chains. [b] The experimental error is about ±1.7 %.

Table S5. ITAMeD diffusion values (x 10^{-10} m^2 s^{-1}), hydrodynamic radii (Å) and M_W UCC-predicted (Da) for P1-P6 and Myoglobin at 294 K. The viscosity η used in the Stokes-Einstein equation was 0.9976 cP, obtained by applying the equation $\log_{10}\eta = A + B/T + CT + DT^2$ at 294 K given in reference 6.

	M_W[a]	D[b]	r_H	Predicted M_W	diff (%)
Ubiquitin (P1)	8938	1.212	18.2	9280	3.8
Lysozyme (P2)	14889	1.002	22.1	14300	4.0
α-chymotrypsinogen (P3)	26051	0.801	27.6	23756	8.8
β-lactoglobulin (P4)	37226	0.667	33.1	35991	3.3
Ovalbumin (P5)	45049	0.601	36.8	45821	1.7
Albumin (P6)	67474	0.502	44.1	68952	2.2
Myoglobin	17900	0.947	23.3	16937	5.4

[a] Considering masses that arise from the exchange by deuterium of labile protons in the backbone and side chains. [b] The experimental error is about ±1.7 %.

Protein sequence of ubiquitin (227 aa)

1 mawksggash selihnlrkn giiktdkvfe vmlatdrshy akcnpymdsp qsigfqatis

61 aphmhayale llfdqlhega kaldvgsgsg iltacfarmv gctgkvigid hikelvddsv

121 nnvrkddptl lssgrvqlvv gdgrmgyaee apydaihvga aapvvpqali dqlkpggrli

181 lpvgpaggnq mleqydklqd gsikmkplmg viyvpltdke kqwsrwk

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Table S6. Number of labile protons exchanged by deuterium in ubiquitin.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	14	14
Threonine	t	1	7	7
Cysteine	c	1	3	3
Methionine	m	1	9	9
Glutamic acid	e	1	9	9
Aspartic acid	d	1	16	16
Tritophan	w	1	3	3
Tyrosine	y	1	7	7
Aspargine	n	2	6	12
Glutamine	q	2	10	20
Histidine	h	2	8	16
Lysine	k	3	16	48
Arginine	r	6	8	48
Amide protons backbone				226
TOTAL				**438**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

Protein sequence of lysozyme (147 aa)

1 mkaliilgfl flsvavqgkv fercelartl kklgldgykg vslanwlclt kwessyntka

61 tnynpssest dygifqinsk wwcndgktpn avdgchvscs elmendiaka vacakhivse

121 qgitawvawk shcrdhdvss yvqgctl

//

Table S7. Number of labile protons exchanged by deuterium in lysozyme.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	14	14
Threonine	t	1	8	8
Cysteine	c	1	8	8
Methionine	m	1	2	2
Glutamic acid	e	1	7	7
Aspartic acid	d	1	7	7
Tritophan	w	1	6	6
Tyrosine	y	1	5	5
Aspargine	n	2	8	16
Glutamine	q	2	4	8
Histidine	h	2	4	8
Lysine	k	3	12	36
Arginine	r	6	3	18
Amide protons backbone				146
TOTAL				**289**

a Assuming carboxylic acid, amine and imine groups as protonated.

Protein sequence of α-chymotrypsinogen (245 aa)

1 cgvpaiqpvl sglsrivnge eavpgswpwq vslqdktgfh fcggslinen wvvtaahcgv

61 ttsdvvvage fdqgssseki qklkiakvfk nskynsltin nditllklst aasfsqtvsa

121 vclpsasddf aagttcvttg wgltrytnan tpdrlqqasl pllsntnckk ywgtkikdam

181 icagasgvss cmgdsggplv ckkngawtlv givswgsstc ststpgvyar vtalvnwvqq

241 tlaan

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Table S8. Number of labile protons exchanged by deuterium in α-chymotrypsinogen.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	28	28
Threonine	t	1	23	23
Cysteine	c	1	10	10
Methionine	m	1	2	2
Glutamic acid	e	1	5	5
Aspartic acid	d	1	9	9
Tritophan	w	1	8	8
Tyrosine	y	1	4	4
Aspargine	n	2	14	28
Glutamine	q	2	10	20
Histidine	h	2	2	4
Lysine	k	3	14	42
Arginine	r	6	4	24
Amide protons backbone				244
TOTAL				**451**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

Protein sequence of β-lactoglobulin (2*162 aa)

1 livtqtmkgl diqkvagtwy slamaasdis lldaqsaplr vyveelkptp egdleillqk

61 wendecaqkk iiaektkipa vfkidalnen kvlvldtdyk kyllfcmens aepeqslvcq

121 clvrtpevdd ealekfdkal kalpmhirls fnptqleeqc hi

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Table S9. Number of labile protons exchanged by deuterium in β-lactoglobulin.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence*2	Total
Serine	s	1	14	14
Threonine	t	1	16	16
Cysteine	c	1	10	10
Methionine	m	1	8	8
Glutamic acid	e	1	32	32
Aspartic acid	d	1	22	22
Tritophan	w	1	4	4
Tyrosine	y	1	8	8
Aspargine	n	2	10	20
Glutamine	q	2	18	36
Histidine	h	2	4	8
Lysine	k	3	30	90
Arginine	r	6	6	36
Amide protons backbone				322
TOTAL				**626**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

Protein sequence of ovalbumin (386 aa)

1 mgsigaasme fcfdvfkelk vhhanenify cpiaimsala mvylgakdst rtqinkvvrf

61 dklpgfgdsi eaqcgtsvnv hsslrdilnq itkpndvysf slasrlyaee rypilpeylq

121 cvkelyrggl epinfqtaad qarelinswv esqtngiirn vlqpssvdsq tamvlvnaiv

181 fkglwekafk dedtqampfr vteqeskpvq mmyqiglfrv asmasekmki lelpfasgtm

241 smlvllpdev sgleqlesii nfekltewts snvmeerkik vylprmkmee kynltsvlma

301 mgitdvfsss anlsgissae slkisqavha ahaeineagr evvgsaeagv daasvseefr

361 adhpflfcik hiatnavlff grcvsp

//

Table S10. Number of labile protons exchanged by deuterium in ovalbumin.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	38	38
Threonine	t	1	15	15
Cysteine	c	1	6	6
Methionine	m	1	17	17
Glutamic acid	e	1	33	33
Aspartic acid	d	1	14	14
Tritophan	w	1	3	3
Tyrosine	y	1	10	10
Aspargine	n	2	17	34
Glutamine	q	2	15	30
Histidine	h	2	7	14
Lysine	k	3	20	60
Arginine	r	6	15	90
Amide protons backbone				385
TOTAL				**749**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

Protein sequence of albumin (609 aa)

1 mkwvtfisll flfssaysrg vfrrdahkse vahrfkdlge enfkalvlia faqylqqcpf

61 edhvklvnev tefaktcvad esaencdksl htlfgdklct vatlretyge madccakqep

121 ernecflqhk ddnpnlprlv rpevdvmcta fhdneetflk kylyeiarrh pyfyapellf

181 fakrykaaft eccqaadkaa cllpkldelr degkassakq rlkcaslqkf gerafkawav

241 arlsqrfpka efaevsklvt dltkvhtecc hgdllecadd radlakyice nqdsissklk

301 eccekpllek shciaevend empadlpsla adfveskdvc knyaeakdvf lgmflyeyar

361 rhpdysvvll lrlaktyett lekccaaadp hecyakvfde fkplveepqn likqncelfe

421 qlgeykfqna llvrytkkvp qvstptlvev srnlgkvgsk cckhpeakrm pcaedylsvv

481 lnqlcvlhek tpvsdrvtkc cteslvnrrp cfsalevdet yvpkefnaet ftfhadictl

541 sekerqikkq talvelvkhk pkatkeqlka vmddfaafve kcckaddket cfaeegkklv

601 aasqaalgl

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Table S11. Number of labile protons exchanged by deuterium in albumin.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	28	28
Threonine	t	1	29	29
Cysteine	c	1	35	35
Methionine	m	1	7	7
Glutamic acid	e	1	62	62
Aspartic acid	d	1	36	36
Tritophan	w	1	2	2
Tyrosine	y	1	19	19
Aspargine	n	2	17	34
Glutamine	q	2	20	40
Histidine	h	2	16	32
Lysine	k	3	60	180
Arginine	r	6	27	162
Amide protons backbone				608
TOTAL				**1274**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

Protein sequence of myoglobin (154 aa)

1 mglsdgewqq vlnvwgkvea diaghgqevl irlftghpet lekfdkfkhl kteaemkase

61 dlkkhgtvvl talggilkkk ghheaelkpl aqshatkhki pikylefisd aiihvlhskh

121 pgdfgadaqg amtkalelfr ndiaakykel gfqg

//

Table S12. Number of labile protons exchanged by deuterium in myoglobin.

Aminoacid	Code	Labile protons in side chain[a]	Number of aa in sequence	Total
Serine	s	1	5	5
Threonine	t	1	7	7
Cysteine	c	1	0	0
Methionine	m	1	3	3
Glutamic acid	e	1	13	13
Aspartic acid	d	1	8	8
Tritophan	w	1	2	2
Tyrosine	y	1	2	2
Aspargine	n	2	2	4
Glutamine	q	2	6	12
Histidine	h	2	11	22
Lysine	k	3	19	57
Arginine	r	6	2	12
Amide protons backbone				153
TOTAL				**300**

a Assuming carboxylic acid, amine and imine functional groups as protonated moieties.

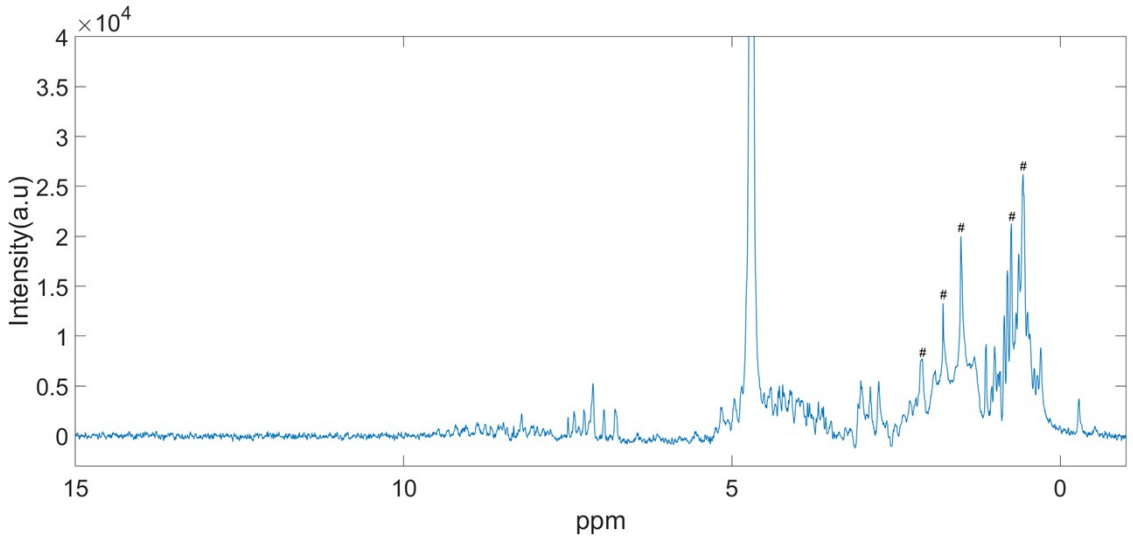

Figure S4. ^{1}H NMR spectrum (500.13 MHz) of ubiquitin in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 2.12, 1.78, 1.51, 0.75, 0.57 ppm.

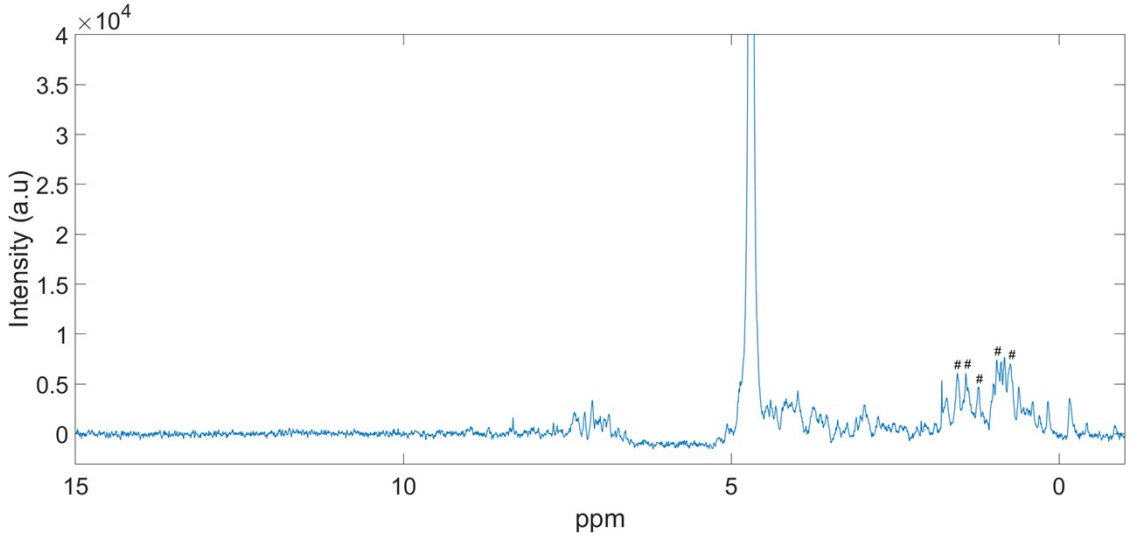

Figure S5. ^{1}H NMR spectrum (500.13 MHz) of lysozyme in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 1.54, 1.42, 1.23, 0.83, 0.73 ppm.

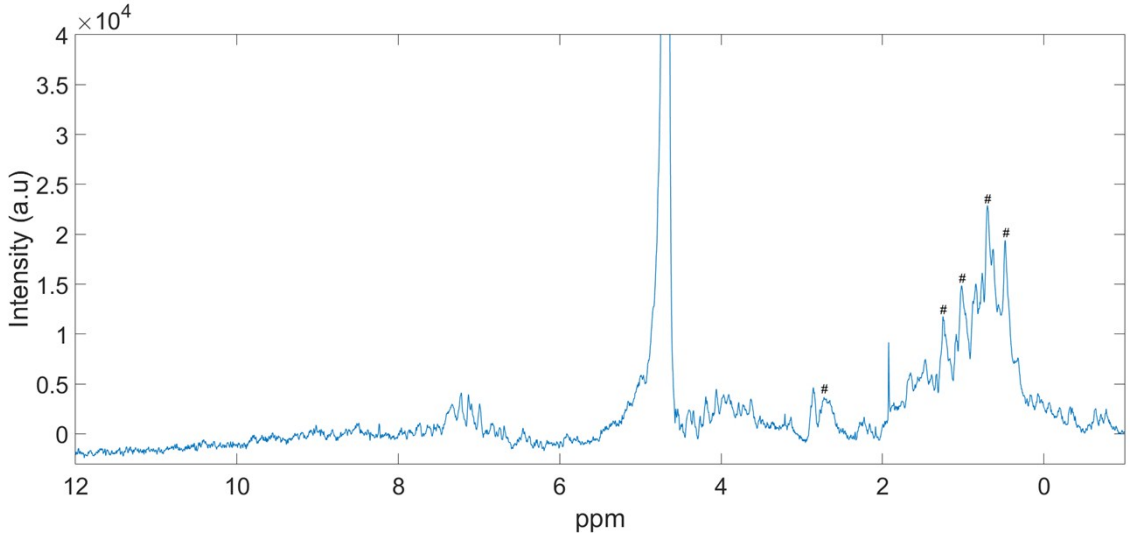

Figure S6. ^{1}H NMR spectrum (500.13 MHz) of α-chymotrypsinogen in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 2.70, 1.25, 1.02, 0.70, 0.48 ppm.

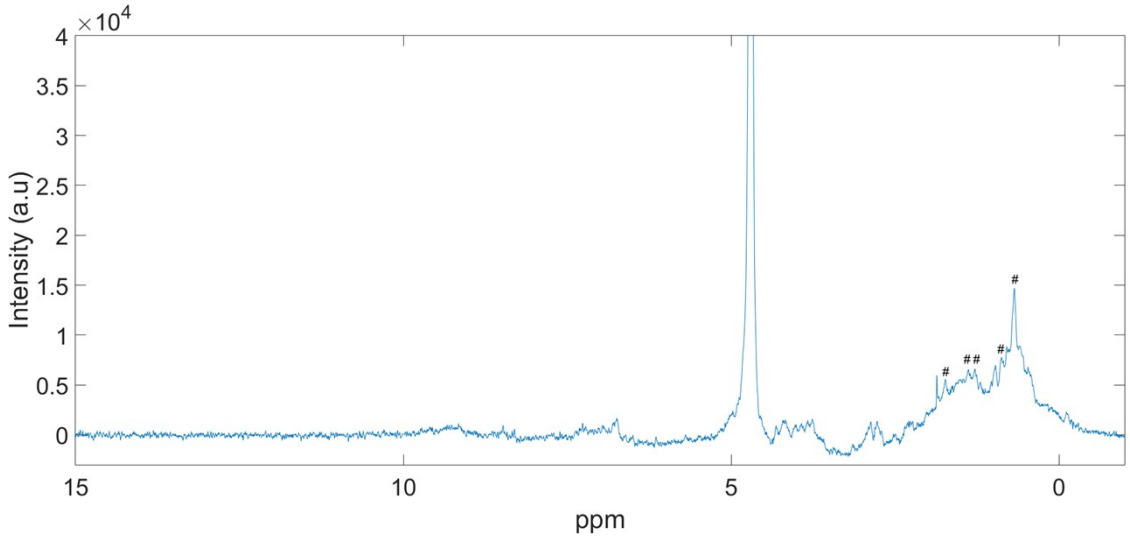

Figure S7. ^{1}H NMR spectrum (500.13 MHz) of β-lactoglobulin in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 1.73, 1.37, 1.28, 0.86, 0.68 ppm.

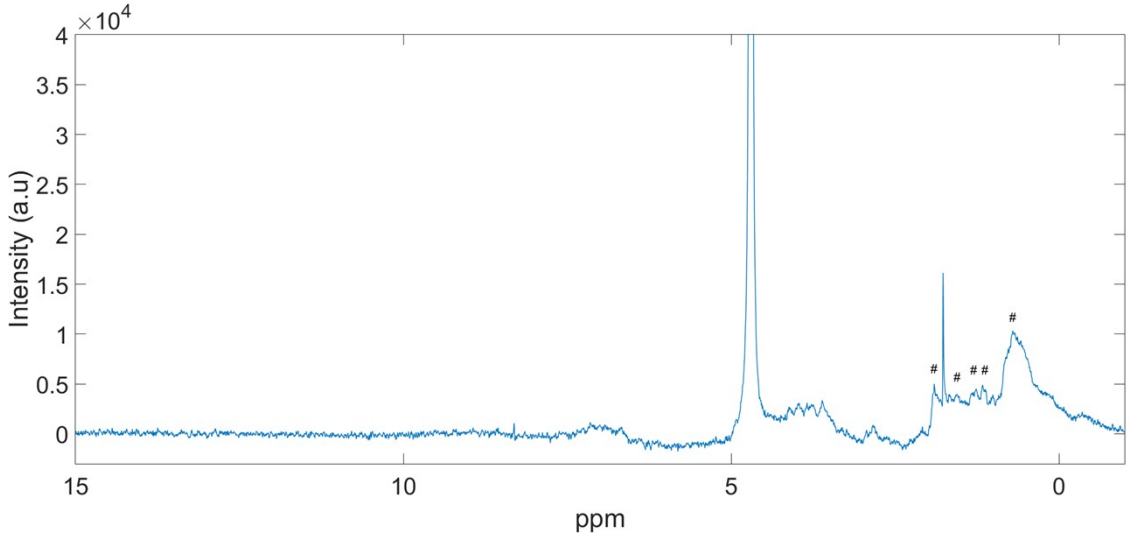

Figure S8. ^{1}H NMR spectrum (500.13 MHz) of ovalbumin in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 1.89, 1.56, 1.27, 1.16, 0.71 ppm.

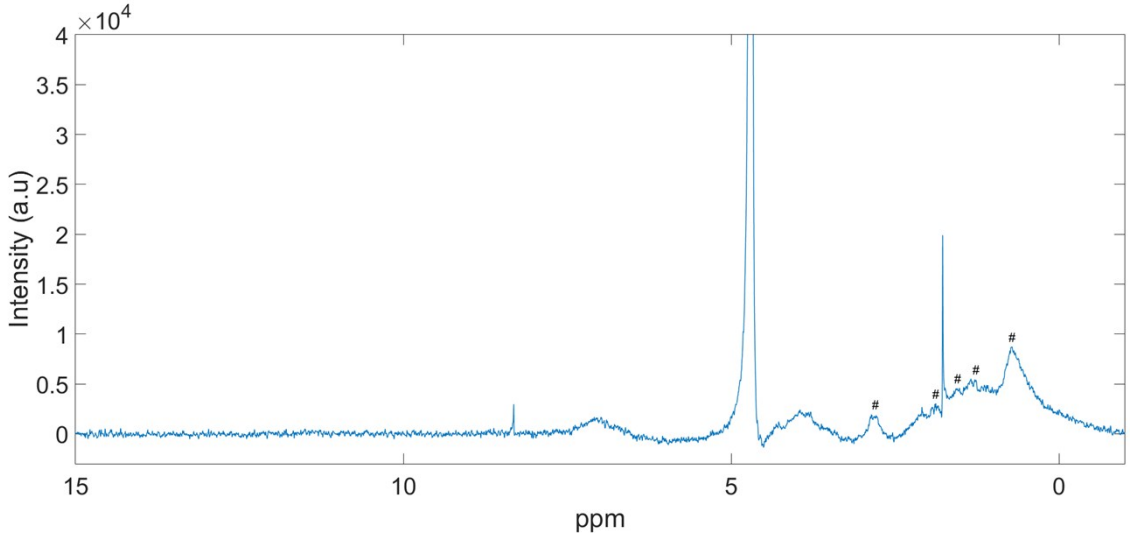

Figure S9. ^{1}H NMR spectrum (500.13 MHz) of albumin in D_2O at 294 K. The following resonances were monitored in the diffusion experiments: 2.80, 1.89, 1.56, 1.26, 0.69 ppm.

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