

1 Structural features of the glutamate-binding protein from
2 *Corynebacterium glutamicum*
3

4 Alessandro Capo¹, Antonino Natalello², Jan Marienhagen^{3,4}, Angela Pennacchio¹, Alessandra
5 Camarca¹, Stefano Di Giovanni¹, Maria Staiano¹, Sabato D'Auria^{1*}, Antonio Varriale¹
6

7 ¹Institute of Food Science CNR, via Roma 64, 83100 Avellino, Italy

8 ²Department of Biotechnology and Biosciences University of Milano-Bicocca P.zza della Scienza
9 2, 20126 Milano, Italy

10 ³Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich,
11 Germany

12 ⁴Institute of Biotechnology, RWTH Aachen University, Worringer Weg 3, D-52074 Aachen,
13 Germany
14

15
16
17
18 *Correspondence to:

19 Dr. Sabato D'Auria

20 Institute of Food Science, CNR

21 Via Roma, 64, 83100 Avellino

22 Italy

23 Phone: +39-0825299101

24 Fax: +39-0825299100

25 Email: sabato.dauria@cnr.it
26
27
28

29 **Abstract**

30 L-glutamate (Glu) is the major excitatory transmitter in mammalian brain. Inadequate concentration
31 of Glu in the brain correlates to mood disorder. In industry, Glu is used as a flavour enhancer in
32 food and in foodstuff processing. A high concentration of Glu has several effects on human health
33 such as hypersensitive effects, headache and stomach pain. The presence of Glu in food can be
34 detected by different analytical methods based on chromatography, or capillary electrophoresis or
35 amperometric techniques. We have isolated and characterized a glutamate-binding protein (GluB)
36 from the Gram-positive bacteria *Corynebacterium glutamicum*. Together with GluC protein, GluD
37 protein and the cytoplasmic protein GluA, GluB permits the transport of Glu in/out of cell. In this
38 study, we have investigated the binding features of GluB as well as the effect of temperature on its
39 structure both in the absence and in the presence of Glu. The results have showed that GluB has a
40 high affinity and selectivity versus Glu (nanomolar range) and the presence of the ligand induces a
41 higher thermal stability of the protein structure.

42 **Keywords**

43 Glutamate binding protein, *Corynebacterium glutamicum*, Fluorescence spectroscopy, FT-IR

44 **1 Introduction**

45 Glu is the major excitatory neurotransmitter of the human brain [1]. Different studies have
46 demonstrated a correlation between anomalies in Glu signalling and different neuro-generative
47 psychiatric disorders such as schizophrenia [2], alcohol dependence [3], and obsessive-compulsive
48 disorder [4] and, more in general, with mood-related disorders. These studies have demonstrated
49 that the increase of plasma Glu level is associated with mood disorders. On the contrary, Glu is
50 extensively used in the food industry as a flavor enhancer of various foodstuffs. High
51 concentrations of Glu could have hypersensitizing effects on human health such as headache,
52 stomach pain [5]. High Glu level has been also linked to Chinese Restaurant Syndrome (CRS) [5].
53 CRS refers to a group of symptoms that some people experience after eating food from a Chinese
54 restaurant. It is known as monosodium glutamate MSG symptom complex, and often it includes
55 headache, skin flushing, and sweating.

56 In biomedicine and food safety, different analytical methods have been developed for detection of
57 Glu. These methods are based on amperometric [6], chromatography [7] and capillary
58 electrophoresis [8] techniques. Due to the high operational complexity of these methods, there is an
59 urgent need to monitor the presence of Glu in a more fast and easy way.

60 The development of advanced optical biosensors based on fluorescence and/or surface plasmon
61 resonance techniques can overcome these drawbacks. The central core of these kind of biosensors is
62 the molecular recognition element (MRE) that provides high specificity and sensibility to the
63 device. In this context, we explored the possibility to use a specific substrate-binding protein (SBP)
64 as molecular recognition element MRE for the detection of Glu. In all kingdoms of life, cells
65 interact through an extremely elegant transport system, defined ATP-binding cassette (ABC)
66 transporters. The function of this system is to uptake nutrients such as ions, cations and anions,
67 amino acids, peptides, proteins, vitamins, drugs, fatty acids, and lipids into and out of cell. Different
68 proteins constitute a typical ABC transporter: the protein soluble portion is named substrate-binding
69 protein (SBP) in both the Gram-negative and Gram-positive bacteria. In particular, members

70 belonging this class of proteins are located in the periplasm of Gram-negative bacteria. Instead, they
71 are lipid-anchored or fused to the two transmembrane domains (TMD) in Gram-positive bacteria
72 and Archaea [9]. The common features of this class of protein family are low sequence similarity
73 and high conserved structural fold, with two similarly folded domain connected with two-three
74 stranded β -sheet [10]. SBPs are extensively used for biotechnological applications. Different studies
75 showed the applicability of proteins belonging to this family as MREs in protein-based biosensor
76 development [11-13]. In fact, different research groups are involved in the development of
77 extremely sensitive biosensors, using SBPs for detection of small analytes with implication in food
78 safety, environment and medical diagnostics. In the last two decades, our lab has contributed to
79 enrich the knowledge in this field, with isolation and characterization of different substrate-binding
80 proteins for application in fluorescence protein-based biosensors [11, 14].

81 With the aim to identify a possible candidate of MRE, we selected as SBP, a *putative* glutamate-
82 binding protein (GluB) from the Gram-positive bacteria *Corynebacterium glutamicum*. This protein
83 is involved in the uptake of Glu in this bacterium [15]. *Corynebacterium glutamicum* is a well-
84 studied platform organism in industrial biotechnology [16, 17]. The most prominent application of
85 this Gram-positive microorganism is the fermentative production of proteinogenic amino acids.
86 Among them, L-glutamate and L-lysine that are used as flavour enhancer and the feed additive,
87 respectively, are produced at a multimillion-ton-scale annually [18]. Furthermore, *C. glutamicum*
88 strains have been engineered to produce numerous compounds of biotechnological interest such as
89 organic acids [19], diamines and terpenes [20] and more recently diols [21] and polyphenols [22].
90 Recently GluB was crystallized, X-ray diffraction data set was collected and a structure model to a
91 resolution of 1.9Å could be calculated [23].

92 In this study, we present the cloning, purification and a spectroscopic characterization of GluB. In
93 particular, we explored the binding features and effect of temperature on the protein stability in the
94 absence and in the presence of Glu in order to obtain information on GluB structural and functional

95 properties. The obtained data showed a high affinity of GluB versus Glu (nanomolar range) and the
96 binding of Glu to GluB induced more stability to the protein structure.

97

98 2 Materials and Methods

99 2.1 Materials

100 Glu and DNase purchased from Sigma. Ampicillin, IPTG, lysozyme and all the reagents used in the
101 preparation of LB medium and buffers were from AppliChem. All the other chemicals used were
102 commercial samples of the purest quality. 2, 5-diphenyl-1, 3, 4-oxidiazole was purchased from
103 Invitrogen.

104

105 2.2 Construction of pET22b (+)-*gluB* for the heterologous expression of *gluB* in *E. coli*

106 Recombinant DNA work, such as PCR, DNA restriction, or DNA ligation were carried out
107 according to standard molecular cloning protocols [24]. If not stated otherwise, all enzymes were
108 obtained from Thermo Scientific (Schwerte, Germany). The plasmid pET22b (+)-*gluB* was
109 constructed for the heterologous expression of *gluB* from *C. glutamicum* in *Escherichia coli* BL21
110 (DE3). For this purpose, the *gluB* gene without the N-terminal signal sequence of 78 bps for the
111 translocation into the periplasm was isolated by PCR using genomic DNA of *C. glutamicum* ATCC
112 13032 as a template. Necessary oligonucleotides (*PgluB_C_his_for*: 5'-
113 AGCTGACATATGTGTGGTGATTCAAGCGGTGGCG-3' (*NdeI* restriction site underlined) and
114 *PgluB_C_his_rev*: 5'-GACAGTGCGGCCGCGCCTTGCGTCGAGGAAGGAGAGGTCACC-3'
115 (*NotI* restriction site underlined) were ordered from Eurofins MWG Operon (Ebersberg, Germany)
116 and the employed KOD Hot Start DNA polymerase was purchased from Merck (Darmstadt,
117 Germany). The PCR program for the amplification of the desired *gluB* DNA sequence started with
118 an initial denaturation step at 94°C for 2 min. Subsequently, 30 cycles (94°C, 30s; 60°C, 30s; and
119 72°C, 3s) and one fill-up cycle (72°C, 5 min) were performed. The resulting PCR product of
120 approximately 850 bps in size was purified using the PCR purification kit (Macherey–Nagel,
121 Dueren, Germany) according to manufacturer's instructions and quantified using a NanoDrop
122 instrument (NanoDrop Technologies, Wilmington, DE, USA). Afterwards, the PCR-product was
123 subjected to *NdeI/NotI* restriction and cloned into the equally digested pET22b (+) expression

124 vector. By design, gene expression from the resulting vector pET22b (+)-*gluB* attaches a C-terminal
125 His₆-tag for the possibility of performing affinity purification of the truncated GluB protein. Finally,
126 the agreement of the constructed plasmid with the *in silico* design was verified by DNA sequencing
127 at Eurofins MWG Operon (Ebersberg, Germany).

128

129 2.3 Heterologous *gluB*-expression and protein purification

130 *E. coli* BL21 (DE3) was transformed with pET22b(+)-*gluB* and cultivated in LB medium in
131 presence of 50µg/ml Ampicillin, until reaching an optical density at 595nm (OD₅₉₅) of 0.6, and
132 GluB expression was induced with 0.8mM isopropyl β-D-1-thiogalactopyranoside (IPTG)
133 overnight at 22°C. The cells were harvested by centrifugation at 4000 rpm at 4°C for 30 min, then
134 they were suspended in Lysis buffer (20mM NaH₂PO₄/Na₂HPO₄, 500mM NaCl, 20mM imidazole,
135 pH 7.4) with lysozyme (1mg/ml) and incubated for 30 minutes at 30°C. The suspension was
136 sonicated (10 cycles, 30s on/30s off at 38% power), DNase (0.05mg/ml) and MgCl₂ (5mM) were
137 added and incubated for 1h in ice. The lysate was centrifuged at 15000g at 4°C for 30 minutes. The
138 obtained supernatant was filtered with a 0.22µm polyethersulfone (PES) syringe filter and purified
139 using an AKTA FPLC system equipped with a HisTrap HP column (GE Healthcare). The flow-
140 through was collected and the column washed with lysis buffer until the absorbance baseline was
141 stable; then the protein was eluted at a flow rate of 5 ml/min with increasing concentration of
142 imidazole (50-100-250mM imidazole). The purity of the eluted fractions was verified by gel
143 electrophoresis (SDS-PAGE).

144 The fractions eluted at 50mM imidazole were concentrated with a centrifugal filter device
145 (Vivaspin Turbo-Sartorius 30,000 MWCO) and were loaded onto a HiLoad 16/600 Superdex75
146 column equilibrated in PBS solution (6mM KH₂PO₄/Na₂HPO₄, 140mM NaCl, 3mM KCl, pH 7.4).
147 The protein was eluted with PBS solution at a flow rate of 1ml/min, monitoring the absorbance of
148 the eluate at 280nm. In order to verify the degree of purity of the obtained fractions, an SDS-PAGE
149 (15%) was performed. The protein concentration was calculated on the absorbance values at 280nm

150 by using Lambert and Beer's law. The molar extinction coefficient, calculated using Expasy
151 ProtParam, tool that follow the Edelhoch [25] method, is equal to $27515 \text{ M}^{-1} \text{ cm}^{-1}$; the molecular
152 weight is 32490 Da. Before to start experiments it was essential to remove the Glu bound to the
153 protein following the purification. For this purpose, extensive dialysis of the isolated protein was
154 performed against PBS pH 8.0 for five days.

155

156 2.4 Protein Analysis by MALDI-TOF MS

157 Identity of the truncated and C-terminal His-6-tagged GluB protein was confirmed by mass
158 spectrometry. Therefore, proteins to be analysed by MS were excised in-gel trypsinized as
159 described previously [26]. For protein identification by peptide mass fingerprinting, the tryptic
160 peptides were subjected to MALDI-TOF MS using an Ultraflex III TOF/TOF mass spectrometer
161 (Bruker Daltonics, Billerica, MA, USA). Mascot algorithm (Matrix Science, London, UK) was used
162 to compare the experimentally determined peptide mass patterns with those of the entire *C.*
163 *glutamicum* proteome.

164

165 2.5 Circular dichroism measurements

166 Circular dichroism (CD) measurements were performed on homogeneous samples of GluB (20 mM
167 sodium phosphate, pH 7.4), with a protein amount of 0.15mg/mL in the absence or in the presence
168 of the specified amounts of Glu.

169 We used the J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with the Neslab RTE-110
170 temperature-controlled liquid system (Neslab Instruments, Portsmouth, NH). The instrument was
171 calibrated with a standard solution of (+)-10-camphorsulfonic acid. Sealed cuvettes with a 0.1cm
172 path length (Helma, Jamaica, NJ) were used. Photomultiplier voltage never exceeded 600V in the
173 measured spectral region (190 to 250nm). Each spectrum was averaged five times and smoothed
174 with spectropolarimeter system software, version 1.00 (Jasco, Japan). All measurements were

175 performed under nitrogen flow. Before undergoing CD analyses, all samples were kept at the
176 temperature being studied for 10 min. The results are expressed in terms of molar Ellipticity $[\theta]_{\text{mrw}}$.

177

178 2.6 Steady-State fluorescence spectroscopy measurements

179 Steady-state emission experiments were performed on ISS-K2 fluorometer (ISS, Urbana-
180 Champaign) equipped with a cell temperature-controlled sample holder. To selectively excite Trp
181 residues, the excitation wavelength was set at 295nm. Excitation and emission slit widths were
182 fixed at 0.5nm and 1.0nm respectively. Emission spectra recorded from 310nm and 410nm, at
183 1.0nm intervals. Samples were placed in the thermostatic holder and temperature of samples was
184 measured directly in the cuvette with an accuracy of $\pm 0.2^\circ\text{C}$. The buffer alone was used as blank
185 and its emission contribution was subtracted from the experimental spectra [27]. GluB binding
186 capability was studied by a fluorescence titration approach. For this purpose, recombinant GluB
187 (10 μM) was titrated with increasing concentrations of Glu (0-100 μM). In order to verify the GluB
188 binding specificity the binding of L-aspartate (Asp) was been investigated. The binding of amino
189 acids to GluB was estimated by a variation of the intrinsic protein fluorescence emission. The
190 maximal decrease of the protein fluorescence due to the saturation of the binding sites by the ligand
191 (F_{max}) was estimated from the titration data. Experimental data were processed by a non-linear
192 regression analysis computed with the OriginPro 8.0.

193

194 2.7 Fluorescence Quenching

195 Acrylamide quenching of the Trp fluorescence was observed at fluorescence maximum and
196 analysed by the Stern-Volmer equation:

197

$$198 \quad F_0/F = 1 + K_D[Q] = 1 + k_q\tau_0[Q]$$

Equation 1

199

200 F_0 and F are the fluorescence intensities in the absence and presence of acrylamide, respectively; k_q
 201 is the bimolecular quenching constant; τ_0 is the lifetime of the fluorophore in the absence of
 202 quencher, and Q is the concentration of acrylamide. The Stern-Volmer quenching constant is given
 203 by $K_d = k_q \tau_0$. If the quenching is dynamic K_d will represent the Stern-Volmer constant. All
 204 fluorescence experiments were done in PBS buffer at pH 8.0 with the protein concentration of
 205 0.05mg/mL. Quenching data are presented as plots of F_0/F versus $[Q]$. F_0/F is expected to be
 206 linearly dependent upon the concentration of quencher. A plot of F_0/F versus $[Q]$ yields an intercept
 207 of one on the y -axis and a slope equal to K_{sv} . A linear Stern-Volmer plot is generally indicative of a
 208 single class of fluorophores, all equally accessible to the quencher [28].

209

210 2.8 Time-resolved fluorescence measurements

211 Fluorescence decay measurements were performed with a Chronos-ISS fluorometer, (ISS,
 212 Champaign, IL, USA) using the phase shift and demodulation technique. The excitation source (295
 213 nm) was a Laser diode and the emission was collected through a 337 long-pass filter (Edmund
 214 Optics, USA). As lifetime reference was used the 2, 5-diphenyl-1, 3, 4-oxadiazole and intensity
 215 decays were analyzed in terms of the multi-exponential model:

$$216 \quad I(t) = I_0 e^{-t/\tau} = \sum \alpha_i e^{-t/\tau_i} \quad \text{Equation 2;}$$

217 where I_0 is the intensities at $t = 0$, τ is the lifetime of the fluorophore, α_i are the pre-exponential
 218 factors, and $\sum \alpha_i = 1.0$. For multi-component decays, the fractional intensity f_i of each decay time is
 219 given by

$$220 \quad f_i = \alpha_i \tau_i / \sum \tau_i \alpha_i \quad \text{Equation 3;}$$

221 and the mean lifetime can be calculated as:

$$222 \quad \tau = \sum f_i \tau_i \quad \text{Equation 4 [28].}$$

223

224 2.9 FT-IR measurements

225 Fourier transform infrared (FTIR) characterizations were performed on GluB in 20mM of
226 deuterated sodium phosphate buffer pH 8.0, with a protein concentration of 6mg/mL in the absence
227 and in the presence of Glu or Asp at 5mM concentration. FTIR spectra were collected and analyzed
228 as previously described [29]. Briefly, 20 μ L of the sample solution was placed in a temperature-
229 controlled transmission cell (Wilmad, Buena, NJ, U.S.A.) with two BaF₂ windows and a 100 μ m
230 Teflon spacer. Absorption spectra were collected using the Varian 670-IR (Varian, Mulgrave,
231 Australia) spectrometer, equipped with a nitrogen-cooled mercury cadmium telluride detector,
232 under the following conditions: 2cm⁻¹ spectral resolution, 25kHz scan speed, 500 scan co-addition,
233 and triangular apodization. For the thermal denaturation experiments, sample solutions in the
234 temperature-controlled cell were heated at 0.4°C/min from 25°C to 100°C, each spectrum being
235 collected every ~1.74°C. The protein FTIR absorption spectra were obtained after subtraction of the
236 appropriate reference spectra strictly collected under the same conditions. When necessary, the
237 subtraction of residual vapour absorption was also performed [29, 30]. Second derivative spectra
238 were obtained following the Savitsky-Golay method [31]. The Resolutions-Pro (Varian, Mulgrave,
239 Australia) software was employed for spectra collection and analyses.

240

241 2.10 Statistical analysis

242 All measurements were been collect in triplicate and were been calculated the mean value and
243 standard deviation. All fluorescence data were been normalized by the maximum and smoothed by
244 Savitzky-Golay algorithm applied as a point of window 5 and as polynomial order 2.

245

246 **3 Results and Discussion**

247 *3.1 Cloning, expression, and purification of GluB*

248 GluB is a monomeric protein of 34kDa, involved in the uptake of the Glu inside cell. Recently, its
249 three-dimensional structure was resolved at 1.9Å from the group of the Prof. Wang [23]. As a
250 member of SBPs family, the GluB structure contains two similarly folded domains connected by a
251 two or three-stranded β -sheet hinge, a characteristic folding structure feature of this class of protein
252 (Fig. 1). The *gluB* gene was isolated from *C. glutamicum* ATCC 13032 by PCR without the N-
253 terminal signal sequence of 78 bps necessary for protein translocation into the periplasm and cloned
254 into the expression vector pET22b(+). The resulting plasmid pET22b(+)-*gluB*, conferring a C-
255 terminal His₆-tag to GluB for affinity chromatography, was transformed into *E. coli* BL21(DE3) for
256 heterologous gene expression. After cultivation of *E. coli* BL21 (DE3) pET22b(+)-*gluB* and
257 expression of *gluB*, the resulting GluB protein was purified by two steps of purification (an affinity
258 chromatography followed by a gel filtration chromatography). Starting from 4g of the humid pellet,
259 12mg of protein have been purified. The main steps of the purification procedure and the
260 homogeneity of purified GluB are reported in Fig. 2A, 2B, 2C. The identity of GluB has been
261 successfully verified by MALDI-TOF MS (data not shown).

263 *3.2 CD results*

264 CD experiments in Far-UV region were performed to investigate the temperature effect on GluB
265 secondary structure. Fig. 3 shows GluB Far-UV spectra at 25°C in the absence and presence of
266 100μM Glu. The data shows that the Far-UV CD spectrum of GluB is characterized by the presence
267 of two absorption bands: a positive band at 192nm related to the α -helix content and a negative
268 band at 208 nm related to the β -sheet structure that change as consequence of the Glu binding. The
269 thermal stability of the GluB in the absence and in the presence of Glu, in the range of temperatures
270 25-90°C, was studied. CD signal at 222nm was plotted as function of temperature and the data were

271 fitted with a single step transition curve. The obtained data show that the addition of Glu to the
272 protein solution results in a marked increase of GluB melting temperature (T_m); then the T_m value
273 increases from 40 to 53°C (Fig. 3 inset). These results suggest an increase of the stability of the
274 secondary structure as a consequence of Glu binding. Similar effect was found for the binding of
275 the Asp to GluB (data not shown).

276

277 3.3 Fluorescence results

278 The fluorescence steady state and fluorescence decay experiments were performed. In particular the
279 effect of the binding Glu at GluB was estimated by a variation of its intrinsic tryptophan (W58 and
280 W65) fluorescence emission [32]. The fluorescence emission spectrum (Fig. S1 of supplementary
281 materials) of GluB in absence of Glu presents a fluorescence emission maximum peak centered at
282 333nm. This position is blue-shifted respect to the emission maximum of N-acetyl-
283 tryptophanamide (NATA) centred at 350nm (data not shown), suggesting that the tryptophanyl
284 residues of GluB at 25°C are in buried and/or un-relaxed microenvironments. On contrary the
285 fluorescence emission maximum of GluB/Glu complex reveals that the binding of 100μM Glu
286 results in quenching of the protein fluorescence intensity about 30%. A fluorescence titration was
287 performed in order to evaluate the affinity of GluB for Glu. The experiments were performed at
288 25°C and in Fig. 4A is reported the variation of fluorescence emission associated to the increase
289 concentration of Glu in solution. The obtained variation of the fluorescence emission intensity at
290 333nm as function of the Glu concentration can be fit with a single binding isotherm model and we
291 can determine, for Glu, an apparent $K_{d\ app}$ of 0.6nM at 25°C.

292 Also, we evaluated the effect of the temperature on the stability of GluB in the absence and in the
293 presence of ligands. In Fig. 4B are reported the variations of maximum of the fluorescence intensity
294 in function of the temperature (25-90°C) at pH 8.0. The result shows that as consequence of the
295 binding of Glu the tertiary structure of GluB was stabilized and the T_m value of GluB increases
296 from 40 °C to 50°C.

297 Since is well reported in literature that the binding capability of periplasmic glutamate binding
298 protein [20, 21] is often associate to the binding and transport of the Asp, as for the CD experiments
299 (Table 1), we have investigated the binding this amino acid to the GluB. In Fig. S2B of the
300 supplementary material is reported the variation of fluorescence intensity as consequence of
301 increase concentration of the Asp. Plotting the variation of fluorescence intensity with the increase
302 of Asp concentration, the apparent $K_{d\text{ app}}$ values calculated result to be 0.014nM. Analogous
303 thermo-stability effect was obtained as consequence of ASP binding. The T_m value calculated for
304 the GluB/Asp complex was 55°C (Fig. S2B, Table 1).

305

306 **3.4 Stern-Volmer quenching results**

307 With the aim to evaluate ligand binding-induced changes of Trp residue accessibility to the
308 quencher, we performed, in the absence and in the presence of Glu and Asp, acrylamide-quenching
309 experiments at 25°C. In Fig. 5 are reported the obtained results and Table 2 reports the values of the
310 Stern-Volmer quenching constant (K_{sv}) calculated for the GluB in the absence and in the presence
311 of Glu and Asp. At 25°C in the absence and in presence of the Glu, the K_{sv} values are 7,26 and
312 5,26 respectively. The data show a decrease of acrylamide quenching of Trp emission as
313 consequence of Glu addition. This result suggests that the Trp residues become less solvent
314 accessible following ligand bound. Similar effects have obtained with the Asp (Table 2). The linear
315 Stern-Volmer plot obtained data suggests that only one class of fluorophore is involved in the
316 quenching, as reported in Lakowicz [28].

317

318 **3.5 Lifetime measurements**

319 Finally, we have performed fluorescence decay experiments of GluB in the absence and in the
320 presence of Glu and Asp (Fig. 6). The data show that the best mathematical fit applicable with the
321 lifetime is a bi-exponential discrete distribution. The mean lifetime values reported in Table 3 do
322 not change in the absence and in the presence of the tested ligands [33].

323 3.6 FT-IR results

324 The secondary structures and thermal stability of GluB in the absence and in the presence of Glu or
325 Asp were also investigated by FTIR spectroscopy. The second derivative spectra, whose minima
326 correspond to absorption maxima, of GluB in the deuterated buffer are reported in Fig. 7A in the
327 1700-1500cm⁻¹ spectral region, where the Amide I' band (1700-1600cm⁻¹) and the tyrosine side
328 chain peak (around 1515cm⁻¹) can be observed. The Amide I' band is mainly due to the C=O
329 peptide bond absorption whose peak position is sensitive to the protein's secondary structure [30,
330 34]. In order to resolve this band into its overlapping components, the second derivative analysis of
331 the absorption spectra was performed [31]. The second derivative spectrum of GluB at 25°C
332 displayed the main Amide I' component around 1638cm⁻¹ that, along with the ~1694cm⁻¹ peak, is
333 assigned to the native β-sheets of the protein. The components at ~1656cm⁻¹ and ~1675cm⁻¹ are due
334 to α-helical and turn structures, respectively. Finally, the peak at ~1615cm⁻¹ can be assigned to
335 amino acid side chains and/or to β-sheet structures in protein-protein interactions [30, 34]. Fig. 7A
336 also shows second-derivative FTIR spectra of GluB as a function of temperature. No significant
337 spectral changes were observed up to ~52°C. Above this temperature, the Amide I' components
338 assigned to the protein native secondary structures (at ~1638, ~1656, ~1694, and ~1675cm⁻¹)
339 decreased in intensity and disappeared. At 100°C, the GluB second derivative spectrum is
340 dominated by a broad minimum at ~1646cm⁻¹, assigned to random coil structures [30, 34],
341 indicating a thermal unfolding of the protein. The ~1615cm⁻¹ peak slightly increased with the
342 temperature at became more evident after cooling of the sample from 100°C to 25°C. These results
343 indicated that a partial aggregation of the protein took place during the thermal treatments and that
344 the thermal unfolding was irreversible, under our experimental conditions. Similar spectral
345 behaviors were found in the thermal unfolding of GluB in the presence of 5mM of Glu (Fig. 7B), or
346 Asp (data not shown).

347 In all cases, changes in the peak position of the IR tyrosine response around 1515cm⁻¹ were
348 observed also in the thermal treatments, as reported in Fig. 7C. These results indicated that Glu and

349 Asp induced a stabilization of GluB protein toward thermal unfolding. For a quantitative
350 comparison, the intensity variation in the second derivatives of the native β -sheet component at
351 $\sim 1638\text{cm}^{-1}$ as a function of the temperature was employed for the T_m estimation (Fig. 7D, 7E). In
352 the presence of Glu or Asp, the T_m value of GluB increased from ~ 68 to $\sim 78^\circ\text{C}$ (Fig. 7E).
353 Therefore, these results indicated that the two amino acids increased the thermal stability of GluB
354 both considering the protein secondary (Fig. 7D and 7C) structures.

355 4 Conclusion

356 Glu is the major excitatory neurotransmitter in the brain and it is used in food industry to enhance
357 the food taste. Glu is routinely detected through analytical methods, with all the disadvantages
358 (time-consuming, high cost etc.) associated with their application. So, the application of the
359 biosensor technologies could be an alternative for the *on-field* Glu sensing. In this work we have
360 performed a preliminary structural characterization of the GluB isolated from *C. glutamicum*. We
361 explored the binding features and the effect of temperature on the protein stability in the absence
362 and in the presence of Glu by CD, FT-IR and fluorescence techniques. The obtained results show a
363 high affinity of Glu to GluB ($K_d = 0,6\text{nM}$) and the binding is associated with substrate-induced
364 stability. Only in the FT-IR experiments, difference in the T_m values were registered respect the
365 other two techniques and it is probably related to the different concentrations of the GluB used.
366 Since in nature the transport of Glu into the cells is often associated also with the L-aspartate
367 transport we have studied the binding of this aminoacid to GluB. Surprisingly, the results showed a
368 high affinity of Asp respect the Glu and its binding is associated to a stabilizing effect on GluB
369 structure.

370 Therefore, our experiments have shown that GluB have affinity for the Asp and this data suggest
371 that GluB could be involved in both aminoacids uptake in the cell and GluB is a Glu/Asp binding
372 protein (Glu/AspBP). In the future studies, we have planned to investigate in details the binding
373 discrimination between the two ligands (pH, ionic strength, etc.), but meantime we can conclude
374 that GluB could be a good candidate as MRE to develop and optical reagentless biosensor for the
375 detection of the Glu.

376

377 **5 Acknowledgments**

378 This project was partially funded by the CNR project NUTR-AGE (SD; MS; AC; AV).

379

380

381 **6 References**

- 382 [1] E. Palsson, J. Jakobsson, K. Sodersten, Y. Fujita, C. Sellgren, C.J. Ekman, H. Agren, K.
383 Hashimoto, M. Landen, Markers of glutamate signaling in cerebrospinal fluid and serum from
384 patients with bipolar disorder and healthy controls, *Eur. Neuropsychopharmacol.* 25(1) (2015) 133-
385 40.
- 386 [2] D.C. Javitt, S.R. Zukin, Recent advances in the phencyclidine model of schizophrenia, *Am. J.*
387 *Psychiatry* 148(10) (1991) 1301-8.
- 388 [3] D.M. Lovinger, G. White, F.F. Weight, Ethanol inhibits NMDA-activated ion current in
389 hippocampal neurons, *Science* 243(4899) (1989) 1721-4.
- 390 [4] B.O. Rothbaum, Critical parameters for D-cycloserine enhancement of cognitive-behavioral
391 therapy for obsessive-compulsive disorder, *Am. J. Psychiatry* 165(3) (2008) 293-6.
- 392 [5] K. Niaz, E. Zaplatic, J. Spoor, Extensive use of monosodium glutamate: A threat to public
393 health?, *EXCLI journal* 17 (2018) 273-278.
- 394 [6] O.V. Soldatkina, O.O. Soldatkin, B.O. Kasap, D.Y. Kucherenko, I.S. Kucherenko, B.A. Kurc,
395 S.V. Dzyadevych, A Novel Amperometric Glutamate Biosensor Based on Glutamate Oxidase
396 Adsorbed on Silicalite, *Nanoscale research letters* 12(1) (2017) 260.
- 397 [7] E. Swanepoel, M.M. de Villiers, J.L. du Preez, Fluorimetric method of analysis for d-
398 norpseudoephedrine hydrochloride, glycine and l-glutamic acid by reversed-phase high-
399 performance liquid chromatography, *J. Chromatogr. A* 729(1) (1996) 287-291.
- 400 [8] S. Tucci, C. Pinto, J. Goyo, P. Rada, L. Hernández, Measurement of Glutamine and Glutamate
401 by Capillary Electrophoresis and Laser Induced Fluorescence Detection in Cerebrospinal Fluid of
402 Meningitis Sick Children, *Clin. Biochem.* 31(3) (1998) 143-150.
- 403 [9] R.P. Berntsson, S.H. Smits, L. Schmitt, D.J. Slotboom, B. Poolman, A structural classification
404 of substrate-binding proteins, *FEBS Lett.* 584(12) (2010) 2606-17.

405 [10] F.A. Quioco, Atomic structures of periplasmic binding proteins and the high-affinity active
 406 transport systems in bacteria, *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 326(1236) (1990) 341-51;
 407 discussion 351-2.

408 [11] M. Strianese, M. Staiano, G. Ruggiero, T. Labella, C. Pellecchia, S. D'Auria, Fluorescence-
 409 based biosensors, *Methods Mol. Biol.* 875 (2012) 193-216.

410 [12] R.M. de Lorimier, J.J. Smith, M.A. Dwyer, L.L. Looger, K.M. Sali, C.D. Paavola, S.S. Rizk, S.
 411 Sadigov, D.W. Conrad, L. Loew, H.W. Hellenga, Construction of a fluorescent biosensor family,
 412 *Protein Sci.* 11(11) (2002) 2655-75.

413 [13] V. Scognamiglio, M. Staiano, M. Rossi, S. D'Auria, Protein-based biosensors for diabetic
 414 patients, *J Fluoresc* 14(5) (2004) 491-8.

415 [14] M. Staiano, V. Scognamiglio, G. Mamone, M. Rossi, A. Parracino, M. Rossi, S. D'Auria,
 416 Glutamine-binding protein from *Escherichia coli* specifically binds a wheat gliadin peptide. 2.
 417 Resonance energy transfer studies suggest a new sensing approach for an easy detection of wheat
 418 gliadin, *J. Proteome Res.* 5(9) (2006) 2083-6.

419 [15] W. Kronemeyer, N. Peekhaus, R. Kramer, H. Sahm, L. Eggeling, Structure of the *gluABCD*
 420 cluster encoding the glutamate uptake system of *Corynebacterium glutamicum*, *J. Bacteriol.* 177(5)
 421 (1995) 1152-8.

422 [16] L.E. Eggeling, M.E. Bott, *Handbook of Corynebacterium glutamicum*, Boca Raton: CRC Press
 423 (2005).

424 [17] H. Yukawa, M. Inui, *Corynebacterium glutamicum Biology and Biotechnology Microbiology*
 425 *monographs*, Springer, Heidelberg. (2013).

426 [18] V.F. Wendisch, J.M.P. Jorge, F. Perez-Garcia, E. Sgobba, Updates on industrial production of
 427 amino acids using *Corynebacterium glutamicum*, *World J. Microbiol. Biotechnol.* 32(6) (2016) 105.

428 [19] S. Wieschalka, B. Blombach, M. Bott, B.J. Eikmanns, Bio-based production of organic acids
 429 with *Corynebacterium glutamicum*, *Microb. Biotechnol.* 6(2) (2013) 87-102.

430 [20] S.A. Heider, V.F. Wendisch, Engineering microbial cell factories: Metabolic engineering of
431 *Corynebacterium glutamicum* with a focus on non-natural products, *Biotechnol. J.* 10(8) (2015)
432 1170-84.

433 [21] M. Vogt, C. Brusseler, J.V. Ooyen, M. Bott, J. Marienhagen, Production of 2-methyl-1-butanol
434 and 3-methyl-1-butanol in engineered *Corynebacterium glutamicum*, *Metabolic engineering* 38
435 (2016) 436-445.

436 [22] N. Kallscheuer, M. Vogt, A. Stenzel, J. Gatgens, M. Bott, J. Marienhagen, Construction of a
437 *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2S)-flavanones,
438 *Metabolic engineering* 38 (2016) 47-55.

439 [23] Q. Liu, D. Li, Y. Hu, D.C. Wang, Expression, crystallization and preliminary crystallographic
440 study of GluB from *Corynebacterium glutamicum*, *Acta crystallographica. Section F, Structural*
441 *biology and crystallization communications* 69(Pt 6) (2013) 657-659.

442 [24] J. Sambrook, D. Russell, *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold
443 Spring Harbor, NY. vol. 1–3. (2001).

444 [25] H. Edelhoch, Spectroscopic determination of tryptophan and tyrosine in proteins, *Biochemistry*
445 6(7) (1967) 1948-54.

446 [26] J. Marienhagen, N. Kennerknecht, H. Sahm, L. Eggeling, Functional analysis of all
447 aminotransferase proteins inferred from the genome sequence of *Corynebacterium glutamicum*, *J.*
448 *Bacteriol.* 187(22) (2005) 7639-46.

449 [27] M.M. Moghaddam, M. Pirouzi, M.R. Saberi, J. Chamani, Comparison of the binding behavior
450 of FCCP with HSA and HTF as determined by spectroscopic and molecular modeling techniques,
451 *Luminescence* 29(4) (2014) 314-31.

452 [28] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer, New York, (3rd edition)
453 (2006).

454 [29] D. Ami, S. Ricagno, M. Bolognesi, V. Bellotti, S.M. Doglia, A. Natalello, Structure, stability,
455 and aggregation of beta-2 microglobulin mutants: insights from a Fourier transform infrared study
456 in solution and in the crystalline state, *Biophys. J.* 102(7) (2012) 1676-84.

457 [30] A. Natalello, S.M. Doglia, Insoluble protein assemblies characterized by fourier transform
458 infrared spectroscopy, *Methods Mol. Biol.* 1258 (2015) 347-69.

459 [31] H. Susi, D.M. Byler, Resolution-enhanced Fourier transform infrared spectroscopy of
460 enzymes, *Methods Enzymol* 130 (1986) 290-311.

461 [32] N. Akbay, J.R. Lakowicz, K. Ray, Distance-dependent intrinsic fluorescence of proteins on
462 aluminum nanostructures, *Proc SPIE Int Soc Opt Eng* 8234 (2012) 823417.

463 [33] P. Herman, M. Staiano, A. Marabotti, A. Varriale, A. Scire, F. Tanfani, J. Vecer, M. Rossi, S.
464 D'Auria, D-trehalose/D-maltose-binding protein from the hyperthermophilic archaeon
465 *Thermococcus litoralis*: the binding of trehalose and maltose results in different protein
466 conformational states, *Proteins* 63(4) (2006) 754-67.

467 [34] A. Barth, Infrared spectroscopy of proteins, *Biochim. Biophys. Acta* 1767(9) (2007) 1073-101.
468
469

470 **Table 1.** Melting temperature of GluB in the absence and in the presence of Glu and Asp.

471

	Circular Dichroism T _m (°C)	Steady-state Fluorescence T _m (°C)
GluB	40 ± (0.8)	40 ± (0.8)
GluB + 100μM Glu	53 ± (0.3)	50 ± (0.4)
GluB + 100μM Asp	53 ± (1.2)	55 ± (1.4)

472

473

474 **Table 2.** Stern-Volmer analysis.

475

	K_d	R-Square
GluB	7.04±0.23	0.99652
GluB + 100μM Glu	5.26±0.13	0.99837
GluB + 100μM Asp	5.26±0.12	0.99853

476

477

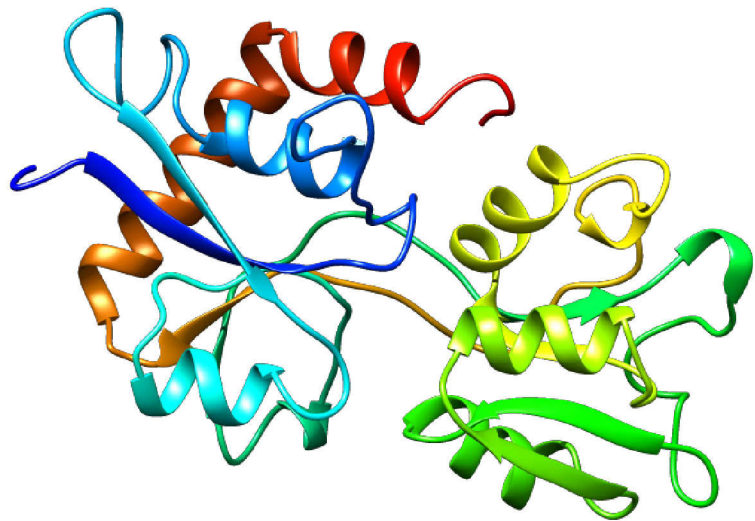
478 **Table 3.** Lifetime analysis.

479

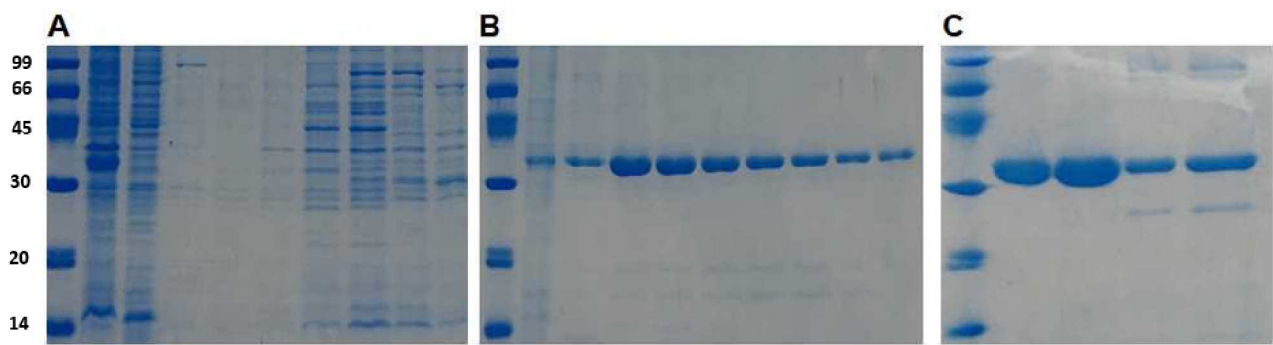
	Average Lifetime [ns]	χ^2
GluB	3.40	2.40
GluB + 100μM Glu	3.40	2.14
GluB + 100μM Asp	3.37	1.78

480

481 **Figures**

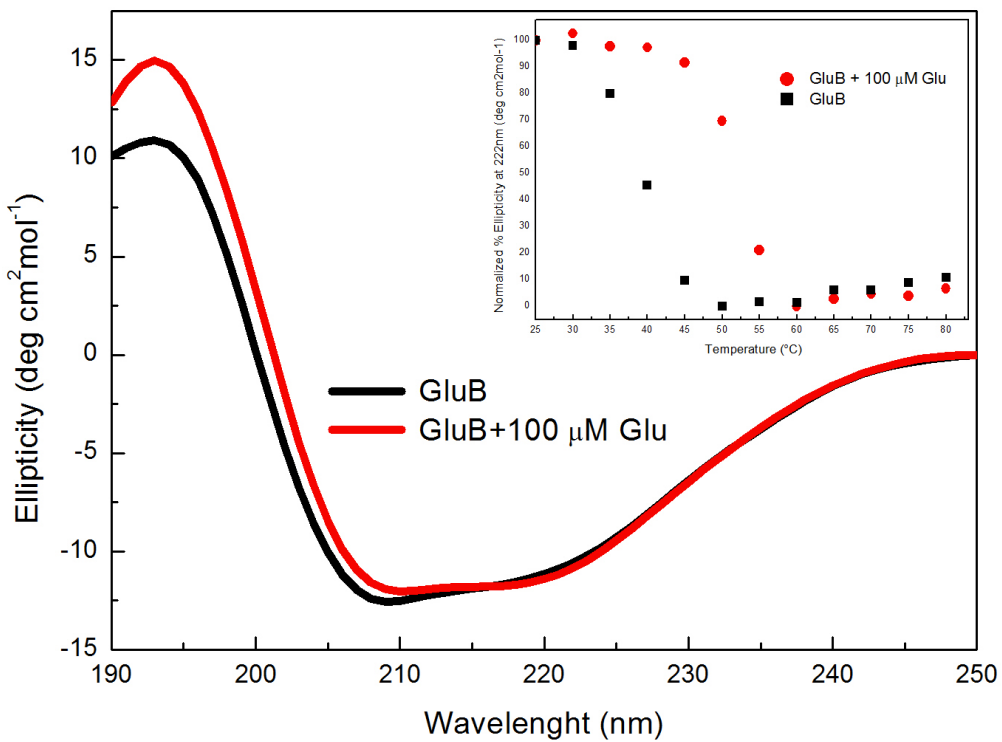


482
483 **Figure 1. GluB homology model structure.** The picture shows the GluB homology model
484 calculated by Swiss-Model software and collected in Swiss-Model repository database
485 (<https://swissmodel.expasy.org/repository/uniprot/P48242>).
486

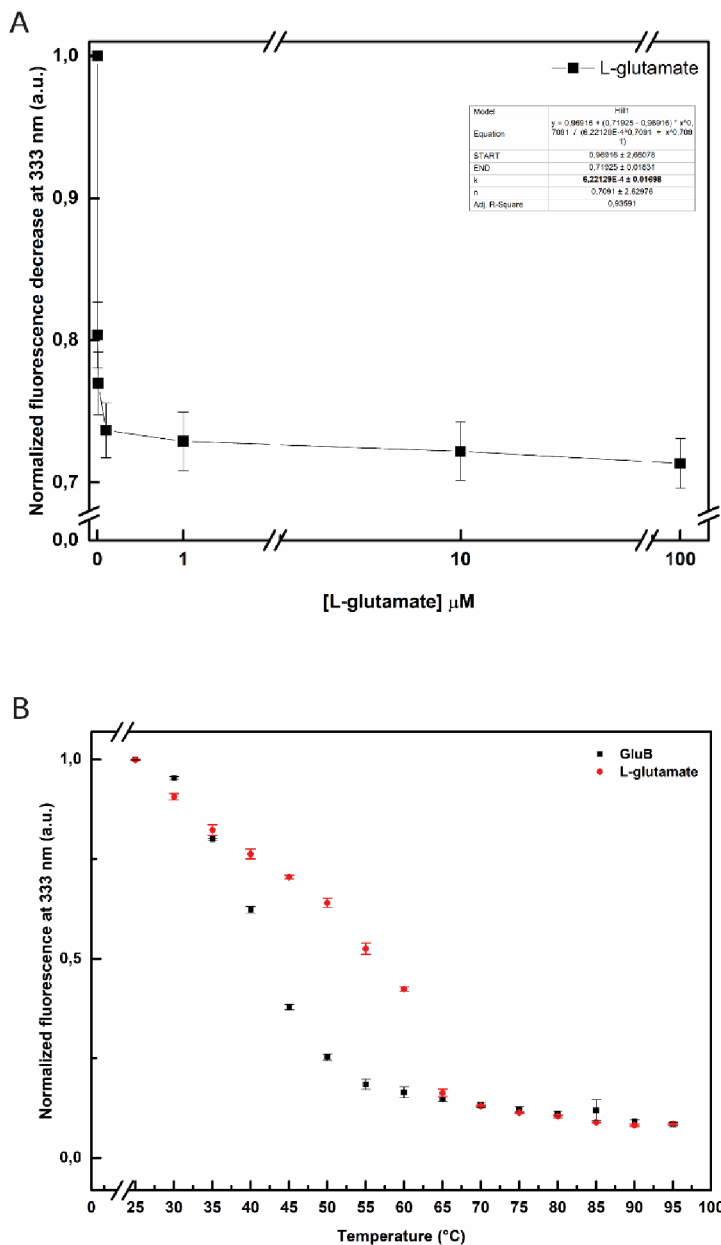


487
488 **Figure 2. SDS-PAGE of the fractions obtained from chromatographic purification. (A)**
489 Affinity chromatography: (molecular weight standard, input, flow-through, wash, fractions 1 to 5
490 eluted at 50mM imidazole). **(B)** Affinity chromatography: (molecular weight standard, fractions 10,
491 11, 15, 20, 25, 30, 35, 40, 45 eluted at 50mM imidazole). **(C)** Size exclusion chromatography

492 (molecular weight standard, collected protein fractions after (line 2 and 3) and before (line 4 and 5)
493 the gel filtration step).
494



495
496 **Figure 3. Circular dichroism results.** Far-UV CD spectra of GluB in the absence and in presence
497 of 100 μ M of Glu at 25 $^{\circ}$ C. Inset: variation of the molar ellipticity at 222nm in the function of
498 temperature in the absence and in the presence of Glu.
499



500

501 **Figure 4. Fluorescence steady-state measurements: ligand binding and thermal stability**

502 **evaluation. (A)** Variation of the GluB fluorescence emission at 333nm in the presence of increase

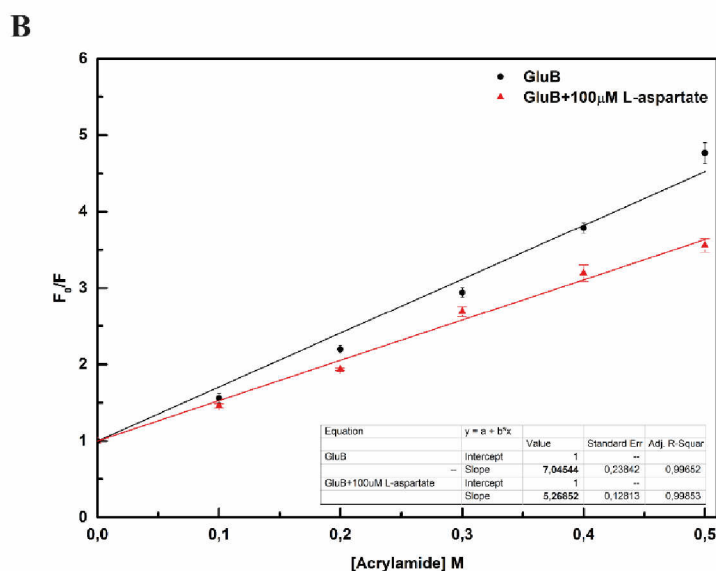
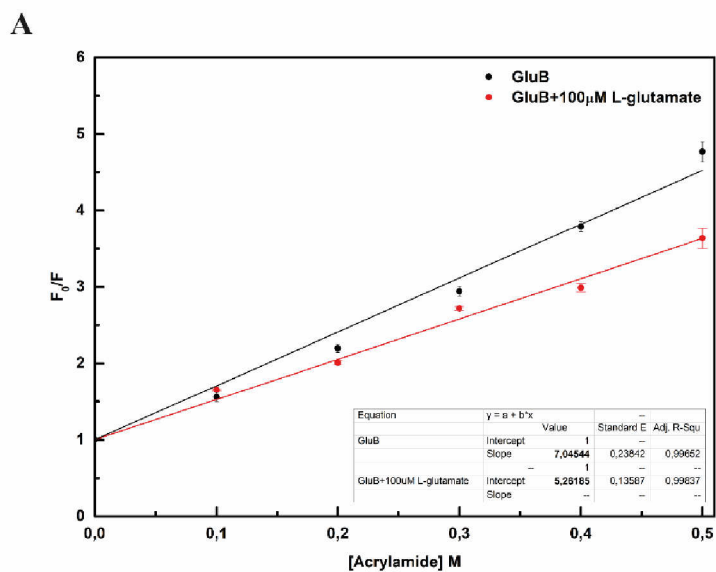
503 concentration of Glu. Excitation of two Trp residues (W58 and W65) was performed at 295 nm in

504 5mM phosphate buffer, pH 8.0. **(B)** GluB thermal unfolding: variation of the Trp emission in the

505 absence and in presence of 100μM Glu. Fluorescence measurements obtained at an excitation

506 wavelength of 295 nm in 5 mM phosphate buffer at pH 8.0.

507



508

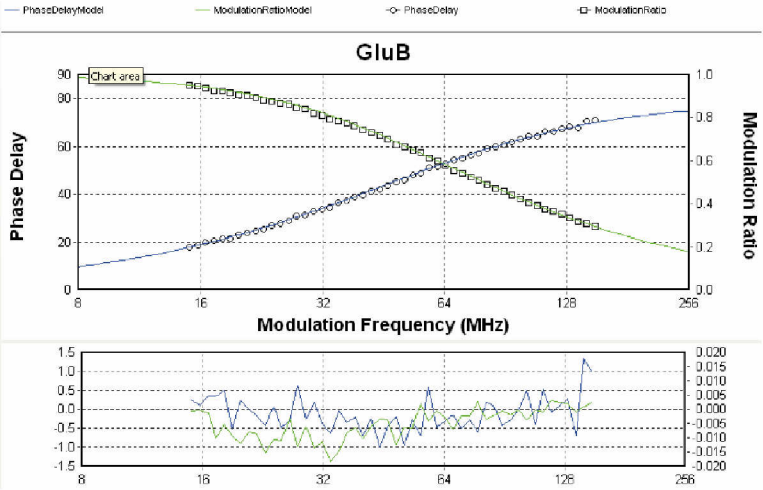
509 **Figure 5. Quenching measurements.** Steady-state acrylamide quenching of GluB in the absence

510 (closed circles) and in the presence (red circles) of 100 μM Glu, and in the presence of 100 μM Asp

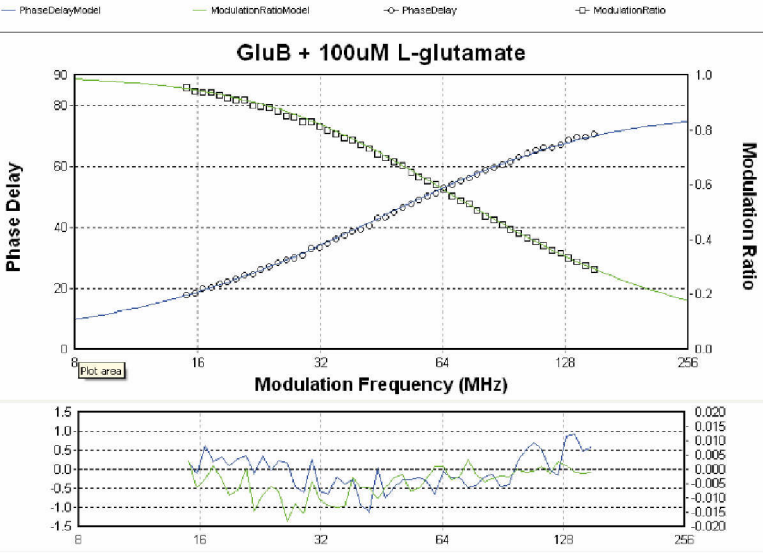
511 (red triangles). The measurements performed at 25°C.

512

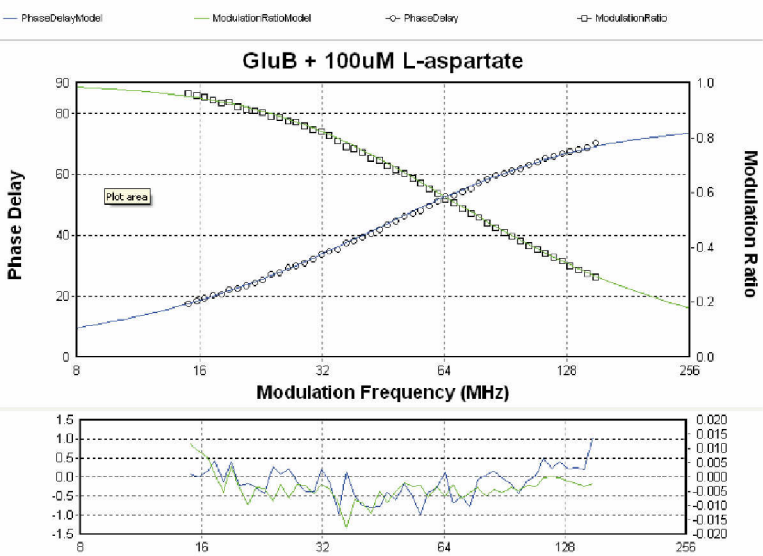
A



B



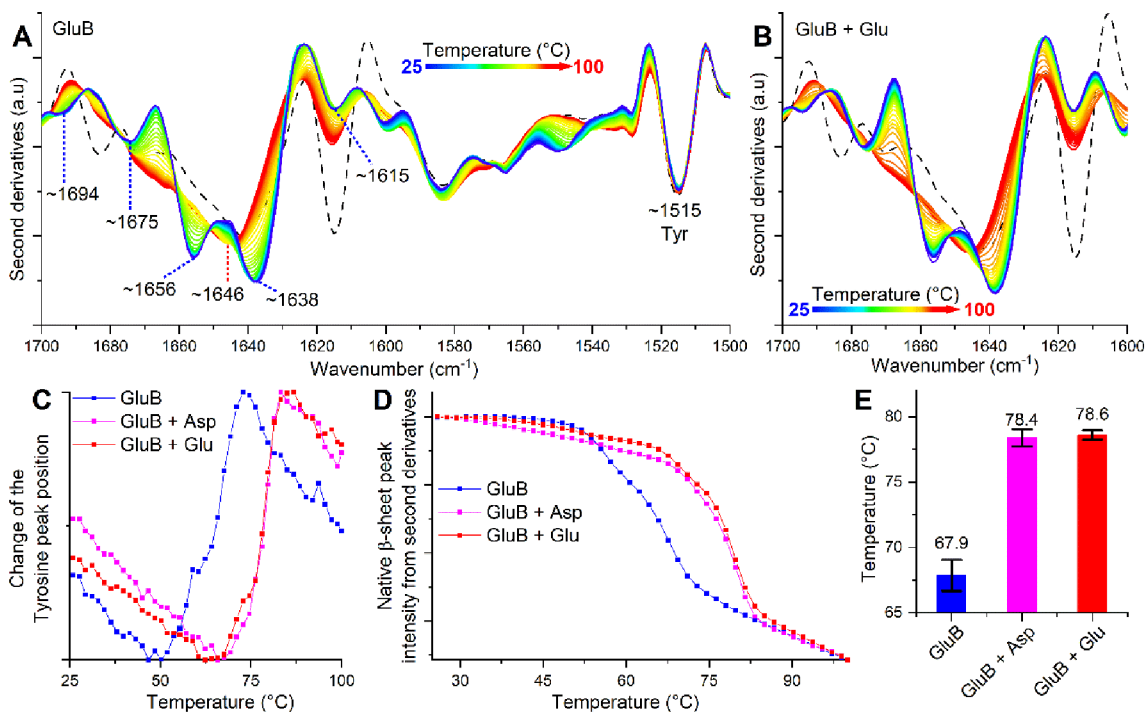
C



514 **Figure 6. Lifetime measurements.** In the figures are reported the bi-exponential fitting analysis for
 515 (A) GluB, (B) GluB/Glu, and (C) GluB/Asp. The bottom part of each panel reports the residual
 516 analysis of the fitting procedure.

517

518



519

520 **Figure 7. GluB thermal unfolding monitored by FTIR.** (A) Second derivative FTIR spectra of 6
 521 mg/mL GluB in deuterated phosphate buffer at different temperatures between 25 and 100°C. The
 522 spectrum taken at 25°C after the thermal treatment (dashed line) is also reported. Spectra are shown
 523 in the 1700-1500 cm^{-1} regions. The peak position of the main components is indicated. (B) Second
 524 derivative FTIR spectra of 6 mg/mL GluB and 5mM Glu in deuterated phosphate buffer at different
 525 temperatures between 25 and 100°C. The spectrum taken at 25°C after the thermal treatment
 526 (dashed line) is also reported. Spectra are shown in the 1700-1600 cm^{-1} region. (C) The temperature
 527 dependence of the peak position of the ~1515 cm^{-1} tyrosine band, taken from second derivative
 528 spectra, is reported for GluB in the absence and in the presence of 5mM of Glu or Asp. (D) The
 529 temperature dependence of the native β -sheet peak intensity at ~1638 cm^{-1} , taken from second
 530 derivative spectra, is reported for GluB in the absence and in the presence of 5mM of Glu or Asp.

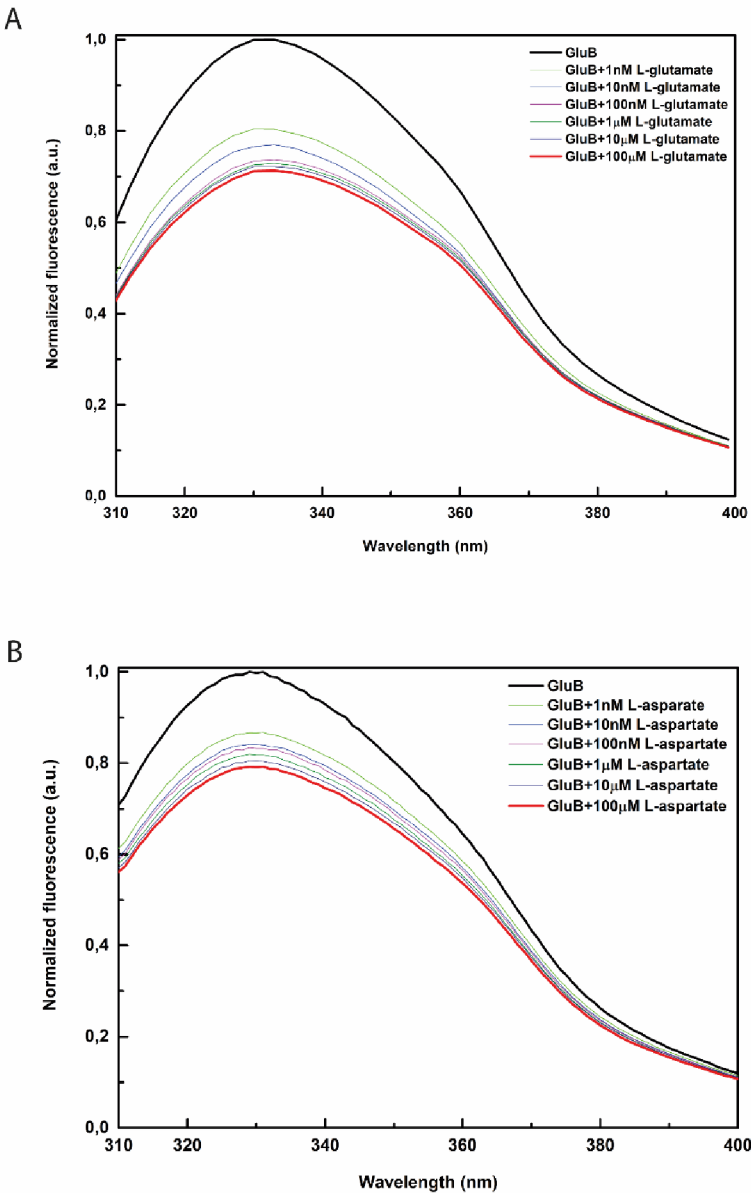
531 **(E)** T_m values obtained from the data of panel **(D)**. Error bars are the standard deviations of
532 independent thermal unfolding experiments.

533

534 **7 Supplementary materials**

535 **Fig. S1.** Steady state fluorescence measurements: **(A)** normalized fluorescence emission spectra of
536 the L-glutamate titration experiments, and **(B)** normalized fluorescence emission spectra of the L-
537 aspartate titration experiments.

538



539

540

541 **Fig. S2.** Steady state fluorescence measurements: **(A)** GluB/Asp binding curve and **(B)** GluB/Asp
542 thermal stability.

