1   **Kinetic mechanism of coupled binding in sodium-aspartate symporter GltPh**

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6

7 **Abstract**

8 Many secondary active membrane transporters pump substrates against concentration gradients

9 by coupling their uptake to symport of sodium ions. Symport requires the substrate and ions to

10 be always transported together. Cooperative binding of the solutes is a key mechanism

11 contributing to coupled transport in the sodium and aspartate symporter from *Pyrococcus*

12 *horikoshii* Glt_{Ph}. Here, we describe the kinetic mechanism of coupled binding for Glt_{Ph} in the

13 inward facing state. The first of the three coupled sodium ions, binds weakly and slowly,

14 enabling the protein to accept the rest of the ions and the substrate. The last ion binds tightly, but

15 is in rapid equilibrium with solution. Its release is required for the complex disassembly. Thus,

16 the first ion serves to "open the door" for the substrate, the last ion "locks the door" once the

17 substrate is in, and one ion contributes to both events.

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Introduction

21   Glt_{Ph} is an archaeal homologue of glutamate transporters (excitatory amino acid

22   transporters, EAATs), which move the neurotransmitter from the synaptic cleft into the

23   cytoplasm of glial cells and neurons, thereby terminating neurotransmission events (Danbolt,

24   2001). Glutamate transport by EAATs is driven by symport of three sodium (Na^+) ions and one

25   proton and by antiport of a potassium ion (Zerangue and Kavanaugh, 1996, Levy et al., 1998,

26   Owe et al., 2006). Under pathophysiologic conditions such as ischemia or stroke, ionic gradients

27   across membranes dissipate, and EAATs operate in reverse, releasing glutamate from the cells.

28   Sustained elevated glutamate concentrations lead to excitotoxicity and ultimately to cell death

29   and brain damage (Yi and Hazell, 2006, Sheldon and Robinson, 2007).

30   Glt_{Ph}, which shares ~35 % amino acid sequence identity with EAATs, has served as a

31   structural and mechanistic model for this family of transporters (Yernool et al., 2004, Boudker et

32   al., 2007, Reyes et al., 2009, Reyes et al., 2013, Akyuz et al., 2013, Akyuz et al., 2015, Verdon et

33   al., 2014, Scopelliti et al., 2018). Like EAATs, it symports the substrate aspartate (L-asp)

34   together with three Na^+ ions (**Figure 1 - figure supplement 1**), but is independent of protons or

35   potassium ions (Ryan et al., 2009, Groeneveld and Slotboom, 2010, Boudker et al., 2007).

36   Structural studies on Glt_{Ph}, a closely related Glt_{Tk}, and human EAAT1 showed that these proteins

37   are homotrimers (Yernool et al., 2004, Canul-Tec et al., 2017, Guskov et al., 2016). Each

38   protomer consists of a peripheral transport domain, which is a mobile unit harboring the

39   substrate- and ion-binding sites, and a rigid central scaffold or trimerization domain (**Figure 1 -**

40   **figure supplement 1**) (Reyes et al., 2009). The conformational transitions between the outward-

41   and inward-facing states involve translocation of the transport domain across the membrane by

42   ~15 Å in a stochastic, thermally driven process that occurs independently in individual subunits

43   (Reyes et al., 2009, Akyuz et al., 2013, Hanelt et al., 2013, Erkens et al., 2013, Georgieva et al.,

44   2013, Ruan et al., 2017, Verdon and Boudker, 2012).

45   When substrate binds to the transporter in the outward-facing state, a helical hairpin (HP)

46   2 acts as a gate (Verdon et al., 2014, Akyuz et al., 2013, Focke et al., 2011, Jensen et al., 2013).

47   In the apo transporter and in the transporter bound to both L-asp and Na^+ ions, HP2 closes down

48   over the substrate-binding site, but it remains open when the transporter is bound to Na^+ ions

49   only (**Figure 1 – figure supplement 2**). Inability of HP2 to close in the absence of substrate may

50   prevent uncoupled ion transport (Verdon et al., 2014). HP2 assumes an even more open

51 conformation in the structures of Glt$_{Ph}$ and EAAT1 bound to a blocker DL-*threo*-β-

52 benzyloxyaspartic acid (DL-TBOA) and to other non-transportable inhibitors (Boudker et al.,

53 2007, Canul-Tec et al., 2017, Scopelliti et al., 2018). The conformations of the apo and substrate-

54 bound transport domains are identical in the outward- and inward-facing states (Reyes et al.,

55 2009, Verdon et al., 2014), though what constitutes a gate in the latter state is unclear. HP1,

56 related to HP2 by the structural pseudo-symmetry, was considered as a possible gate (Reyes et

57 al., 2009), but it lacks the abundance of glycine residues that confers flexibility to HP2 (Verdon

58 et al., 2014). HP2 itself is constrained at the interface between the transport domain and the

59 scaffold. Nevertheless, inward-facing Glt$_{Ph}$ binds DL-TBOA and the structurally related inhibitor

60 L-*threo*-β-benzylaspartic acid (L-TBA), suggesting that a conformation with an open HP2 exists

61 (Reyes et al., 2013). Consistently, a crystal structure of an inward-facing mutant R276S/M395R,

62 dubbed "fast mutant" because it transports L-asp about four times faster than the wild type Glt$_{Ph}$

63 (Ryan et al., 2010), pictured the transport domain leaning away from the scaffold leaving

64 sufficient space for HP2 to open (Akyuz et al., 2015).

65 The linchpin of the coupled transport in Glt$_{Ph}$ is the cooperative binding of L-asp and

66 three Na$^+$ ions, such that alone the solutes bind with low affinity, but together they bind tightly

67 (Reyes et al., 2013). Both outward- and inward-facing transporter shows this property as has

68 been demonstrated using conformationally constrained variants. These were generated by cross-

69 linking double cysteine mutants strategically placed into the scaffold and transport domains

70 (Reyes et al., 2013). Consequently, when the substrate-binding site faces the extracellular

71 solution containing high Na$^+$ concentration, the substrate affinity is high and when the site is

72 exposed to the low cytoplasmic Na$^+$ concentrations, the affinity drops and the substrate is

73 released. While the thermodynamics of binding is well characterized, what is the kinetic role of

74 Na$^+$ ions is less well understood. Equilibrium studies showed that at least two Na$^+$ ions are able

75 to bind weakly to both outward- and inward-facing transporter in the absence of L-asp.

76 Therefore, it was postulated that their binding preceded L-asp binding (Reyes et al., 2013).

77 Consistently, kinetic studies on conformationally unconstrained Glt$_{Ph}$ variants showed that

78 binding of Na$^+$ ion or ions preceded binding of the substrate (Hänelt et al., 2015, Ewers et al.,

79 2013). Notably, these studies were difficult to interpret mechanistically because reorientation of

80 the transport domain might have contributed to the observed events. Nevertheless, they likely

81   approximated binding in the outward-facing state because the unconstrained Glt_{Ph} samples this

82   state preferentially (Georgieva et al., 2013, Akyuz et al., 2013, Ruan et al., 2017).

83   Here we describe a kinetic mechanism underlying the thermodynamically coupled

84   binding and release of three Na^+ ions and L-asp in the inward-facing state of the transporter.

85   Using a tryptophan mutant of the conformationally constrained inward facing transporter, we

86   find that both binding and dissociation of L-asp are slow and controlled by Na^+ ions.

87   Specifically, we show that binding of the first Na^+ ion is rate limiting to the complex formation.

88   We further show that once the complex is assembled, at least one Na^+ ion is in rapid equilibrium

89   with solution, and its dissociation is pre-requisite for the complex disassembly. Based on these

90   findings, we construct a minimal kinetic model and show that there are no unique binding and

91   disassembly mechanisms, but rather probabilistically weighted reaction paths. Which path is

92   taken is controlled by Na^+ concentration in solution. However regardless of the path,

93   combination of the very slow weak binding of the first Na^+ ion and very rapid and tight binding

94   of the last Na^+ ion ensures that neither equilibrium nor kinetic intermediates are populated during

95   Na^+ and L-asp unloading and loading, provided both solutes are encountered by the transporter at

96   the same time.

97

98   **Results**

99   **Na^+ ions control binding and unbinding rates of L-asp.**

100   To detect ligand binding to the inward-facing state of Glt_{Ph}, we introduced a tryptophan

101   mutation, P11W in the trans-membrane (TM) segment 1 of the scaffold domain within the

102   context of Hg^{2+}-crosslinked K55C/C/321A/A364C mutant (Glt_{Ph}^{IFS}), referred to as $P11W^{IFS}$

103   (**Figure 1a**). Notably, Glt_{Ph} contains no native tryptophans. The fluorescence of P11W increases

104   upon coupled binding of Na^+ ions and L-asp or blocker DL-TBOA (**Figure 1b** and **Figure 1 –**

105   **figure supplement 3**). The affinity of $P11W^{IFS}$ for L-asp was similar to that of the wild type

106   Glt_{Ph}^{IFS} over a range of Na^+ concentrations (**Figure 1c**). The slope of the log-log plot of L-asp

107   dissociation constants as a function of Na^+ concentration was 2.5, similar to that of the wild type

108   transporter, suggesting a nearly complete coupling between L-asp and three Na^+ ions. Taken

109   together, these results show that the binding process is not significantly affected by P11W

110   mutation.

The tight thermodynamic coupling has to manifest in strong dependence of L-asp binding on- and/or off-rates on Na^+ concentration. If all three Na^+ ions were coupled to L-asp through accelerating binding, the L-asp binding rate would show cubic dependence on Na^+ concentration. Upon mixing the transporter with Na^+ ions and L-asp, the equilibration process would be described by a single exponential yielding a rate constant, k_{obs}:

$$k_{obs} = k_{off} + k_{on}[Na^+]^3[asp] \qquad (1)$$

where k_{on} and k_{off} are binding and dissociation rate constants, respectively. Addition of L-asp to the apo P11W^{IFS} in the presence of variable concentrations of Na^+ ions led to a time-dependent increases of fluorescence signal (**Figure 1 – figure supplement 3c**) that were well described by single exponentials, yielding k_{obs} values. As expected, the equilibration rates increased with increased Na^+ concentration (**Figure 2a**), but not as steeply as Equation 1 requires, showing quadratic dependence at low L-asp concentrations and linear dependence at high L-asp concentrations (**Figure 2 – figure supplement 1**). These observations suggest that binding of one or two Na^+ ions facilitate the amino acid binding.

These data further suggest that the role of Na^+ ions cannot be limited to accelerating L-asp binding and that the ions should also slow L-asp dissociation. To test this hypothesis, we prepared a P11W^{IFS} stock pre-bound to L-asp and Na^+ ions. We then diluted the stock to the final concentration of 0.25 µM transporter, 0.25 µM L-asp and Na^+ ions varying between 50 µM and 1 mM. Under these conditions, L-asp is expected to dissociate from the protein nearly completely as the K_D for L-asp is ~100 µM at 1 mM Na^+ (**Figure 1c**). Upon dilution, we observed gradual decreases of fluorescence reflecting L-asp and Na^+ dissociation, which were well described by single exponential decay functions (**Figure 1 – figure supplement 3d**). The obtained $k_{obs,d}$ decreased steeply with increased Na^+ concentrations (**Figure 2b**). This behavior is in contrast to Equation 1, which presumes that k_{off} is Na^+-independent. To explain it, we hypothesized that one or more Na^+ ions were in rapid equilibrium between the complex and solution and that L-asp dissociated only when the ions were off (**Figure 2c**). If so, the rapidly equilibrating Na^+ ions would respond to the changes of the ion concentration in the media, and the rate of L-asp dissociation would depend on the fraction of the transporter free of the ions:

141

$$k_{obs,d} = k_{off,asp} \frac{K_{D,Na}^n}{[Na^+]^n + K_{D,Na}^n} \qquad (2)$$

143

144 where $k_{off,asp}$ is the intrinsic rate constant of L-asp dissociation once the rapidly equilibrating Na^+

145 ions are released, $K_{D,Na}$ is the dissociation constant of the equilibrating Na^+ ions and n is the Hill

146 coefficient. Hill coefficients between 1 and 3 described data similarly, producing fitted $K_{D,Na}$

147 values between 30 and 150 μM, respectively. The $k_{off,asp}$ is between 0.5 and 0.2 s^{-1} suggesting

148 that even when the locking Na^+ ion(s) are released, it still takes approximately 2 to 5 seconds for

149 the rest of the complex to disassemble.

150   These experiments demonstrate that as the concentration of Na^+ ions in the media

151 increases, L-asp release slows down. Consistently, we observed that in proteoliposomes

152 containing Glt_{Ph}, the rate of concentrative Na^+-driven uptake of radiolabeled L-asp into vesicles

153 containing Na^+-free buffer was significantly higher than the rate of L-asp exchange when both

154 vesicle internal and external solutions contained L-asp and Na^+ ions (**Figure 2d**). In the latter

155 experiment, the proteoliposomes are loaded with high Na^+ and cold L-asp that exchanges with

156 radiolabeled L-asp in the external solution. Under these conditions, high Na^+ concentrations on

157 both sides of the membrane slow the release of the substrate from the transporter, resulting in a

158 slow exchange process. The Na^+-dependent release mechanism is also consistent with kinetic

159 measurements on the mammalian EAAT4, for which the transport cycle was highly sensitive to

160 the cytoplasmic Na^+ ions concentrations (Wadiche et al., 2006). These results further explain

161 why the measured Km values for Glt_{Ph}, ~100 nM at 100 mM NaCl (Ryan et al., 2009), are

162 significantly higher than L-asp K_D of ~1 nM measured in detergent solutions: L-asp bound to the

163 transporter is more likely to be translocated into the vesicle and released into Na^+-free lumen

164 than to dissociate back into the media containing high Na^+ concentration.

165   Collectively, these experiments show that one or two Na^+ ions control the on-rate of L-

166 asp binding and one or more ions control the off-rate. The importance of the Na-dependent off-

167 rate in cooperative Na^+ and L-asp binding is consistent with the observation that introducing

168 M311A mutation, which leads to largely Na^+-independent L-asp binding (Verdon et al., 2014),

169 into P11W[IFS] eliminates Na^+ dependence of L-asp dissociation rates (**Figure 2b**).

170

171 **Na^+ ions drive conformational transition into a binding competent state.**

172   Plotting equilibration k_{obs} constants as a function of L-asp concentration shows that the
173   dependence is not linear, as Equation 1 would predict, but that instead k_{obs}-s plateau at high L-
174   asp concentrations (**Figure 3a**). Thus, slow conformational transitions independent of L-asp
175   become rate limiting. Typically, two mechanisms are invoked to explain such behavior: induced
176   fit and conformational selection. According to induced fit mechanism, initial weak binding is
177   followed by a slow isomerization into a high affinity state. In conformational selection, the slow
178   isomerization into a high affinity state precedes binding (Vogt and Di Cera, 2012). Either of
179   these mechanisms can lead to k_{obs} values that level off with increased ligand concentration
180   approaching a maximum. Notably, the observed plateaus are Na^+-dependent; therefore, the rate-
181   limiting step involves Na^+ binding.

182   To further probe the mechanism of complex formation, we examined the kinetics of DL-
183   TBOA binding to P11W^{IFS} because it is thought to recapitulate early events of substrate binding
184   that precede closure of HP2 and Na^+ binding to Na2 site (**Figure 1 – figure supplement 2**)
185   (Reyes et al., 2013). Consistently in equilibrium experiments, binding of L-TBOA to
186   unconstrained Glt$_{Ph}$ and of L-TBA to Glt$_{Ph}$^{IFS} showed reduced Na^+ coupling (Boudker et al.,
187   2007, Reyes et al., 2013). Addition of DL-TBOA to P11W^{IFS} at variable Na^+ concentrations led
188   to time-dependent increases of tryptophan fluorescence that were fitted to single exponentials
189   (**Figure 1 – figure supplement 3e**). Strikingly, equilibration rate constants decreased with
190   increased concentrations of DL-TBOA, asymptotically approaching minima (**Figure 3b**). Such
191   behavior is a hallmark of conformational selection mechanism (Vogt and Di Cera, 2012). It
192   occurs because the binding-competent conformation is a minor state sampled by the protein. The
193   higher is the concentration of the added ligand, the larger proportion of the protein slowly
194   isomerizes into the binding-competent state that is then rapidly sequestered by the ligand.
195   Therefore, it takes longer for the system to equilibrate at higher ligand concentrations until
196   saturation is reached. Because the plateau values increased at increased Na^+ concentrations, the
197   slow isomerization must be coupled to Na^+ binding. Thus, we envision that one or more Na^+ ions
198   bind slowly and weakly to the transporter, so that only a small fraction of the protein is bound to
199   the ion alone. Under 'rapid equilibrium approximation', where binding and unbinding of DL-
200   TBOA are assumed to be faster than the preceding conformational changes, associated with Na+
201   ion binding (**Figure 3c**), we can fit the data to an equation:
202

203 $$k_{obs} = k_r + k_{-r} \frac{K_{D,TBOA}}{K_{D,TBOA} + [TBOA]} \qquad (3)$$

204

205 where k_r and k_{-r} are the forward and reverse rate constants of the conformational change and

206 $K_{D,TBOA}$ is the affinity of the inhibitor for the high affinity state of the transporter. Notably, k_r are

207 Na⁺-dependent since they reflect Na⁺ ion binding. The asymptotic values of k_r are resolved by

208 the data, but k_{-r} and $K_{D,TBOA}$ are correlated and could not be determined. Plotted as a function of

209 Na⁺ concentration, k_r values show linear dependence (**Figure 3d**) consistent with binding of one

210 Na⁺ ion coupled to the slow conformational transition.

211   Based on these data, we hypothesized that L-asp and DL-TBOA share the same rate-

212 limiting Na⁺ binding event that is prerequisite for the amino acid binding. In contrast to DL-

213 TBOA, the rates of L-asp binding increased with ligand concentration. This is expected to be the

214 case when the rate of L-asp dissociation, k_{off} is slower than k_{-r} (**Figure 3c**). In this case 'rapid

215 equilibrium approximation' is not valid, and the data must be described by a full equation (Vogt

216 and Di Cera, 2012):

217

218 $$k_{obs} = \frac{k_{-r} + k_r + k_{off} + k_{on}[asp] - \sqrt{(k_{off} + k_{on}[asp] - k_{-r} - k_r)^2 + 4k_{-r}k_{on}[asp]}}{2} \qquad (4)$$

219

220 Most of the rate constants in the above equation are not resolved by the data, but k_r values are

221 well determined as they correspond to the plateaus reached at high ligand concentrations (see

222 Methods for details). The obtained k_r values for L-asp binding also depended linearly on Na⁺

223 concentration with a slope of 36 M⁻¹ s⁻¹, similar to that of DL-TBOA (**Figure 3d**). We note that

224 while similar, the slope for DL-TBOA k_r values is smaller than that for L-asp, and the linear fit

225 does not pass through zero when unconstrained. This might be because at higher Na⁺

226 concentrations used in DL-TBOA binding experiments, Na⁺ binding itself might become rate-

227 limited by a Na⁺-independent conformational transition.

228

229 **Kinetic model of coupled Na⁺ and L-asp binding**

230 Our data suggest that binding of the first Na⁺ ion is rate limiting and drives a conformational

231 transition of the transporter into a state competent to bind the remaining Na⁺ ions and L-asp or

232 DL-TBOA. Once the complex is fully formed, at least one Na⁺ ion is able to rapidly equilibrate

- 9 -

233   with the solution; its release is prerequisite for L-asp dissociation. The role of the third Na^+ ion

234   remains ambiguous and it may contribute to both processes. Putting these considerations

235   together, we have constructed the most parsimonious kinetic model compatible with the

236   experimental data. The proposed model (**Figure 4a**) includes the initial slow Na^+ binding with

237   the rate constant of 36 M^{-1} s^{-1}, final Na^+ binding rapidly with a rate constant set arbitrarily to 10^7

238   M^{-1} s^{-1}, and two alternative branches reflecting the ambiguous role of the second Na^+ ion. The

239   model contains twelve rate constants, which we estimated by fitting raw binding and dissociation

240   data globally after fixing parameters for Na^+ binding (see Methods for details). The constants

241   that describe the rate limiting steps in the binding and dissociation reactions are well determined

242   by the data. Other rate constants are only defined in terms of the lower limits (i.e., faster than the

243   rate-limiting steps). The obtained rate constants (**Figure 4a** and **Figure 4 – figure source data**

244   **1**) recapitulate well most of the kinetic data for L-asp binding and dissociation (**Figure 4 –**

245   **figure supplement 1a-c**). Only minor changes were required to reproduce DL-TBOA binding

246   kinetics limited to about 20-fold increase in the rate of DL-TBOA unbinding (**Figure 4 – figure**

247   **supplement 2** and **Figure 4 – figure source data 1**). As expected simulated data were in good

248   agreement with experiment at lower Na^+ concentrations, but diverged somewhat at higher Na^+

249   concentrations. The fitted rate constants of L-asp binding and unbinding also yield L-asp

250   equilibrium dissociation constant similar to experimental at 0.5 mM Na^+ (**Figure 4 – figure**

251   **supplement 1d**). However, the predicted Na^+ dependence of L-asp binding is steeper than the

252   experimentally measured, and there is an increasing divergence between the predicted and

253   measured constants at increased Na^+ concentrations. This discrepancy is likely due to our model

254   predicting that the L-asp unbinding rates continue to decrease at increasing Na^+ concentrations

255   (**Figure 3** and **Figure 4 – figure supplement 1b**). It is possible, however, that an alternative

256   disassembly mechanism becomes significant at higher Na^+ concentrations.

257   The relative fluxes through the two alternative reaction branches, "second Na^+ ion binds

258   first" (reactions 2 and 4 in **Figure 4a**) and "L-asp binds first" (reactions 3 and 5), determine the

259   shape of dependencies of k_{obs} and $k_{obs,d}$ on Na^+ concentration and constrain the ratios of several

260   rate constants (Equations 7 and 8 in Methods). Error analysis shows that excluding either of the

261   branches from the fit leads to 20 - 40 % increase in the sums of χ^2, confirming that both branches

262   contribute to the reaction. The flux through "second Na^+ ion binds first" both during binding and

263   dissociation of the complex is proportional to Na^+ concentration and is the predominant

264   mechanism at high ion concentration (Equation 7 and 8 and **Figure 4b**). However, the alternative

265   route (reactions 3 and 5) becomes dominant at Na$^+$ concentrations below ~3.6 mM (**Figure 4b**).

266   Therefore, both complex assembly and disassembly mechanisms should be described as

267   probabilistic reaction paths rather than unique mechanisms. Regardless of the reaction path used,

268   the three Na$^+$ ions and L-asp bind to P11W^{IFS} in a concerted manner with no equilibrium or

269   kinetic intermediates (**Figure 4c**). The disassembly of the complex also proceeds without

270   intermediates at Na$^+$ concentrations of ~1 mM and above (**Figure 4c**). Only when Na$^+$

271   concentration approaches or falls below the dissociation constant for the rapidly equilibrating

272   Na$^+$ ions, a kinetic intermediate TANa becomes populated.

273

Increased rate of substrate binding to the 'fast mutant' of Glt$_{Ph}$

275   A potential concern with our experiments is the use conformationally constrained transporter

276   mutants. To test whether cross-linking limits the rates of substrate gating, we examined binding

277   to the so-called "fast mutant" R276S/M395R (FM), which shows about four times faster turnover

278   rate compared to the wild type Glt$_{Ph}$ (Ryan et al., 2010). The increased transport rate was

279   attributed to the increased frequency of transitions of the transport domain from the outward to

280   the inward facing position (Akyuz et al., 2015). The crystal structure of the mutant captured a

281   distinct inward-facing conformation that was termed "unlocked" because the transport domain

282   leaned away from the scaffold, potentially providing space for HP2 to open (**Figure 5a**). To

283   examine whether binding and unbinding events are accelerated in this mutant, we introduced

284   R276S/M395R mutations within P11W^{IFS} background (FM-P11W^{IFS}). We also determined the

285   crystal structure of crosslinked K55C/C321A/A364C/R276S/M395R Glt$_{Ph}$ (FM-Glt$_{Ph}$^{IFS}). The

286   structure was very similar to the structure of Glt$_{Ph}$^{IFS}, i.e. none of the protomers were in the

287   "unlocked" conformation and packed well against the scaffold (**Figure 5a** and **Table 1**).

288   Nevertheless, the affinity of FM-P11W^{IFS} for L-asp was similar to that of the unconstrained FM-

289   Glt$_{Ph}$ (Akyuz et al., 2015) and about 100 fold weaker than that of the P11W^{IFS}: 23 ± 7 and 0.23 ±

290   0.1 µM, respectively, at 10 mM Na$^+$. Kinetics of L-asp binding to FM-P11W^{IFS} revealed that k_{obs}

291   plateaued at elevated L-asp concentrations and that these plateaus were Na$^+$-dependent,

292   suggesting that the overall mechanism of L-asp and Na$^+$ binding is similar in FM-P11W^{IFS} and

293   P11W^{IFS} (**Figure 5c**). The binding rates were about four times higher than those of P11W^{IFS} at

294   equivalent Na$^+$ ion concentrations. Our kinetic model described the data well. The key

295   adjustments included the 4-fold increase of the rate of binding of the first Na^+ ion; modestly

296   increased Na^+ affinity; and approximately thousand-fold increase of L-asp dissociation rates

297   (accounting for lower affinity) (**Figure 5 – figure supplement 1a** and **Figure 4 – source data**

298   **1**). The reduced affinity and correspondingly increased dissociation rate might be due to distinct

299   position of guanidinium group of R276 in the wild type transporter and R395 in FM (**Figure 5b**

300   and **Figure 5 – figure supplement 1a**). Computational studies showed that in the wild type

301   transporter, R276 spends a significant proportion of the time hydrogen-bonded to D394, which

302   coordinates the amino group of the bound L-asp. In FM protein, R395 does not show such

303   propensity, perhaps explaining a more labile nature of L-asp complex (Akyuz et al., 2015).

304   The key conclusion from these experiments is that cross-linking does not limit the rate of

305   the first Na^+ ion binding or the rate of substrate release. Furthermore, the increased rate of the

306   first Na^+ ion binding in FM-P11W^{IFS} suggests that the propensity to sample the unlocked

307   conformation might increase the rate of the slow Na^+-dependent gating event.

308

Discussion

310   L-asp and Na^+ ions bind to Glt$_{Ph}$ cooperatively, with essentially no equilibrium

311   intermediates in the lower Na^+ concentration range (Reyes et al., 2013), which mandates that Na^+

312   ions determine on- and /or off-rates of L-asp binding. Here we show that in the inward-facing

313   state, Na^+ controls both on- and off-rates of the amino acid binding. Furthermore, we show that

314   the slow low affinity binding of the first Na^+ ion is the rate-limiting step of the complex

315   assembly, which is completed upon tight and rapid binding of the last Na^+ ion. Dissociation of

316   one or two ions controls the kinetics of complex disassembly. Collectively, these features ensure

317   that binding and unbinding of L-asp and Na^+ ions proceeds with no kinetic intermediates.

318   Notably, when the transporter in the outward facing state is exposed to high extracellular Na^+

319   concentration in the absence of substrate Na^+-only bound states will occur. Opening of HP2 tip,

320   observed in the structure of Na^+-only bound transporter may serve as an additional structural

321   safeguard to prevent uncoupled Na^+ uptake (Verdon et al., 2014)

322   We do not have direct evidence to show that L-asp binding and dissociation in the

323   outward-facing state follows the same kinetic mechanism. Indeed, the structural mechanism of

324   gating in the inward-facing state has not yet been resolved. However, it seems likely that the

325   energetic penalty paid by the first Na^+ ion to restructure the binding sites is similar, because the

326   geometry of the sites is essentially identical in the two states both in the apo and fully bound

327   states. Previous kinetic studies on unconstrained Glt_{Ph} variants reported some of the same

328   features that we have observed in the inwardly constrained transporter. In one study, a slow low

329   affinity Na^+ binding was found to preceded binding of L-asp (Hanelt et al., 2015). In the other,

330   binding of two Na^+ ions was postulated to precede binding of the amino acid (Ewers et al.,

331   2013). Interestingly, in this latter study the amino acid binding rates were also found to plateau at

332   high concentrations. These plateaus occurred at distinct levels for L-asp, D-aspartate and L-

333   cysteine sulfinic acid, suggesting that the slow step occurred after the initial low affinity binding,

334   consistent with induced fit mechanism. In our studies, the maximal equilibration rates are Na^+-

335   dependent and similar for L-asp and DL-TBOA, suggesting that the same Na^+ binding event

336   occurs before binding of both ligands consistent with Na^+-dependent conformational selection

337   mechanism. These findings are not contradictory, however. In our experiments, binding of the

338   first Na^+ ion is rate limiting. Therefore, the detailed kinetic mechanism of L-asp binding to the

339   transporter already bound to two Na^+ ions is not resolved, and may occur with additional steps

340   that are all fast compared to the rate of binding of the first Na^+ ion.

341   Detailed kinetic schemes describing glutamate and Na^+ ions binding to EAATs have been

342   proposed (Watzke et al., 2001, Bergles et al., 2002, Mim et al., 2005, Zhou et al., 2014). Most of

343   them are consistent with the mechanism proposed here for Glt_{Ph} in that one or two ions are

344   believed to bind before glutamate and the remaining Na^+ ion(s) bind. Similarly Na^+ ion

345   dissociation is thought to precede amino acid release. Such binding mechanism, in which both

346   substrate binding and unbinding rates are controlled by Na^+ ions, provides significant

347   physiological advantages. A transporter facing extracellular solution where Na^+ concentration is

348   high will show rapid binding and slow release rate. One might envision that following glutamate-

349   mediated neurotransmission, glutamate could bind to the transporters and remain bound for

350   comparatively long times even as the concentration of the transmitter in and near the synapse

351   falls below the dissociation constant. Thus, the slow release would be beneficial not only to

352   increase the efficiency of transport, but also to retain glutamate bound to the transporter as its

353   concentration drops to resting levels of ca 25 nM, significantly below the transporter equilibrium

354   affinity (Chiu and Jahr, 2017). Once the amino acid is translocated into the cell and the inward-

355   facing transporter encounters low Na^+ concentration, glutamate would be rapidly released. In

356 contrast, an unbinding rate that is intrinsically slow and independent of Na^+ concentration would

357 have resulted in slow release of the amino acid into the cytoplasm and low transport efficiency.

358 Structural interpretation of the kinetic data is difficult. Crystallographic, computational

359 and mutational studies have located the three Na^+ binding sites in Glt_{Ph} and closely related Glt_{Tk}

360 (Boudker et al., 2007, Huang and Tajkhorshid, 2010, Bastug et al., 2012, Guskov et al., 2016).

361 Two sites, called Na1 and Na3, are located deep in the core of the protein below the substrate-

362 binding site (**Figure 1 - figure supplement 1**). The third Na^+ binding site, Na2, is at the surface

363 of the protein between HP2 and the rest of the transport domain and is partially exposed to the

364 solvent. In the structure of Glt_{Ph} R397A mutant with drastically reduced substrate affinity that

365 was crystallized in the presence of Na^+ ions only, the tip of HP2 is open and the Na2 site is

366 unoccupied (**Figure 1 – figure supplement 2**) (Verdon et al., 2014). Similarly, in Glt_{Ph} variants

367 bound to non-transportable blockers, including L-TBOA, HP2 is open and Na2 site unoccupied

368 (Boudker et al., 2007, Scopelliti et al., 2018). Interestingly, in none of the three sites, the ions are

369 coordinated by the bound L-asp directly; therefore all sites are coupled allosterically.

370 It is likely that the first Na^+ ion binds (step 1 in **Figure 4**) to a site with the highest

371 intrinsic affinity, which according to computational studies is Na3 site, buried at the core of the

372 transport domain (Heinzelmann et al., 2011, Heinzelmann et al., 2013). Its intrinsic affinity has

373 been estimated at -16 kcal/mol. In contrast, experimental data suggest that the first Na^+ ion binds

374 with affinity of no more than -0.5 kcal/mol. This large disparity is consistent with the

375 observation that the geometry of all three Na^+ and substrate binding sites are drastically distorted

376 in the apo protein (Jensen et al., 2013, Verdon et al., 2014). Thus, binding of the first Na^+ is

377 likely slow and weak, because the protein undergoes significant remodeling, which must be

378 energetically costly. Notably, this initial binding event is faster in the so-called fast mutant

379 R276S/M395R variant FM-P11W[IFS], suggesting that the rate of the first ion binding is not

380 limited by cross-linking used to conformationally constrain the transporter. Thus, the rate is

381 likely controlled by the local restructuring events, even if it is affected by the propensity for the

382 global transport domain movements. Once remodeling occurs, binding of the remaining ligands

383 occurs much faster and with high affinity. It is difficult to establish unambiguously, which two

384 Na^+-binding sites are vacated first during complex disassembly, but it seems likely that the

385 surface Na2 site is one of them. In our experiments, the two "locking" ions bind with high

386 affinity around 100 µM. The tight binding appears to contradict computational studies that found

387   Na$^+$ ion in Na2 site unstable or only weakly bound (DeChancie et al., 2011, Heinzelmann et al.,

388   2011, Zomot and Bahar, 2013). The origin of this discrepancy is currently unclear.

389   In conclusion, we have examined the kinetic mechanism of binding and release of the

390   substrate and coupled ions in the inward facing state of Glt$_{Ph}$. We show that the mechanism

391   ensures lack of equilibrium and kinetic intermediates and that rates of both substrate binding and

392   release are controlled by Na$^+$ ions.

Methods

Mutagenesis, protein expression, and purification

All Glt$_{Ph}$ mutants were prepared by site-directed mutagenesis using cysteinless seven-histidine mutant of Glt$_{Ph}$ that has been used in several previous studies (Reyes et al., 2009, Akyuz et al., 2013, Akyuz et al., 2015). Proteins were expressed and purified as described previously (Boudker et al., 2007). In brief, Glt$_{Ph}$ mutants were expressed in *Escherichia coli* DH10b strain (Invitrogen, Inc). Small volumes of *E. coli* overnight cultures were diluted into Luria broth media supplemented ampicillin and grown at 37 °C. Cultures were induced at optical density of 0.6 with 0.2 % L-arabinose for 3 hrs at 37 °C. Cells were harvested by centrifugation and pellets frozen in liquid nitrogen and stored at -80 °C until use. Thawed cells were disrupted by using cell disrupter (Avestin, Inc) at ~15,000 psi. Cell debris were removed by centrifugation at 7,700 *g* for 15 min at 4 °C. Crude membranes were pelleted by ultracentrifugation at 180,000 *g* for 1 hr at 4 °C. Membranes were homogenized in 20 mM Hepes/Tris pH 7.4, 200 mM NaCl and pelleted again by ultracentrifugation. Washed membranes were homogenized and then solubilized in the same buffer supplemented with 40 mM dodecyl-β,D-maltopyranoside (DDM) for 1 hr. Solubilized membrane were clarified by ultracentrifugation at 164,000 *g* for 1 hr at 4 °C and incubated with pre-equilibrated Ni-NTA resin (Qiagen) with gentle shaking. Ni-NTA resin was washed with seven column volumes (CV) of 20 mM Hepes/Tris pH 7.4, 200 mM NaCl, 40 mM imidazole and 1 mM DDM. Proteins were eluted with 5 CV of 20 mM Hepes/Tris pH 7.4, 200 mM NaCl, 250 mM imidazole and 1 mM DDM. Eluted proteins were concentrated using concentrators with 100 kDa MWCO (Amicon). The tag was removed by thrombin digestion at room temperature overnight. Cleaved proteins were purified by size exclusion chromatography in 20 mM Hepes/Tris pH 7.4, 300 mM choline chloride and 1 mM DDM.

Crosslinking

Before crosslinking, purified proteins were reduced with 5 mM TCEP at room temperature for 1 hr and purified by SEC. For crosslinking, 10-fold molar excess of HgCl$_2$ was added to protein solutions at concentrations below 1 mg/mL, and incubated for 15 minutes at room temperature. Crosslinked proteins to be used in binding assays were re-purified by SEC. Peak fractions were collected and concentrated. Protein concentration was measured using Nanodrop (Thermo-Fisher Scientific) using an extinction coefficient determined by ProtParam (Gasteiger et al., 2005). To

424   check availability of free thiols after crosslinking, proteins were incubated with 5-fold molar

425   excess of fluoroscein-5-maleimide (F5M). Fluorescent F5M-labeled proteins were imaged on

426   SDS-PAGE under blue illumination and stained with Coomassie blue.

427

428   *Fluorescence-based kinetics assay*

429   For emission scans, protein samples at 1μM were prepared in 2 mL of 20 mM Hepes/Tris pH

430   7.4, 300 mM choline chloride and 1 mM DDM. Intrinsic protein fluorescence was excited at 295

431   nm and emission measured in bulk fluorometer (PTI Inc.). Baseline was determined using buffer

432   only and subtracted from the data. For real-time fluorescence measurements of ligand

433   dissociation, protein samples at 25 μM were prepared in 20 mM Hepes/Tris pH 7.4, 300 mM

434   choline chloride, 10 mM NaCl, 0.5 mM L-asp 1 mM DDM and diluted 100 fold into the

435   fluorometer cell containing the same buffer lacking L-asp and containing variable NaCl

436   concentrations with fast stirring at 25 °C. Data were normalized by the value of the fluorescence

437   after addition of saturating Na^+/L-asp at the end of experiment. Approximately 10 % reduction in

438   protein fluorescence was observed upon dissociation. For real-time fluorescence measurements

439   of ligand binding, protein samples at 1 μM were incubated in 2 mL fluorometer cell containing

440   20 mM Hepes/Tris pH 7.4, 300 mM choline chloride and 1 mM DDM with various NaCl

441   concentrations at 25 °C. Data were normalized by dividing fractional fluorescence change by the

442   maximal change observed upon addition of saturating ligand concentrations at the end of each

443   experiment. After signal equilibration, ligands were added with fast stirring. Excitation and

444   emission wavelengths were 295 and 350 nm, respectively. Here and elsewhere, data were

445   analyzed using SigmaPlot 12.0 (Systat Software, Inc) and GraphPad Prism 7 (GraphPad

446   Software). All kinetic relaxation curves were fitted to single exponentials.

447

448   *Fluorescence-based titration using RH421*

449   For L-asp titration, protein samples at 1 μM concentration were prepared in 20 mM Hepes/Tris

450   pH 7.4, 300 mM ChoCl, and 0.4 mM DDM. After addition of appropriate NaCl concentrations

451   and 200 nM RH421 (Sigma-Aldrich), the samples were incubated at 25 °C until fluorescence

452   signal plateaued. L-asp aliquots were added to the samples with fast stirring and fluorescence

453   emission was recorded following equilibration. Excitation and emission wavelengths were 532

454   and 628nm, respectively.

455

Protein reconstitution into liposomes

Glt$_{Ph}$ protein samples were reconstituted into liposomes as previously described (Boudker et al., 2007, Ryan et al., 2009). In brief, 0.1% L-α-Phosphatidylethanolamine-N-lissamine rhodamine B sulfonyl (Avanti Polar Lipids) was added to with 3:1 (w/w) mixture of *E.coli* polar lipid extract and egg phosphatidylcholine and dried on rotary evaporator and under vacuum overnight. The mixture was hydrated using 20 mM Hepes/Tris, pH 7.4 and 200 mM KCl buffer at final lipid concentration of 5 mg/ml by 10 freeze/thaw cycles. The suspensions were extruded through 400 nm filter membranes (Whatman) 10 times to form unilamellar liposomes. The rhodamine fluorescence of the liposomes was measured to generate a concentration calibration curve; excitation and emission wavelengths were 560 and 583 nm, respectively. The liposomes were destabilized by addition of Triton X-100 0.5:1 (w:w) detergent to lipid ratio. Proteins were added to the destabilized liposome at a ratio of 1:1000 (w/w) of protein to lipid, and incubated for 30 min at room temperature. Detergent was removed by three rounds of incubation with 80 mg/ml of pre-washed SM-2 beads (Bio-Rad): 2 hrs at room temperature, 2 hrs at 4 °C and overnight at 4 °C with gentle agitation. The proteoliposomes were extruded 10 times through 400 nm membranes and concentrated by ultracentrifugation at 86,000 *g* for 45 min at 4 °C. To replace the internal buffer, proteoliposomes were re-suspended in 1 mL of appropriate buffer and subjected to three freeze/thaw cycles followed by extrusion. Final concentrations of proteoliposomes were determined by rhodamine fluorescence.

475

Transport assays

For substrate exchange experiments, the internal proteoliposome buffer was replaced with buffer containing 20 mM Hepes/Tris, pH 7.4 and 200 mM NaCl and 100 µM L-asp. To initiate uptake or exchange, the proteoliposomes were diluted 1,000 fold into reaction buffer containing 20 mM Hepes/Tris pH 7.4, 200 mM NaCl, and 500 nM ^{3}H-L-asp (PerkimElmer, 13.2 mci/mmol) pre-incubated at 35 °C. In uptake experiments external buffer was supplemented with 100 nM cold L-asp to match exchange buffer conditions. At appropriate time points, 200 µl aliquots were removed and diluted into 2 mL of ice-cold quench buffer containing 20 mM Hepes/Tris pH 7.4, 200 mM LiCl. Samples were immediately filtered through 0.22 µM filter membrane (Millipore) and washed with 3 mL of the quench buffer. The washed filter membranes were transferred into

486 scintillation vials, and retained radioactivity was measured using LS6500 scintillation counter

487 (Beckman Coulter). Background uptake was measured in the reaction buffer containing 20 mM

488 Hepes/Tris pH 7.4, 200 mM KCl, 500 nM ^{3}H-L-asp and 100 nM cold L-asp.

489

490 *Protein crystallization, diffraction data acquisition and processing*

491 FM-Glt$_{Ph}$^{IFS} was purified by size exclusion chromatography in 10 mM Hepes/Tris pH 7.4, 100

492 mM NaCl, and 7 mM n-decyl-ß-D-maltopyranoside (Anatrace). Peak fractions were pooled,

493 concentrated and supplemented with 0.4 M NaCl, 0.1 M MgCl$_2$, and 1 mM L-asp.

494 Crystallizations were performed using hanging drop vapor diffusion method by mixing protein

495 solution with well solution containing 0.1 M ADA pH 7.0, 0.4 M NaCl, 30% PEG600 at 1:1

496 ratio. Protein crystals grew at 20 °C. They were cryo-protected by soaking in the well solution

497 supplemented with higher concentrations of PEG 600. Diffraction data were collected at

498 Advance Photon Source beamline 24-ID-C. Data were processed using HKL2000 and CCP4

499 suite (Minor et al., 2000, Winn et al., 2011). Molecular replacement was performed in PHASER

500 (McCoy et al., 2007) using Glt$_{Ph}$ structure in the inward-facing state (PDB code: 3KBC) as a

501 search model. The model from molecular replacement was further refined using REFMAC,

502 PHENIX and COOT (Emsley and Cowtan, 2004, Adams et al., 2010) applying three-fold non-

503 crystallographic symmetry. TLS was used during final refinement (Winn et al., 2001) (**Table 1**).

504 We noted that while Hg^{2+} was used to crosslink the protein, only traces of Hg^{2+} ions could be

505 observed in the structure, and disulfide bonds formed instead. The Hg^{2+} ions appeared to be

506 present at very low occupancy and we did not model them in the structure. All structural figures

507 were prepared using Pymol (Schrodinger, 2015).

508

509 *Kinetic modeling and data fitting*

510 To fit the dependencies of k_{obs} at given Na^{+} ion concentrations on concentrations of L-asp and

511 DL-TBOA (**Figure 3a and 3b**) we need to consider the kinetic equation for the conformational

512 selection mechanism (**Figure 3c**). The full Equation 4 requires fitting of four rate constants, k_r, k_{-r},

513 k_{on} and k_{off} (Vogt and Di Cera, 2012). Of these, k_r is the best-defined constant because k_{obs}

514 approaches k_r asymptotically at high ligand concentrations. Notably, when $k_{off} > k_r$, as is likely

515 the case for DL-TBOA binding, the values of k_{obs} decrease with increased ligand concentration.

516 Conversely, if $k_{off} < k_r$ then the values of k_{obs} increase with increased ligand concentration as is

517 observed for L-asp. The data for DL-TBOA binding were fitted to Equation 3. The data were

518 fitted globally in Prism 7, keeping k_{-r} value shared between datasets obtained at variable Na^+

519 concentrations, as it is not expected to be Na^+-dependent. Nevertheless, k_{-r} and $K_{D,TBOA}$ were

520 correlated and could not be resolved. The data for L-asp binding were fitted to the full Equation

521 4. The k_{off} values were taken from the fit of the dissociation rates with Hill coefficient $n=1$

522 (**Figure 2b**). The k_{-r} and k_{on} rate constants were correlated and could not be resolved, but k_r

523 values were well defined.

524

525   The kinetic models in **Figure 4**, **Figure 4 – figure supplement 4** and **Figure 5 – figure**

526 **supplement 1** were implemented in KinTek program (*KinTek, Inc*) (Johnson, 2009) for global

527 parameter fitting and in Copasi (Hoops et al., 2006) for simulations. For interactions between L-

528 asp and P11W^{IFS} protein, a 6-step kinetic model (**Figure 4**) with 12 rate constants was

529 implemented. Raw relaxation curves, including 21 dissociation and 58 binding reactions were

530 fitted globally in KinTek (**Figure 4 – figure supplements 2 and 3**). The thermodynamic cycle

531 through two alternative branches, "second Na^+ binds first" (reactions 2 and 4 in **Figure 4**) and

532 "L-asp binds first" (reactions 3 and 5), was constrained. Additionally, the parameters were

533 constrained to set the dissociation constant for the first two Na^+ ions (reactions 1 and 2 yielding

534 *TNa2* complex) at 0.04 M^2 according to the previously measured apparent affinity of ~200 mM

535 for Na^+ ions binding to Glt_{Ph}^{IFS} in the absence of L-asp (Reyes et al., 2013). Specifically, the

536 affinities for the first and second Na^+ ion were set at 0.5 M (reaction 1) and 0.08 M (reaction 2),

537 respectively. These values reproduced experimentally measured Na+ binding isotherms (Reyes

538 et al., 2013). The combined affinity of the second and third Na^+ ions for TANa complex

539 (reactions 5 and 6) was set to $5\ 10^{-8}\ M^2$ to fall within the range of apparent Na^+ affinities of 50-

540 130 µM measured in L-asp dissociation experiments (**Figure 2b**). We noted that the quality of

541 the fits was better if the second and third Na^+ bound cooperatively, i.e. the affinity for the third

542 ion was higher than the second. Thus we set the affinities for the second (reaction 5) and third

543 (reaction 6) Na^+ ions at 500 and 10 µM, respectively. The binding rate for the first Na^+ ion is

544 well determined by the data and was fixed at 36 $M^{-1}\ s^{-1}$ (**Figure 3c**). The remaining binding rates

545 for Na^+ ions were set at an arbitrary high value of $10^7\ M^{-1}\ s^{-1}$. Once binding parameters for Na^+

546 ions were set, the remaining rate constants of L-asp binding and unbinding were fitted with

547 acceptable standard errors (**Figure 4 – figure source data 1**). The total sum of χ^2-s obtained

548 during fitting was 4.7 10^4. Excluding "L-asp binds first" branch of the reaction mechanism

549 produced the best χ^2 sum of 6.5 10^4 (an increase of 40 %). Excluding "second Na$^+$ binds first"

550 branch produced best χ^2 sum of 5.5 10^4 (an increase of 17 %).

551   Notably, the flow of the reaction through the alternative branches determines the shapes

552 of the k_{obs} dependences on Na$^+$ concentrations in binding and dissociation reactions (**Figures 2**

553 and **3**). In binding reactions, the probability to go through the "second Na$^+$ binds first" branch

554 (reactions 2 and 4) depends of Na$^+$ concentration as it is determined by the fraction of the

555 transporter bound to the second Na$^+$, F_{TNa2}. Because the binding and unbinding rates of the

556 second Na$^+$ ion to *TNa* complex (reaction 2) are fast compared to the rates of L-asp binding to

557 *TNa* (reaction 3), we can consider this binding reaction in terms of rapid equilibrium. Thus, the

558 fractions of *TNa$_2$* and *TNa* and will be

559

560 $$F_{TNa_2} = \frac{[Na^+]}{K_{D,2}+[Na^+]} \quad \text{and} \quad F_{TNa} = \frac{K_{D,2}}{K_{D,2}+[Na^+]}, \qquad (5)$$

561

562 where $K_{D,2}$ is the dissociation contestant in reaction 2. The corresponding relative fluxes through

563 *TNa$_2$* and *TANa* species will be:

564

565 $$\eta_{TNa_2} = F_{TNa_2} \cdot k_{on,4} \quad \text{and} \quad \eta_{TANa} = F_{TNa} \cdot k_{on,3} \qquad (6)$$

566

567 And the ratio between them

568

569 $$\frac{\eta_{TNa_2}}{\eta_{TANa}} = \frac{[Na^+]}{K_{D,2}} \frac{k_{on,4}}{k_{on,3}} \qquad (7)$$

570

571   In dissociation reaction, the binding and unbinding of Na$^+$ ions to *TANa* complex

572 (reaction 5) can be considered in rapid equilibrium compared to L-asp unbinding in reaction 4.

573 Therefore, similar reasoning can be applied as above, and fluxes through *TNa$_2$* and *TNA* species

574 calculated as:

575

576 $$\frac{\rho_{TNa_2}}{\rho_{TANa}} = \frac{[Na^+]}{K_{D,5}} \frac{k_{off,4}}{k_{off,3}} \qquad (8)$$

577

578   Importantly, these ratios (Equations 7 and 8), and not the individual equilibrium and rate

579   constants, are defined by the experimental data. The fraction of the relative flux going through

580   the "second Na^+ binds first" branch relative to "L-asp binds first" branch in both forward and

581   reverse directions is proportional to Na^+ concentration with the equal fluxes through the two

582   branches occurring at 3.6 mM Na^+. Simulations in Copasi confirmed these conclusions.

583   To fit DL-TBOA binding kinetic data, the same model was implemented, excluding

584   binding of the third Na^+ ion (reaction 6). 68 individual relaxation curves were fitted globally,

585   allowing only the rates of DL-TBOA dissociation to be adjusted (**Figure 4 – figure supplement**

586   **5**). The best χ^2 sum of $2 \ 10^4$ was obtained. Excluding "DL-TBOA binds first" branch or "second

587   Na^+ binds first branch" produced similar χ^2 values, indicating that these data alone are unable to

588   distinguish between the models. Global fitting of 27 relaxation curves for FM-P11W^{IFS} was

589   accomplished in the same manner. The rates for L-asp dissociation and the rates for binding and

590   unbinding of the first Na^+ ion were adjusted.

591

592

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597

Competing financial interests

The authors declare that no competing interests exist.

600

601

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737
738

Figure legends

Figure 1. **Design and characterization of P11W^{IFS}.**
(**a**) Pro11 (cyan) in the trimerization domain (wheat) of Glt$_{Ph}$^{IFS} (PDB ID: 3KBC) is close to substrate binding site (magenta) in the transport domain (blue). (**b**) Intrinsic fluorescence emission spectrum of apo P11W^{IFS} (blue) and P11W^{IFS} in the presence of 10 mM NaCl (red) and DL-TBOA (green) or L-asp (magenta). (**c**) Dependence of L-asp dissociation constant, K$_D$ of P11W^{IFS} on Na$^+$ ion concentration. The data (solid triangles) were fitted to a straight line with slope of 2.5. Binding assay based on RH421 fluorescence was used to follow L-asp binding to P11W^{IFS}. The dashed line represents Na$^+$ dependence of L-asp K$_D$ of Glt$_{Ph}$^{IFS} with slope of 2.6 (Reyes et al., 2013).

Figure 1 – figure supplement 1. Na$^+$-coupled L-asp transport by Glt$_{Ph}$.
(**a**) Transport cycle of Glt$_{Ph}$ consists of four steps. First, three Na$^+$ ions and L-asp bind to the transporter in an outward-facing state (accessible from the extracellular side). Second, the transporter undergoes a conformational change from the outward- to an inward-facing state (accessible from the cytoplasm). Through this conformational change, Na$^+$ ions and L-asp are translocated across the membrane. Third, the substrate and ions are released into the cytoplasm. Lastly, *apo* transporter undergoes a reverse conformational change back to the outward-facing state. (**b**) Residues from HP2 (red), HP1 (yellow), TM3, TM7, and TM8 (light blue) form the three Na$^+$ binding sites (shown as pink spheres; based on PDB codes 2NWX and 5E9S) (Boudker et al., 2007, Guskov et al., 2016). The ions bind proximally to the substrate L-asp (shown in stick representation) but are not directly coordinated by it. (**c**) Structures of single Glt$_{Ph}$ protomers bound to L-asp in the outward- (left, PDB code 2NWX) and inward-facing states (right, PDB code 3KBC). Cα atoms of the cross-linked cysteine residues Cys55 and Cys364 and Hg^{2+} ion are shown as cyan and black spheres, respectively

Figure 1 – figure supplement 2. HP2 acts as a gate for substrate binding.
Shown are structures of the outward-facing Glt$_{Ph}$ free of ligands (Apo), R397A mutant crystallized in high Na$^+$ concentration and no substrate (Na$^+$ only), Glt$_{Ph}$ bound to Na$^+$ ions and L-TBOA (Na$^+$/TBOA) or L-asp (Na$^+$/Asp). The color scheme is as in **Figure 1 - figure supplement 1**. PDB codes are 4OYE, 4OYF, 2NWW and 2NWX, respectively.

Figure 1 – figure supplement 3. Characterization of P11W^{IFS} Glt$_{Ph}$.
(**a**) SDS PAGE analysis of fluorescein-5-maleimide (F5M) labeled uncrosslinked and Hg^{2+}-crosslinked P11W/K55C/A364C Glt$_{Ph}$ (P11W^{IFS}), imaged under blue illumination (left) and stained with Coomassie Blue (right). (**b**) Titration of P11W^{IFS} Glt$_{Ph}$ with L-asp followed by tryptophan fluorescence in the presence of 10 mM Na$^+$. Data were normalized and fitted to a single binding site quadratic equation yielding K$_D$ of 228 ± 85 nM. Representative kinetic experiments of 0.5 mM L-asp binding to P11W^{IFS} in the presence of 4 mM Na$^+$ (**c**), L-asp dissociation from P11W^{IFS} in the presence of 0.2 mM Na$^+$ (**d**), and 8 mM DL-TBOA binding to

779   P11W^{IFS} in the presence of 4 mM Na$^+$ (**e**). Data were normalized as described in Methods. The

780   white lines are fits to single exponential functions yielding k_{obs} values of 0.0712 s^{-1}, 0.027 s^{-1},

781   and 0.2106 s^{-1}, respectively.

782

783   **Figure 2. Na$^+$ ions control on- and off-rates of L-asp binding.**

784   (**a**) The values of k_{obs} for L-asp binding as a function of Na$^+$ concentration. The binding rates

785   were measured as a function of Na$^+$ concentrations upon additions of L-asp to P11W^{IFS} to the

786   following final concentrations in mM: 0.01 (blue), 0.025 (red), 0.05 (green), 0.1 (purple), 0.25

787   (orange), 0.5 (black), 1 (brown) and 2 (dark blue). (**b**) The dissociation rates of L-asp as a

788   function of Na$^+$ concentration in the media measured for P11W^{IFS} (solid squares) and P11W^{IFS}-

789   M311A (open squares). The lines through data for P11W^{IFS} are the fits to Equation 2 with Hill

790   coefficients n fixed to 1 (red), 2 (green) and 3 (blue). The fitted values of $k_{off,asp}$ and $K_{D,Na}$ were,

791   respectively: 0.5 ± 0.2 s^{-1}, 40 ± 20 µM; 0.2 ± 0.02 s^{-1}, 130 ± 20 µM; 0.2 ± 0.01 s^{-1}, 150 ± 13 µM.

792   (**c**) Schematic representation of the mechanism in Equation 2 exemplified for two rapidly

793   equilibrating cooperatively binding Na$^+$ ions. (**d**) Comparison of the rate of L-asp uptake (open

794   circles) and exchange (solid circles) in proteoliposomes containing wild type Glt$_{Ph}$. Background

795   uptake was measured in the absence of the external NaCl and subtracted from the data. All

796   experiments were performed in triplicate and means and standard errors are shown.

797

798   **Figure 2 – figure supplement 1. Na$^+$ dependence of L-asp binding kinetics in low (a) and**

799   **high (b) L-asp.**

800   In the presence of high concentrations of L-asp the dependence of k_{obs} on Na$^+$ concentration is

801   linear. However, at low L-asp concentrations, the dependence is non-linear, and can be

802   approximated by a quadratic function.

803

804   **Figure 3. Na$^+$ ions drive conformational selection.**

805   The rates of L-asp (**a**) and DL-TBOA (**b**) binding to P11W^{IFS} as functions of ligand

806   concentration. The rates of L-asp binding were measured in the presence of 0.5 (blue), 1 (red), 2

807   (green), 4 (purple) or 8 (orange) mM Na$^+$ ions. Data were fitted to Equation 4 to determine

808   plateau k_r values (Methods). DL-TBOA binding experiments were conducted in 4 (blue), 8 (red),

809   12 (green) or 16 (purple) mM Na$^+$ ions and fitted to Equation 3 to obtain plateau k_r values. All

810   experiments were performed in triplicate and means and standard error bars are shown. (**c**)

811   Schematic representation of Na$^+$-driven conformational selection mechanism. In this model, T*

812   is a conformation of the transporter that is unable to bind substrate with appreciable affinity. Na$^+$

813   binding is coupled to a transition to a conformation that binds L-asp and the remaining two Na$^+$

814   ions rapidly. (**d**) Na$^+$ dependence of k_r values for L-asp (open squares) and DL-TBOA (solid

815   squares) binding. Error bars are standard errors from fits in panels **a** and **b**. The k_r values for DL-

816   TBOA at 20 and 24 mM were approximated by measuring k_{obs} of 8 mM DL-TBOA binding

817   (expected to be well at the plateau). Linear fits of L-asp (red) and DL-TBOA (blue) k_r-s were

818   constrained to go through the origin, yielding slopes of 36 ± 0.9 M^{-1} s^{-1} and 26 ± 1.7 M^{-1} s^{-1}.

819

Figure 4. A kinetic model of Na$^+$ and L-asp binding to P11W^{IFS}.
(**a**) The proposed reaction mechanism. The kinetic and equilibrium constants are shown next to the individual reactions (numbered in green). The on- (red) and off-rate constants (blue) are in M^{-1} s^{-1} and s^{-1}, respectively. The equilibrium dissociation constants (black) are in M. Constants that were fixed during data fitting are marked by starts. (**b**) Simulated binding reaction, in which 1 μM apo transporter is mixed with Na$^+$ ions and L-asp. The amounts of ligands are indicated above the graphs that show reaction fluxes through "second Na$^+$ ion binds first" (TNa$_2$, orange) and "L-asp binds first" (TANa, green) reaction pathways. (**c**) Simulated binding and dissociation reactions showing that only apo transporter (T, red) and fully bound transporter (TANa$_3$, blue) become populated, while all potential reaction intermediates (magenta) are present only in miniscule amounts.

Figure 4 – source data 1. Kinetic rate constants summary
* marks the rate constants that were fixed during KinTek analysis. [#] marks the rate constants that were optimized in KinTek during global data fitting are in bold. [$] A stands for the binding ligand, L-Asp or DL-TBOA. Errors are standard errors of the fit.

Figure 4 – figure supplement 1. Fitted rate constants describe experimental data well.
Shown are simulated and experimental data for L-asp binding kinetics at variable Na$^+$ concentrations (**a**); L-asp dissociation as a function of Na$^+$ concentration (**b**); dependence of L-asp binding rates on Na$^+$ ions at high and low L-asp concentrations (**c**); and equilibrium dissociation constants of L-asp binding as a function of Na$^+$ concentration (**d**).

Figure 4 – figure supplement 2. Experimental and simulated kinetics of L-asp binding to P11W^{IFS}. Left panels: Kinetics of L-asp binding measured upon addition of the indicated Na$^+$ (mM) and L-asp (μM) amounts. Data are fitted to single exponential functions. For brevity, single representative experiments are shown from sets of triplicates under each experimental condition. Color scheme is as follows: blue, 10 μM red, 25 μM; green, 50 μM; purple, 100 μM; orange, 250 μM; black, 500 μM; ocher, 1000 μM; navy blue, 2000 μM L-asp. The simulations using values obtained in global fitting of the data are shown on the right panels.

Figure 4 – figure supplement 3. Experimental and simulated kinetics of L-asp dissociation from P11W^{IFS}. (a) Example of an experiment to determine the kinetics of L-asp dissociation from P11W^{IFS}. Protein bound to Na$^+$ and L-asp was diluted into buffer to the final concentration of 0.1 mM Na$^+$ and 0.25 μM L-asp. Excess Na$^+$/L-asp was added at the end of the experiment (marked by asterisk) to saturate P11W^{IFS} and estimate the pre-dilution baseline. (b) Kinetics of L-asp dissociation observed at the indicated final Na$^+$ (mM) concentrations. Data were fitted to single exponential functions. Representative experiments are shown. Color scheme is as follows:

858 red, 0.1 mM; green, 0.15 mM; purple, 0.2 mM; orange, 0.3 mM; black, 0.5 mM; ocher, 1 mM
859 Na^+. (c) The simulations obtained using values from the global fitting of the data.
860

861 **Figure 4 – figure supplement 4. Kinetic modeling of DL-TBOA binding to P11W^{IFS}.**
862 (**a**) The shown kinetic model was implemented in KinTek. Binding (red), unbinding (blue)
863 kinetic constants and equilibrium dissociation constants (black) are in s^{-1} M^{-1}, s^{-1} and M,
864 respectively. All kinetic constants marked by stars were fixed to values used to fit L-asp binding
865 data. Only the rates of DL-TBOA dissociation in reactions 3 and 4 (shown in bold) were fitted.
866 (**b**) The model (red) recapitulates the experimental data (black) for DL-TBOA binding
867 reasonably well.
868

869 **Figure 4 – figure supplement 5. Experimental and simulated kinetics of DL-TBOA binding**
870 **to P11W^{IFS}.** Left panels: Representative experimental data showing kinetics of DL-TBOA
871 binding in the presence of indicated Na^+ (mM) and L-asp (µM) concentrations. Data were fitted
872 to single exponential function. Single representative experiments are shown. Color scheme is as
873 follows: red, 100 µM; green, 250 µM; purple, 500 µM; orange, 1000 µM; black, 2000 µM; ocher,
874 4000 µM; navy blue, 8000 µM DL-TBOA. Simulation using parameters from the global fitting
875 of the data are shown on the right panels.
876

877 **Figure 5. Crystal structure and L-asp binding kinetics of FM-Glt$_{Ph}$^{IFS}.** (**a**) The
878 superimposition of "locked" conformations of FM-Glt$_{Ph}$^{IFS} and Glt$_{Ph}$^{IFS} (PDB code: 3KBC, left)
879 and "unlocked" conformation of FM-Glt$_{Ph}$ (PDB code: 4X2S, right). Single protomers are
880 viewed from the cytoplasmic side of the membrane, rotated approximately 90 °C compared to
881 the representations in Figure 1a that are shown in membrane plane. FM-Glt$_{Ph}$^{IFS}, Glt$_{Ph}$^{IFS} and FM-
882 Glt$_{Ph}$ are colored pink, gray and blue, respectively. HP1 and HP2 of the FM variants are
883 highlighted as yellow and red, respectively, and HP2 is shown in surface representation. A cleft
884 is visible in uncross-linked FM-Glt$_{Ph}$, which may allow HP2 opening. Cα R.M.S.D. between
885 FM-Glt$_{Ph}$^{IFS} and Glt$_{Ph}$^{IFS} structures is 0.4 Å. The Cα atom of residue 55, which is mutated to Cys
886 and cross-linked to 364C in the transport domain of FM-Glt$_{Ph}$^{IFS} is emphasized as a black sphere.
887 (**b**) Close-up of the substrate-binding site in FM-Glt$_{Ph}$^{IFS} (top) and Glt$_{Ph}$^{IFS} (bottom). The key
888 substrate-coordinating residues are highlighted as sticks, and the bond between the carboxyl
889 group of D394 and amino group of the substrate is shown as a black dotted line. (**c**) Rate
890 constants of L-asp binding to FM-P11W^{IFS} in the presence of 4 (green) and 8 (blue) mM Na^+
891 ions. Solid lines through the data have been generated using the rate constant and kinetic model
892 shown in Figure 5 – figure supplement 1. Open green squares and dotted line show the rate
893 constant of L-asp binding to P11W^{IFS} in the presence of 4 mM Na^+ for comparison.
894

895 **Figure 5 – figure supplement 1.** (**a**) The kinetic model describing L-asp binding to FM-
896 P11W^{IFS}. The binding (red) and unbinding (blue) rate constants and the equilibrium constants
897 (black) are in M^{-1} s^{-1}, s^{-1} and M. All kinetic constants marked by stars were fixed to values used
898 to fit L-asp binding data and the fitted constants are highlighted in bold. (**b**) Shown is a fragment
899 of TM8 of FM-Gt$_{Ph}$^{IFS} including residue R395 and bound L-asp. The mesh object is the three-
900 fold averaged sharpened 2Fo-Fc electron density contoured at 1.2 σ.
901
902 **Figure 5 – figure supplement 2. Experimental and simulated kinetics of L-asp binding to**
903 **FM-P11W^{IFS}.** Left panels: representative data showing kinetics of L-asp binding in the presence
904 of the indicated Na^{+} (mM) and L-asp (μM) concentrations. Data were fitted to single exponential
905 functions. Color scheme is as follows: blue, 25 μM; red, 50 μM; green, 100 μM; purple, 250 uM;
906 orange, 500 μM; black, 1000 μM; navy blue, 2000 μM L-asp. The simulations obtained using
907 parameters from the global fitting of the data are shown on the right panels.

908   **Table 1. Crystallographic data and structure refinement**

	FM-Glt$_{\text{Ph}}^{\text{IFS}}$
Data collection	
Space group	$C222_1$
Cell dimensions	
a, b, c (Å)	116.48, 196.42, 194.33
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	50.0 – 4.05 (4.19 – 4.05)$^{\$}$
R_{sym} or R_{merge}	0.142 (0.982)
I / σ_I	17.8 (2.2)
Completeness (%)	99.7 (99.7)
Redundancy	12.2 (11.7)
Refinement	
Resolution (Å)	19.9 – 4.05
No. reflections	18216
R_{work} / R_{free}	0.2217/0.2660
No. atoms	
Protein	9138
Ligand/ion	27/6
Water	0
B-factors	
Protein	185.57
Ligand/ion	184.71/180.44
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.989

909    $^{\$}$ Values in the brackets correspond to the highest resolution shell.

a

Ext

Int

P11W

L-asp

60°

P11W

L-asp

b

Fluorescence (arbitrary units)

4.0x10⁵

3.5x10⁵

3.0x10⁵

apo

NaCl

Na⁺/L-asp

Na⁺/DL-TBOA

320   340   360

Wavelengths (nm)

c

log $K_{D,asp}$ (M)

-3

-4

-5

-6

-7

Glt_{Ph}^{IFS}

$P11W^{IFS}$

-3.0   -2.5   -2.0

log [Na⁺] (M)

a

Apo^OFS ⇌ (3 Na⁺, 1 L-asp) Bound^OFS

Apo^IFS ⇌ Bound^IFS (3 Na⁺, 1 L-asp)

b

HP2

L-asp

Na2

Na3

TM3

Na1

HP1

TM7

TM8

c

Ext

HP2

L-asp

Int

Bound, OFS

Hg 364

55

L-asp

HP1

Bound, IFS
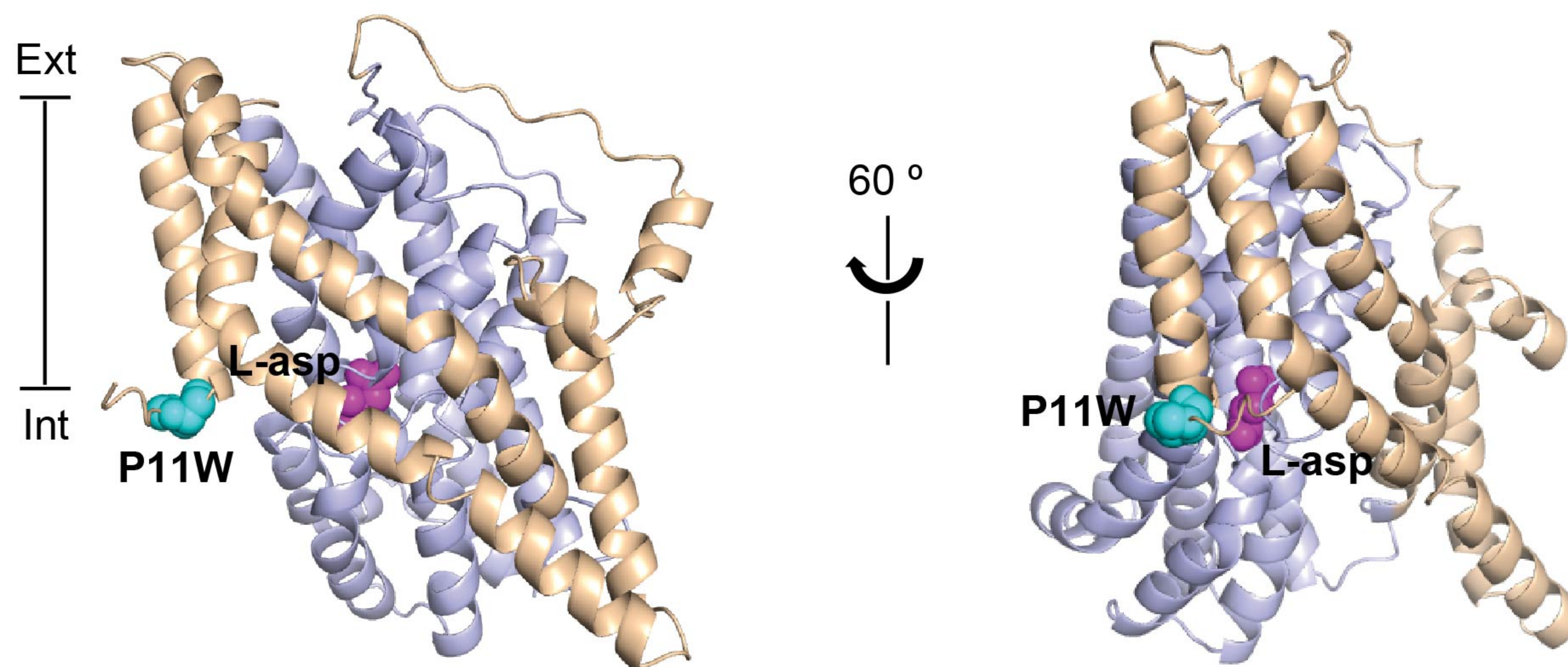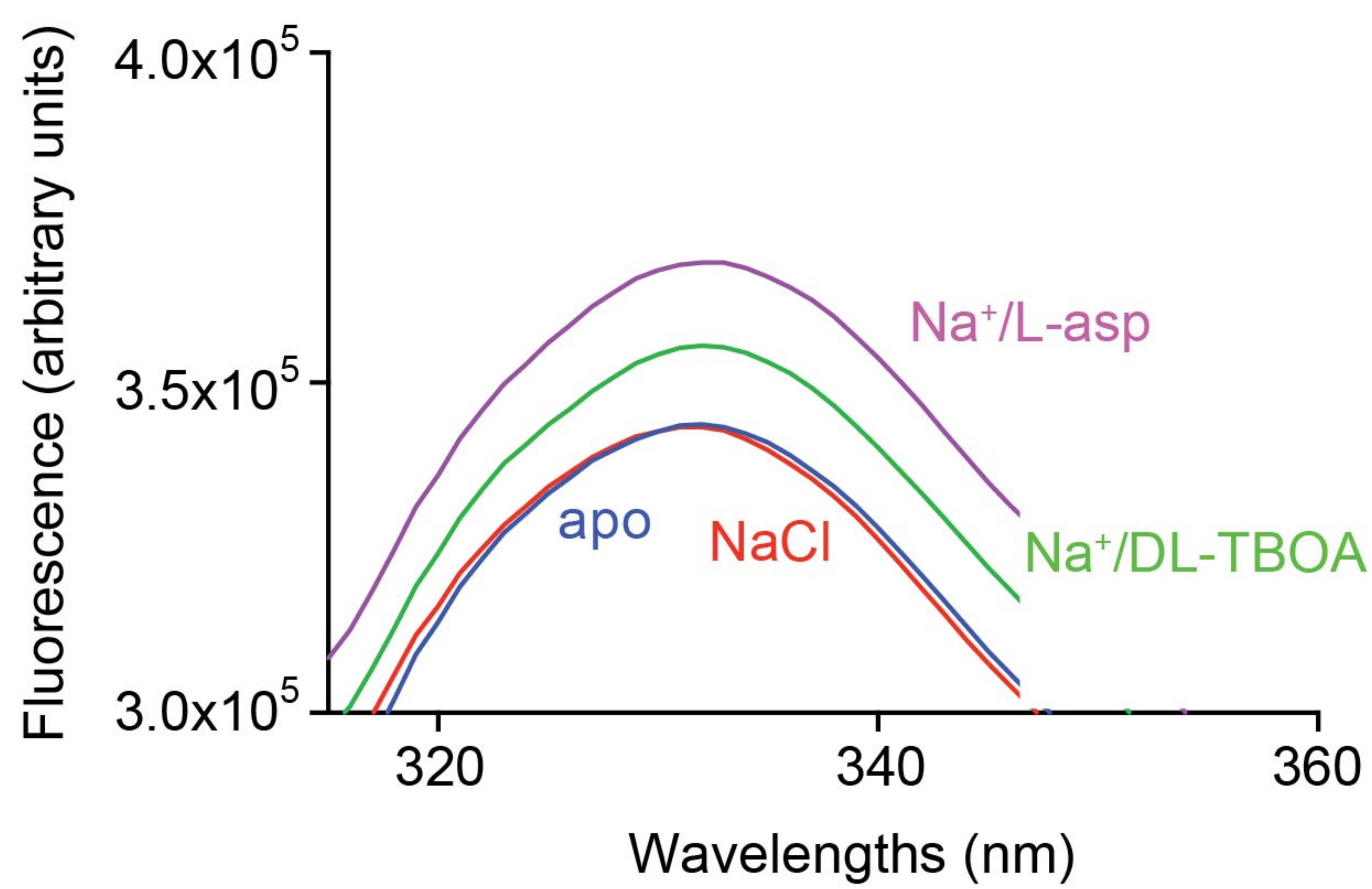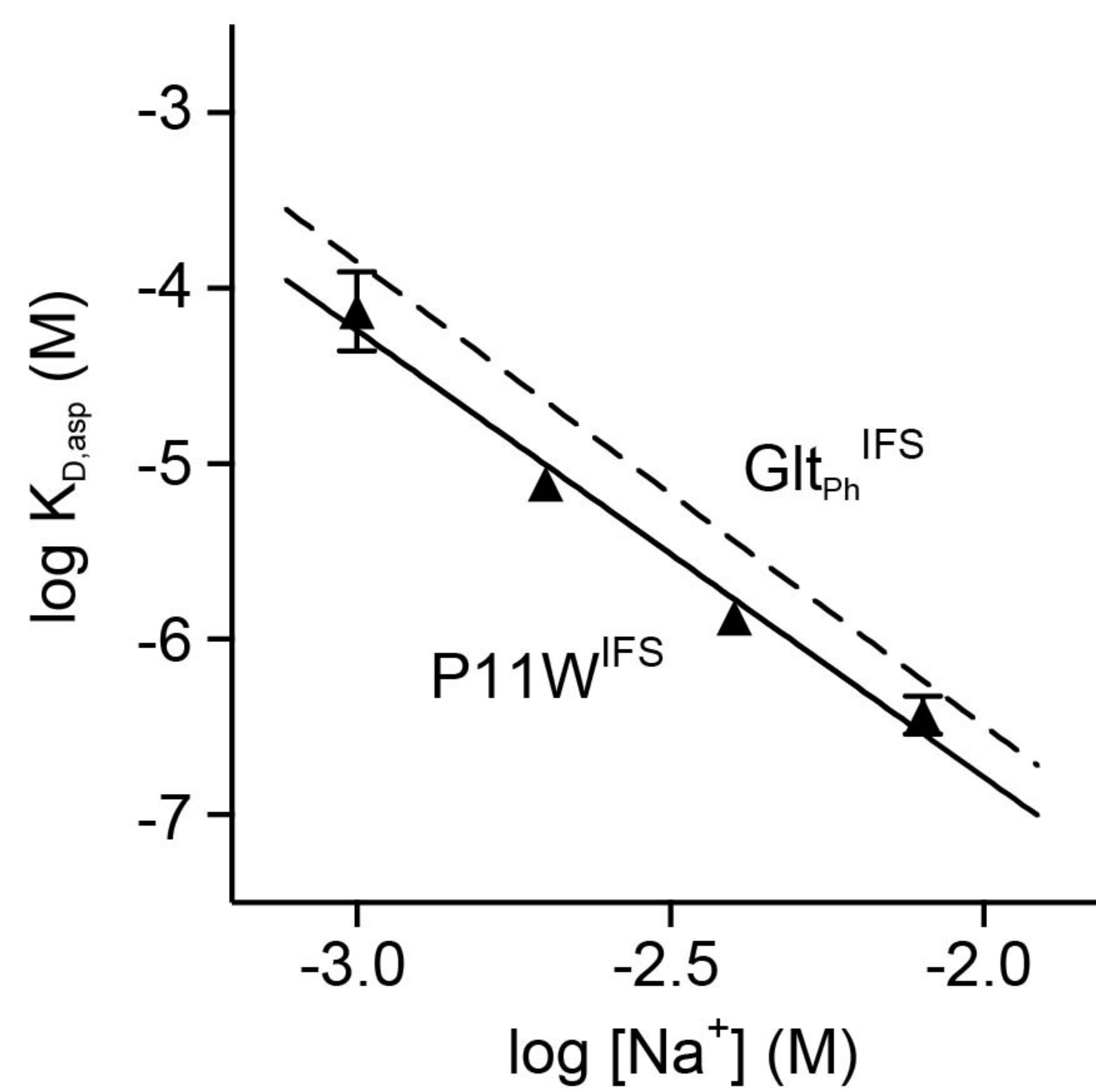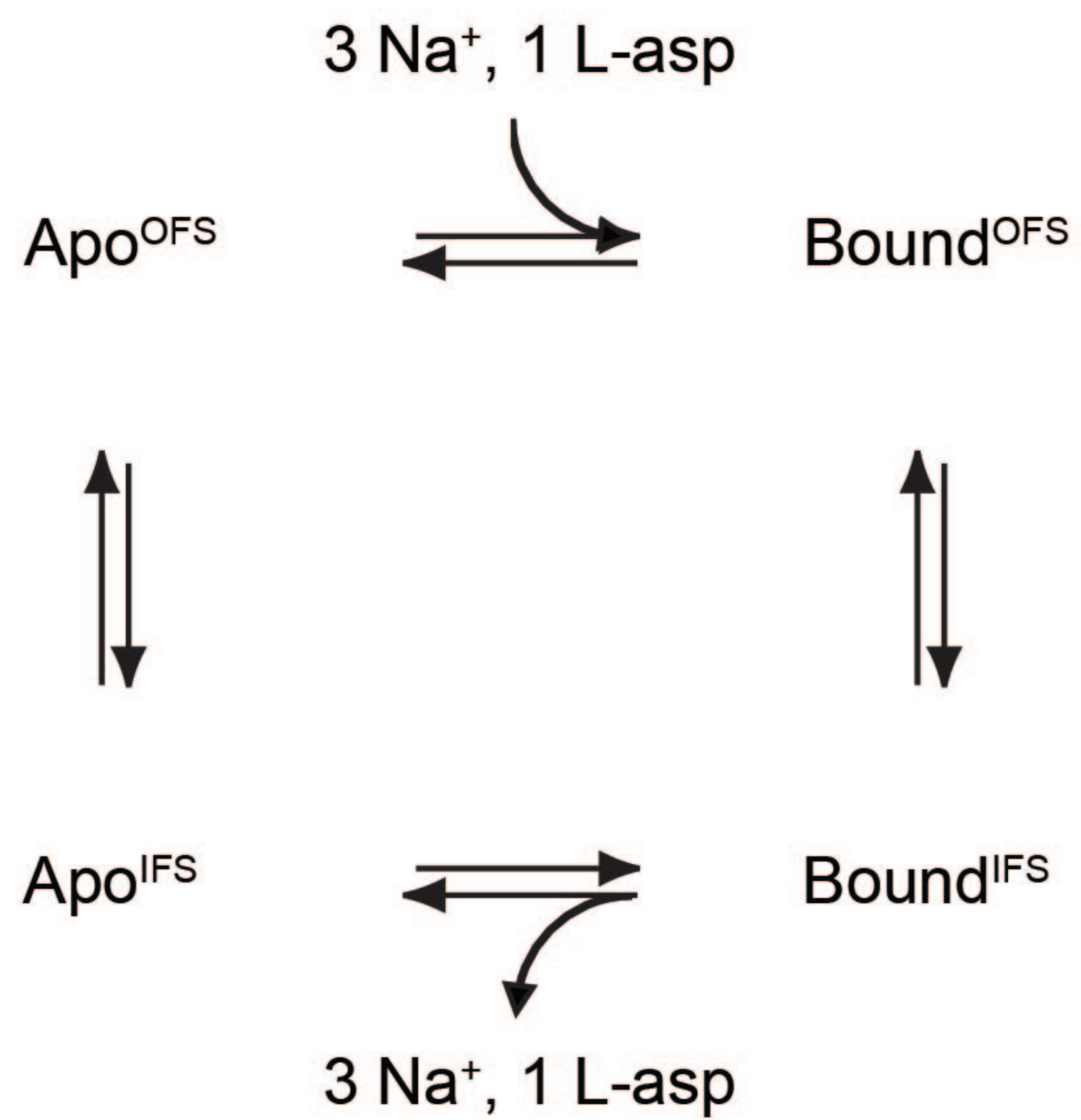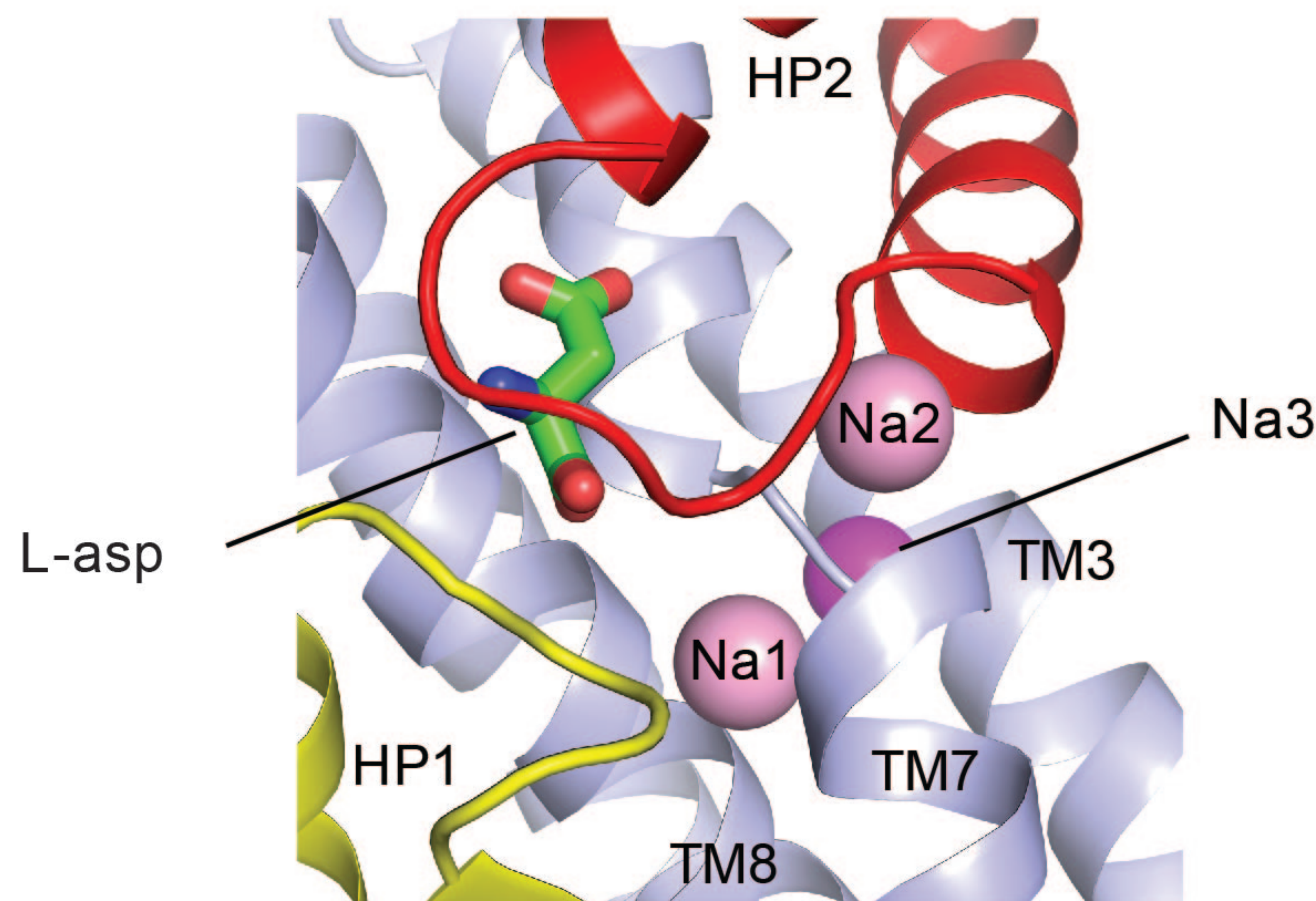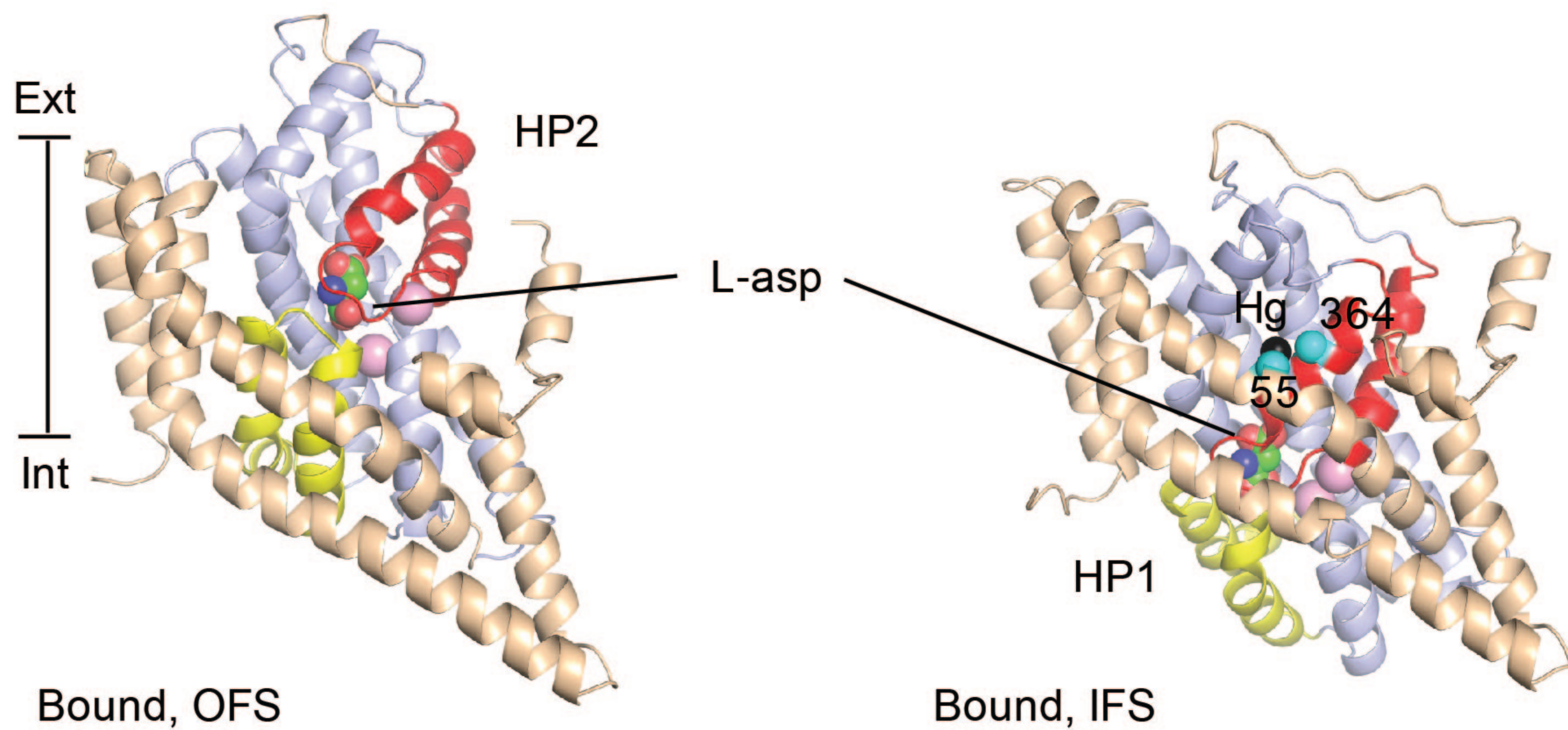
Apo   Na⁺ only   Na⁺/TBOA   Na⁺/Asp

a

F5M   +   +   +   +
HgCl$_2$ −   +   −   +

b

Fraction bound vs [L-asp] (µM)

c Asp binding — normalized fluorescence vs time (sec)

d Asp dissociation — normalized fluorescence vs time (sec)

e TBOA binding — normalized fluorescence vs time (sec)

a

k_{obs} (s^{-1})

[Na$^+$] (mM)

L-asp
mM

2
1
0.5
0.25
0.1
0.05
0.025
0.01

b

k_{obs} (s^{-1})

[Na$^+$] (mM)

c

$$TANa_3 \underset{2Na^+}{\overset{K_{D,Na}}{\rightleftharpoons}} TANa \xrightarrow[Na^+,\ L\text{-}asp]{k_{off,asp}} T$$

d

Asp uptake (mol asp/mol prot)

^{3}H-L-asp
NaCl

KCl
or
NaCl/L-asp

uptake

exchange

time (min)

a

k_{obs} (s^{-1}) vs [L-asp] (mM)

b

k (s^{-1}) vs [DL-TBOA] (mM)

Na$^+$
mM

16
12
8
4

c

$$T^* \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} TNa \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} TANa_3$$

Na$^+$ 2Na$^+$, L-asp

d

k_r (s^{-1}) vs [Na$^+$] (M)

L-asp

DL-TBOA

Lorem ipsum

4 mM Na⁺ (left, data with fit)

Legend:
- 500 TBOA (magenta)
- 1000 TBOA (orange)
- 2000 TBOA (black)
- 4000 TBOA (brown)
- 8000 TBOA (blue)

4 mM Na⁺ (right, fitted curves)

8 mM Na⁺ (left, data with fit)
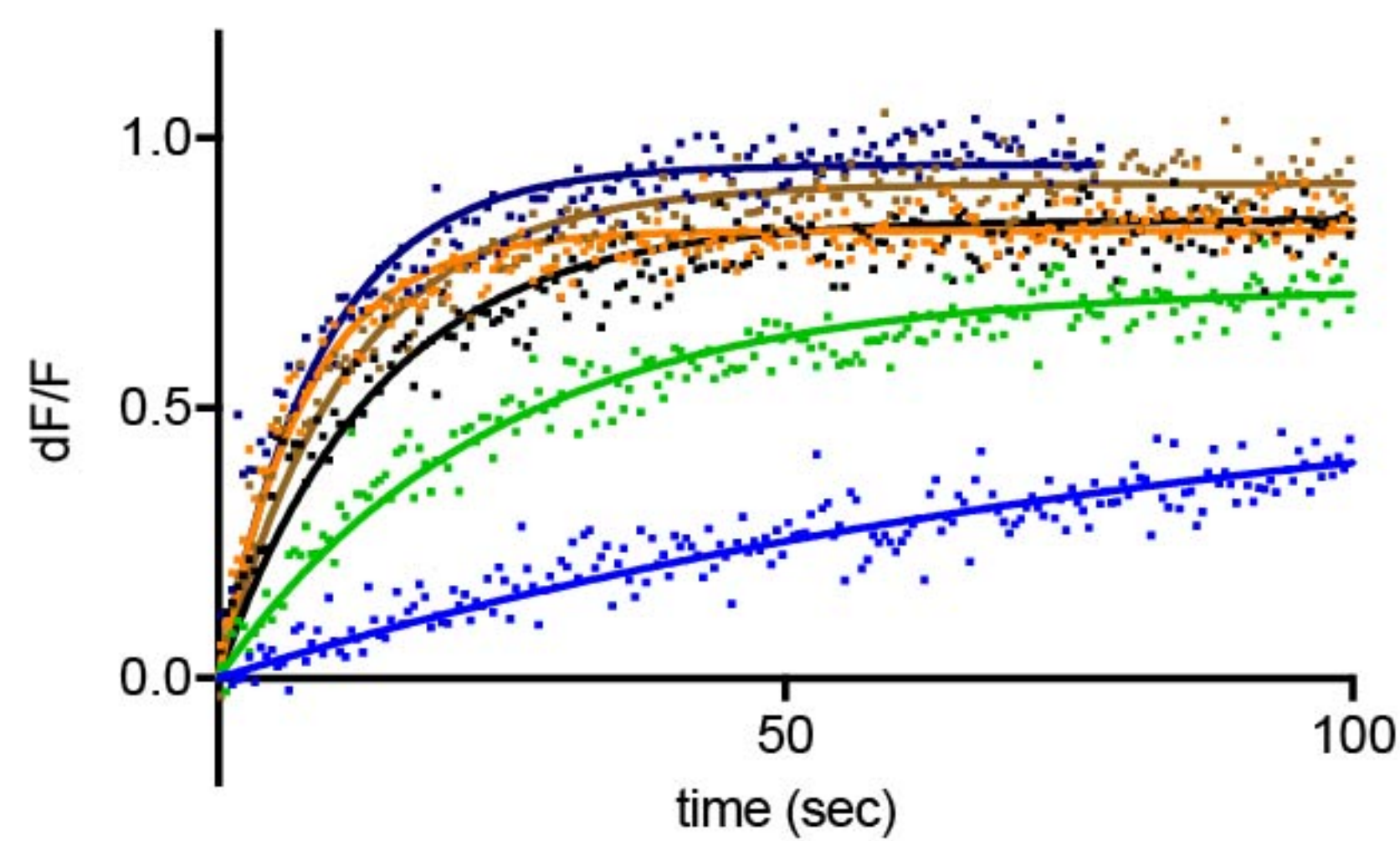
Legend:
- 500 TBOA
- 1000 TBOA
- 2000 TBOA
- 4000 TBOA
- 8000 TBOA

8 mM Na⁺ (right, fitted curves)

12 mM Na⁺ (left, data with fit)

Legend:
- 250 TBOA (green)
- 500 TBOA
- 1000 TBOA
- 2000 TBOA
- 4000 TBOA
- 8000 TBOA

12 mM Na⁺ (right, fitted curves)

16 mM Na⁺ (left, data with fit)
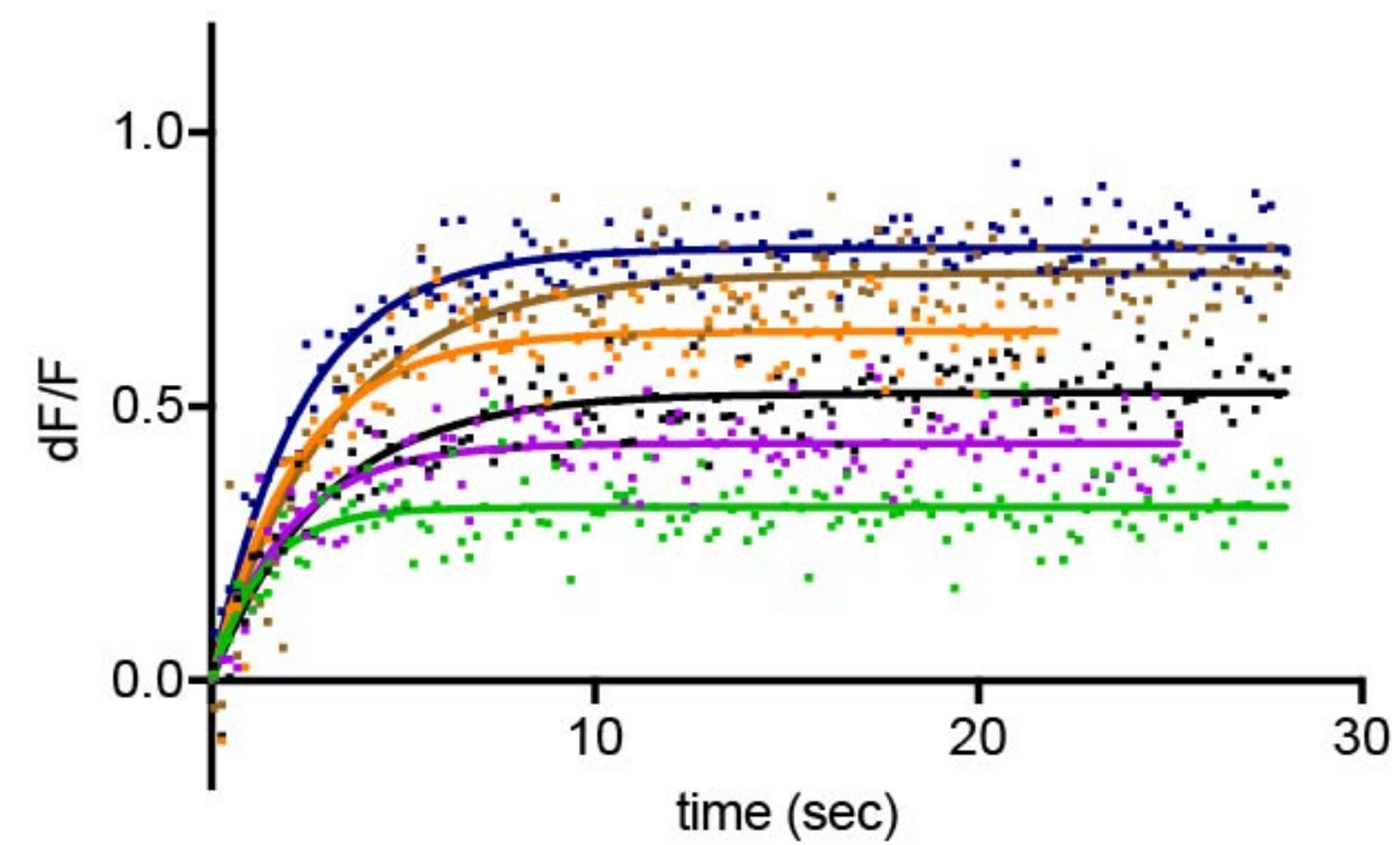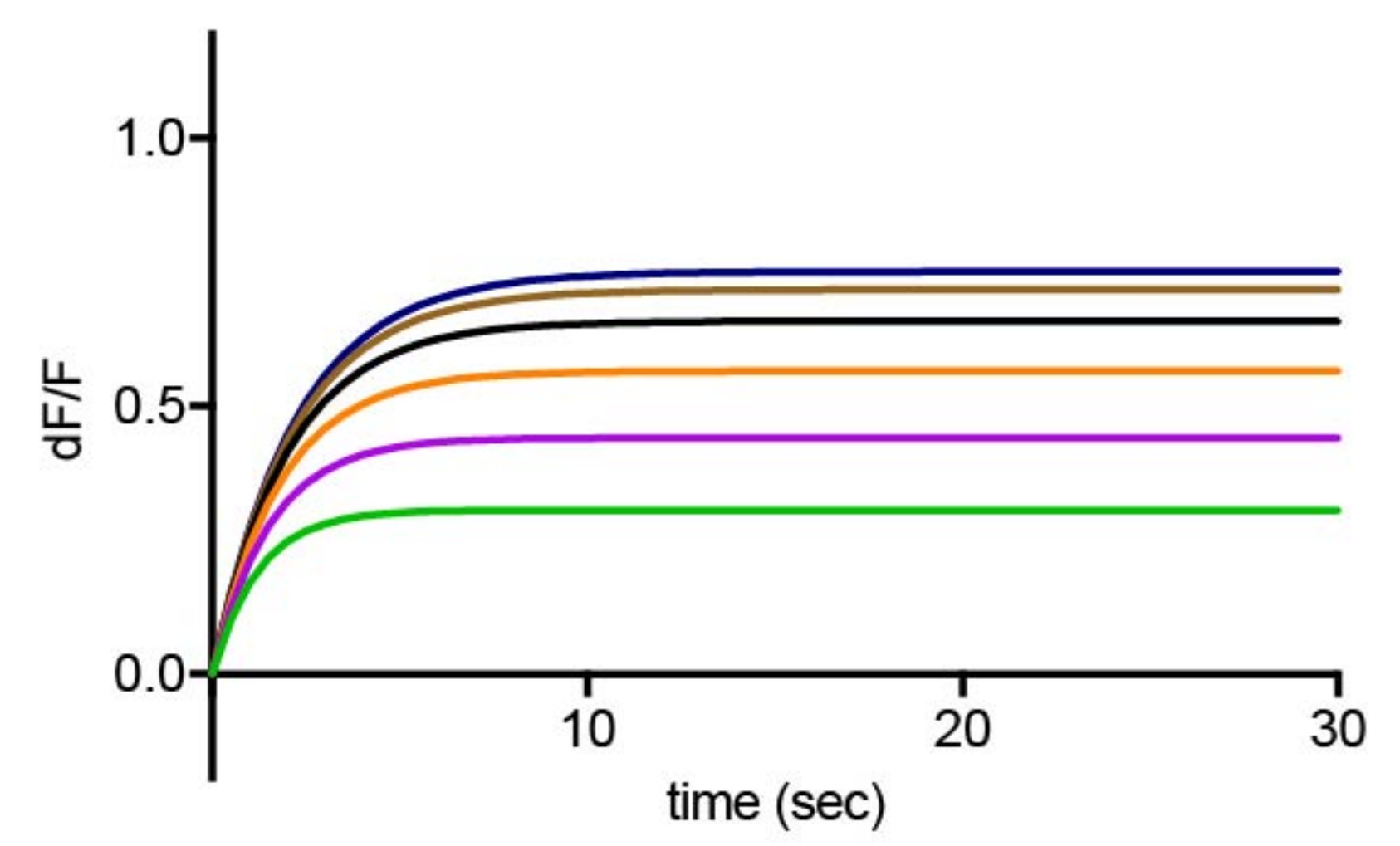
Legend:
- 100 TBOA (red)
- 250 TBOA
- 500 TBOA
- 1000 TBOA
- 2000 TBOA
- 4000 TBOA
- 8000 TBOA

16 mM Na⁺ (right, fitted curves)

a

Locked — Transport, HP1, HP2, C55, Scaffold

Unlocked — Transport, HP1, HP2, K55, Scaffold

b

FM — R397, D394, R395, S276, HP2, L-asp, Na2, Na1

WT — R397, D394, M395, R276, HP2, L-asp, Na2, Na1

c

8 mM Na+

4 mM Na+

WT 4 mM Na+

a

T $\xrightleftharpoons[\textcolor{green}{1}]{Na^+}$ TNa

144
39
0.27 M
Na$^+$

TNa $\xrightleftharpoons[\textcolor{green}{2}]{Na^+}$ TNa$_2$

1 10^7*
8 10^5*
8 10^{-2} M*

TNa$_2$ $\xrightleftharpoons[\textcolor{green}{4}]{L\text{-}asp}$ TANa$_2$

1.2 10^6*
71
6 10^{-5} M

TANa$_2$ $\xrightleftharpoons[\textcolor{green}{6}]{Na^+}$ TANa$_3$

1 10^7*
100*
1 10^{-5} M*

TNa $\xrightleftharpoons[\textcolor{green}{3}]{L\text{-}asp}$ TANa

5.0 10^4*
435
9 10^{-3} M

TANa $\xrightleftharpoons[\textcolor{green}{5}]{Na^+}$ TANa$_2$

1 10^7*
5 10^3*
5 10^{-4} M*

b

D390

R395

D394

L-asp