On the kinetics and conformational dynamics of elevator transporters
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DOI:
10.33612/diss.177792564

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 6

On the role of a conserved methionine in the Na⁺-coupling mechanism of a neurotransmitter transporter homolog

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Abbreviations: TM, transmembrane
Key words: transporter, stoichiometry, molecular dynamics simulations, cation-methionine interactions

This chapter is a modified version of a manuscript published in:
Neurochemical Research (2021)
(https://doi.org/10.1007/s11064-021-03253-w)
Chapter 6. Role of methionine

Abstract

Excitatory amino acid transporters (EAAT) play a key role in glutamatergic synaptic communication. Driven by transmembrane cation gradients, these transporters catalyze the reuptake of glutamate from the synaptic cleft once this neurotransmitter has been utilized for signaling. Two decades ago, pioneering studies in the Kanner lab identified a conserved methionine within the transmembrane domain as key for substrate turnover rate and specificity; later structural work, particularly for the prokaryotic homologs Glt₉₈ and Glt₉₁, revealed that this methionine is involved in the coordination of one of the three Na⁺ ions that are co-transported with the substrate. Albeit extremely atypical, the existence of this interaction is consistent with biophysical analyses of Glt₉₈ showing that mutations of this methionine diminish the binding cooperativity between substrates and Na⁺. It has been unclear, however, whether this intriguing methionine influences the thermodynamics of the transport reaction, i.e., its substrate:ion stoichiometry, or whether it simply fosters a specific kinetics in the binding reaction, which, while influential for the turnover rate, do not fundamentally explain the ion-coupling mechanism of this class of transporters. Here, are presented experimental results to address whether the mutation of the methionine in position 314 influence the stoichiometry of aspartate transport.

Introduction

Glutamatergic synapses are the primary excitatory synapses in the brain and are thought to be essential for learning and memory. In this form of chemical signaling, glutamate is released by the presynaptic nerve terminal, activating receptor proteins on the surface of the post-synaptic neuron. Excessive quantities of glutamate in the synaptic cleft, however, can be cytotoxic and are associated with traumatic events such as stroke (reviewed in [1,2]). Reuptake of glutamate is thus key to maintaining healthy levels, a task that falls largely to membrane transporters belonging to the SLC1 family, also known as excitatory amino acid transporters (EAAT) in the transporter classification database (TCDB) family 2.A.23 [3]. Structures of prokaryotic SLC1 homologues, including Glt₉₈ and Glt₉₁, from Pyrococcus horikoshii and Thermococcus kodakarensis, respectively [4–6], and of a thermally-stabilized mutant of EAAT1 (SLC1A1 or GLAST) [7], reveal a trimeric assembly, where each protomer contains eight transmembrane (TM) segments and two helical hairpins, HP1 and HP2. The three protomers interact through their so-called trimerization domains (Figure 1A), whose relative orientation remains constant during transport [8]. By contrast, the substrate-binding transport domains, in the periphery of the complex, (Figure 1A) undergo dramatic conformational changes, often referred to as elevator-like movements, in order to allow alternating access to the binding sites to each side of the membrane [9–13].

Like their mammalian cousins, Glt₉₈ and Glt₉₁ both require three sodium ions to drive transport of the substrate dicarboxylic acid [14–17]. However, they also differ in that they have a higher affinity for L-aspartate than for L-glutamate, and do not require antiport of potassium nor co-transport of a proton [5,18,19]. The binding sites for sodium and substrate are buried beneath HP2 in the core of the transport domain, and in close proximity to one another (Figure 1B, 1C) but not in direct contact. The Na⁺ binding site furthest from the extracellular surface of the protein, denominated Na₃, is formed by Tyr91, Thr94 and Ser95 in TM3 (residue numbering in Glt₉₁ will be used hereafter) as well as Asn313 and Asp315 from TM7 (Figure 1C). The latter are part of a conserved sequence motif, NMDGT, which the Kanner lab revealed to be essential for the transport functionality of mammalian transporters [20–23]. Asn313 also contributes a backbone carbonyl atom to the so-called Na₁ site, as does Gly309 from TM7, the side chain of Asp309, and the backbone carbonyl of Asn405, both in TM8 (Figure 1C). The Na₂ site is the most proximal to the extracellular space, and also to the substrate binding site. Ion coordination at this site differs from
that of Na1 and Na3, in that it does not involve a direct contact with an acidic sidechain, as is typical among Na+-coupled transporters [24–28]; instead, it involves only four backbone carbonyl oxygens from residues in HP2 (Ser352, Ile353 and Thr355) and TM7 (Thr311), and most strikingly, the sidechain of Met314, of the NMDGT motif (Figure 1B).

This methionine is particularly intriguing. In the known EAAT structures, it separates the Na+ ion in the Na2 site from the substrate, when both are bound. However, such direct methionine-Na+ contacts are extraordinarily rare: a search of the Protein Data Bank [29] reveals only three other structures that appear to feature this interaction, but none of those proteins are known to be Na+ dependent, so these assignments are not conclusive (Table 1). Yet Met314 is conserved in all known sodium-coupled transporters in the SLC1 family; interestingly, though, it is substituted by Leu in proton-coupled transporters such as *E. coli* GltP [30] and *B. subtilis* DctA [31]. Studies in the Kanner lab have examined the effects resulting from mutation of this conserved methionine in Na+-dependent mammalian transporters (Met397 in EAAT2, SLC1A2 or GLT-1, and Met367 in EAAT3, SLC1A3 or EAAC1). Interestingly, though, it is substituted by Cys, Leu, Ala or Ser were observed to severely diminish the rate of substrate uptake and to alter its dependence on Na+ concentration [21,22,32], in a manner that suggests Na+ binding is partially impaired. More recent studies of GlkP and GlkH have dissected the interdependence between substrate and Na+ binding in detail, concluding that these processes are highly cooperative. Specifically, existing data indicates that two Na+ ions must bind before the substrate can be recognized [33–35], presumably to sites Na1 and Na3 in the outward-facing conformation. Binding of substrate and of the third ion to the Na2 site would follow. However, this cooperativity is greatly suppressed when Met314 (Met311 in GlkPh) is replaced by Ala or Leu [36], as is the substrate affinity. Although no structures have been reported for any of these mutants, Trp fluorescence measurements also show that the pattern of conformational changes in HP2 in response to ion and substrate binding [19,36] is also subtly altered upon mutation of Met314 [37].

To our knowledge, the precise explanation for these intriguing effects remains unclear. A plausible hypothesis is that Met314 contributes to defining the Na+ stoichiometry of the transport reaction; by impairing the Na2 site, mutation of Met314 would logically slow down the reaction for a given Na+ concentration gradient, and also partially decouple substrate and Na+ binding, as observed. Here we examine the stoichiometry of Met314 mutants by measuring the

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**Figure 1 Structure and binding sites of GlkP**  
(A) The structure of the holo trimer (PDB entry 5E9S), in an outward-facing conformation, is viewed from the extracellular side and shown as cartoon helices, with each subunit in a different color. Dashed lines indicate the boundaries between the transport domain and trimerization domain in each protomer. Sodium ions (green) and L-aspartate (yellow, blue and red) are shown as spheres.  
(B) Close-up view of the binding site for L-aspartate (sticks with carbon atoms in white), and of the Na+ binding site referred to as Na2 (green sphere), with key residues, including Met314, shown as sticks and labelled. Hairpin HP2 (orange) occludes access to these sites from the extracellular side. Thin black lines indicate oxygen- or sulfur-ion coordination.  
(C) Close-up of the Na1 and Na3 sites, deeper into the structure of the transport domain, showing key residues involved in Na+ coordination.
reversal potential for L-aspartate transport. In electrogenic secondary active transporters the translocation of the coupling ion leads to the accumulation of charges which eventually will inhibit transport. The membrane potential generated by living cells dissipates the accumulation of such charges, offering an inward charge gradient for the cotransported ion. Thus, the direction of the membrane potential together with the direction of the chemical gradient of substrate and co-ion influence the direction of substrate flux across the membrane. The reversal potential is the magnitude of electrical potential across the membrane that is needed to counterbalance the transport direction of the substrates gradients in order to achieve thermodynamic equilibrium. The equation derived by Fitzgerald and colleagues allows to calculate the voltage needed to reach thermodynamic equilibrium for a given set of conditions according to the stoichiometry of transport. In systems where the stoichiometry is unknown, multiple reversal potentials need to be tested experimentally [38].

Materials and Methods

Mutagenesis, expression and purification
Mutagenesis was carried out using the QuickChange protocol (using primers CAACCATTAATTGCGATGGCACCGCAC and TCGGTTGCCATCGCA-ATTAAATGGTTG for M314C; and CAACCATTAATAGCGATGGCACCGCAC and TCGGTTGCGATCGCATATTAATGGTTG for M314S) on the expression plasmid described in [6]. The constructs were verified by DNA sequencing and transformed in E. coli MC1061 cells. Expression and purification were carried out as previously described [39].

Reconstitution into proteoliposomes
A solution of E. coli total lipid extract (20 mg ml\(^{-1}\) in 50 mM KPi, pH 7.0) was extruded with a 400-nm-diameter polycarbonate filter (Avestin, 11 passages) and diluted with the same buffer to a final concentration of 5 mg ml\(^{-1}\). The lipid mixture was destabilized with 10% Triton-X100. Purified Glt\(_T\) and the destabilized lipids were mixed in a ratio of 1:250 (protein: lipid) and incubated at room temperature for 30 min. Bio-beads were added four times (25 mg ml\(^{-1}\), 15 mg ml\(^{-1}\), 15 mg ml\(^{-1}\), 29 mg ml\(^{-1}\) lipid solution) after 0.5 hr, 1 hr, overnight and 2 hr incubation, respectively, on a rocking platform at 4°C. The Bio-beads were removed by passage over an empty Poly-Prep column (Bio-Rad). The proteoliposomes were collected by centrifugation (20 min, 298,906 g, 4°C), subsequently resuspended in 50 mM KPi, pH 7.0 to the lipid concentration of 20 mg ml\(^{-1}\) and freeze-thawed for three cycles. The proteoliposomes were stored in liquid nitrogen until subsequent experiments.

Measuring transporter equilibrium potentials
Stored proteoliposomes were thawed and collected by centrifugation (20 min, 298,906 g, 4°C), the supernatant was discarded and the proteoliposomes were resuspended to a concentration of 10 mg ml\(^{-1}\) of lipids in buffer containing 20 mM Hepes/Tris, pH 7.5, 200 mM NaCl, 50 mM KCl, 10 μM L-aspartate (containing 1 μM \([\text{H}]\)-L-aspartate). The internal buffer was exchanged by three cycles of freezing in liquid nitrogen and thawing, and finally extruded through a polycarbonate filter with 400 nm pore size (Avestin, 11 passages). The experiment was started by diluting the proteoliposomes 20 times into a buffer containing 20 mM Hepes/Tris, pH 7.5, 200 mM NaCl, 3 μM valinomycin, and varying concentrations of KCl and Choline Cl were added in order to obtain the membrane potentials –78.06 mV, –39.03 mV and –26.02 mV, which are the calculated reversal potentials for hypothetical 4:1, 3:1 and 2:1 Na\(^+\):L-asp stoichiometries (35.0/26.4/19.2 mM CholineCl, 0/11.1/18.4 mM KCl).

After 1, 2 and 3 min the reaction was quenched with ice-cold quenching buffer containing 20 mM Hepes/Tris, pH 7.5, 250 mM Choline Cl and immediately filtered on nitrocellulose filter (Protran BA 85-Whatman filter). Finally, the filter was washed with 2 ml of quenching buffer. The flux of radiolabeled aspartate was measured by subtracting the amount of radiolabeled aspartate at 1 min from the amount of radiolabeled substrate at 3 min. The filters were dissolved in scintillation cocktail and the radioactivity was measured with a PerkinElmer Tri-Carb 2800RT liquid scintillation counter. The equilibrium, or reversal, potential, \(E_{\text{rev}}\), for each condition was calculated as described in [38].

Results and Discussion

Polarization effects explain atypical methionine coordination of Na\(^+\)
As mentioned above, the observation that the methionine in the conserved NMDG\(_T\) motif coordinates the Na\(^+\) ion in the Na2 site is, statistically speaking, highly atypical, given its hydrophobic character [40]. However, close-range contacts between methionines and the guanidinium and amino groups in arginines and lysines are common in known protein structures [41], as are interactions with carboxyl and carbonyl groups [42], and aromatic rings [42,43]. This striking promiscuity indicates the sulfur atom in the methionine side
chain is highly polarizable, i.e., it can alter its electrostatic character to better match its near environment. To examine whether this polarizability might also explain the interaction observed in the Na2 site in SLC1-family Na+-dependent transporters, a series of MP2-level quantum-mechanical calculations have been carried out [44]. The result shows that the interaction between Na+ and the S in the methionine analog methylthioethane is unequivocally attractive, favoring a contact distance between ion and Sulfur of about 2.8 Å, which closely resembles what is observed in the structure of GltTk, namely 3.0 Å (on average).

All-atom molecular dynamics (MD) simulations have also been used to examine this interaction in the context of the actual structure of the transporter [44]. The simulations however showed irreversible dissociation of the Na+ ion at the Na2 site, in all three protomers. This observation is consistent with the conclusions of an independent simulation study of GltTk reported while this study was underway [45]. Indeed, irreversible dissociation of the Na2 ion appears to have been a common denominator of simulation studies of the mechanisms of both GltPh and GltTk [19,46–48]. It is however important to recognize that this observation is artefactual since the methionine rendered by the simulation software fails to capture the polarization of the S atom. The dissociation constant of the Na2 site, while L-Asp is bound, has been estimated to be 10-100 μM [49], which is 3-4 orders of magnitude smaller than the concentration of free Na+ in simulation systems (~100 mM). The corresponding off-rate was also estimated at 100 per second [49]. It is clear, therefore, that irreversible dissociation of the ion in Na2 in microsecond scale simulations reflects a systematic methodological problem, rather than a mechanistically significant event.

Simulation of holo-state GltTk trimer carried out after correcting this showed stable occupancy of the Na2 site, similar to the ions in the Na2 and Na3 binding sites. The presence of the ion at the Na2 site also stabilizes the L-aspartate in its binding site, unlike in the simulations without the force field correction, where the substrate L-aspartate dissociated subsequent to the Na2 ion.

**Met314 does not define the Na+ stoichiometry of GltTk**

Experimental mutation of the methionine in the NMDGT motif has clear effects on the function of both EAAT2/3 [21,22,32] and GltTk [36,37]. These effects could be interpreted as evidence that this rare interaction is essential for defining the 3:1 stoichiometry of the coupled ion-substrate transport reaction. To examine this hypothesis experimentally, we determined the ion-substrate stoichiometry in wild-type GltTk or mutants in which Met314 was replaced by cysteine, serine, alanine or leucine, using purified protein reconstituted in proteoliposomes. To do so, we quantified the extent of Na+-coupled L-aspartate influx or efflux in conditions that would strongly favor efflux (a 20-fold outward gradient in L-Asp), were it not for an outward transmembrane electrical potential, experimentally generated as potassium diffusion potential using the ionophore valinomycin (Figure 2A). Because co-transport of Na+ and L-Asp moves positive charge across the membrane, this outward potential not only opposes L-Asp efflux, but can also drive uphill influx at sufficiently large (negative) values. The value at which no net flux occurs, referred to as the reversal potential, depends on the number of Na+ ions co-transported with L-Asp, and can be estimated a priori [38,50,51]. Here, we examined the effect of K+ diffusion potentials that would result in a zero-flux equilibrium in hypothetical reactions requiring 4, 3 or 2 Na+ ions in our specific experimental conditions. The results for WT GltTk showed that this ‘reversal potential’ is ~39 mV, which corresponds to a 3:1 stoichiometry (Figure 2B). As expected, the smaller (less negative) potential allows for downhill efflux of L-Asp while the larger (more negative) potential has the opposite effect, and drives L-Asp into the liposomes, uphill. This result reproduces our previous measurements [17,39] and is satisfyingly consistent with the structural data [19]. Surprisingly, however, analogous experiments for M314C and M314S demonstrate that the stoichiometry of the transport reaction is also 3:1 (Figures 2B, C). M314A was only marginally stable in detergent solution and thus difficult to characterize conclusively, but the trends in the data suggest its stoichiometry is also unchanged relative to WT (Figure 2D). Unfortunately, M314L could not be characterized due to low expression levels.

It is not self-evident how to rationalize these observations at the structural level. While serine side chains are often observed in Na+ binding sites coordinating the ion via their hydroxyl oxygen, cysteine and alanine side chains are uncommon [52]. Thus, to clarify these experimental results, a set of simulations was performed with the corrections above mentioned for the M314A and M314C mutants. The results are in agreement with the experimental data showing that the Na+ ion at the Na2 site remained bound throughout the simulation coordinated by water molecules [44].

The measurements of WT and mutant GltTk demonstrate that the Met314 sidechain is not required for occupation of Na2 by Na+, despite its role in ion coordination in the WT transporter. It seems reasonable to conclude, therefore,
that the thermodynamics of the transport mechanism of Glt₆₄ and by extension other Na⁺-coupled transporters in this family, is not fundamentally defined by the conserved methionine in the NMDGT motif. While this residue appears to be key to couple substrate and Na⁺ binding in this class of transporters, this cooperativity does not seem to be essential to enforce the stoichiometry of the transport cycle, implying other factors more fundamentally control the underlying structural mechanism.

**HP2 occlusion as a rationale for stoichiometric coupling**

Previous studies of the Na⁺/Ca²⁺ exchanger NCX_Mj, also a secondary-active transporter, might provide clues into the nature of that molecular mechanism. NCX_Mj translocates either 3 Na⁺ or 1 Ca²⁺ across the membrane [51], and thus functions as an antiporter. A systematic examination of the conformational energy landscape of NCX_Mj, using molecular simulations and X-ray crystallography, concluded that the interconversion between open and occluded states is the process that explains the stoichiometry of the transport cycle, i.e., the origin of the coupling mechanism [53]. Specifically, the authors found that occluded conformations, which are necessary intermediates in the alternating-access transition, are energetically accessible only when 3 Na⁺ or 1 Ca²⁺ are bound [53]; partial or no ion occupancies, or H⁺ binding to the Na⁺/Ca²⁺ sites, do not permit occlusion, and accordingly do not catalyze transport.

Simulations for Glt₆₄ provide support that this notion will apply to this transporter as well. The order and timescale of these events is artefactual, but the resulting configuration, only occupied by Na⁺ in Na1 and Na3, is a mechanistically relevant state in physiological conditions [33–35] (Ewers et al. 2013; Reyes, Oh, and Boudker 2013; Hänelt et al. 2015). In agreement with a structure of inward-facing Glt₆₄ very recently published [12], we observe that this outward-facing partially occupied state preserves many of the key features of the holo-state structure [19]. For example, the ion-pair between Arg401 and Asp394, which primes Arg401 for substrate coordination (Figure 1B, 3A, C) is present; the configuration of Met314 in this state also permits Na⁺ coordination at the Na2 site (Figure 3B, D). Independent simulations show that occupancy of Na3 alone controls the conformation of Arg401 (Figure 3A, C), seemingly through their electrostatic repulsion. However, binding at Na3 alone is not sufficient to reorganize the backbone or side chain of Met314, even though Asn313 and Asp315, which flank Met314, both contribute side chains to the Nat site. Only when Na1 is also occupied does Met314 reorient to match the geometry conducive to ion binding at the Na2 site (Figure 3B, D).

This partially occupied state, however, lacks a central feature of the holo-state structure, namely HP2 in a configuration occluding the binding sites (Figure 3E). Invariably, the absence of L-Asp and the third ion at the Na2 site leads to the opening of this hairpin towards the extracellular space, which after 1 μs of simulation adopts a conformation closely resembling that observed in structures bound to blockers [5,12] (Figures 3G, 3H). By contrast, extended simulations of the fully-occupied transporter demonstrate that the presence of
Figure 3 Conformation of Glt\textsubscript{H} in a partially occupied state with Na\textsuperscript{+} ions at Na\textsubscript{3} and Na\textsubscript{1}. (A) The crystal structure of the apo-state transporter (PDB entry 5E9S, dark blue) is superposed on a snapshot of the simulation of the apo state in which a single sodium ion (green sphere) was added to the Na\textsubscript{3} site (light blue), with key side chains shown as sticks and labelled. (B) The crystal structure of the fully-occupied transporter (PDB entry 5DWY, gray) is superposed on a snapshot of the simulation in which two sodium ions were sequentially added at the Na\textsubscript{3} and Na\textsubscript{1} sites (red). (C) Distance between the side chain terminal carbon atoms of Arg401 and Asp394, shown as a probability histogram over time, for simulations of fully-occupied Glt\textsubscript{H} (black, holo); the apo state (blue); a hypothetical intermediate occupied only at the Na\textsubscript{3} site (light blue); and a state occupied at the Na\textsubscript{3} and Na\textsubscript{1} sites, either after release of Na\textsubscript{2} and L-Asp (orange) or after addition of ions to the apo state (red). The formation of a salt-bridge between Arg401 and Asp394 primes the former to coordinate the substrate and requires binding of Na\textsuperscript{+} to Na\textsubscript{3}. The values of this distance in the crystal structures of apo (blue) and holo (black) transporter are shown as dashed vertical lines. (D) Distance between the C\textsubscript{\textsuperscript{a}} atoms of Met314 and Asn405 in TM8, shown as in (C). Binding of Na\textsuperscript{+} to Na\textsubscript{1}, with Na\textsubscript{3} already occupied, recruits backbone atoms adjacent to Met314, and positions its sidechain for coordination of the third ion, at the Na\textsubscript{2} site. (E) Crystal structure of the outward-facing holo-state of Glt\textsubscript{H}, highlighting the configuration of HP2 that occludes access to the Na\textsuperscript{+} and substrate binding sites. (F) HP2 remains closed during simulations of the holo-state transporter, as observed in the holo-state crystal structure (blue). Simulation snapshots (orange) are shown for each of the three protomers, taken at 1.0, 1.5 and 2.0 μs. (G) The crystal structure of the occluded holo-state of Glt\textsubscript{H} (blue) is compared with the crystal structure of TBOA-bound Glt\textsubscript{H} (gray) (PDB entry 2NWW). (H) HP2 opens during simulations in which only Na\textsubscript{1} and Na\textsuperscript{+} are occupied, adopting a conformation comparable to that observed in the crystal structure of TBOA-bound Glt\textsubscript{H}. Simulation snapshots are shown (orange) for each of the three protomers, taken at 1.0, 1.5 and 2.0 μs, and superposed on the crystal structure of holo Glt\textsubscript{H} (blue).
all ions and substrate correlates with HP2 favoring the occluded conformation (Figures 3F). These results indicate that the energy landscape that governs the dynamics of HP2, and by extension the occlusion of the transporter and the viability of the alternating-access transition, is indeed re-shaped by the occupancy or vacancy of the ion and substrate binding sites. In other words, opening of HP2 does appear to serve as the structural mechanism that prevents uncoupled transport, as suggested elsewhere [36]. Further experimental and computational investigations will be required to continue to examine this hypothesis and fully clarify its molecular basis.

Conclusions

Most secondary-active transporters couple the uptake or efflux of substrates to the co-translocation of ions in well-defined stoichiometries. This coupling is the defining functional feature of this class of proteins, as it permits them to harness transmembrane electrochemical gradients of usually Na⁺ or H⁺ to power substrate transport, even against an opposing concentration gradient. The general principles that explain this coupling, however, remain to be clearly established. In Na⁺-dependent transporters of the SLC1 family, substrate and Na⁺ binding are cooperative, and so it is reasonable to infer that the substrate-ion coupling of the transport reaction stems from the cooperativity of the binding reaction. However, mutation of a conserved methionine in the so-called NMDGT motif greatly impairs this cooperativity, and while it also slows down the turnover rate, our data shows that it does not impact the stoichiometric substrate-ion coupling of the transport cycle. Therefore, we conclude that while cooperativity in binding can be a form of kinetic optimization, the thermodynamics of coupled substrate-ion transport owe to other molecular mechanisms, that are, in our view, yet to be fully delineated.

Acknowledgements

This research was funded by the Divisions of Intramural Research of the National Institute of Neurological Disorders and Stroke (LRF) and of the National Heart, Lung and Blood Institute (WZ and JDFG), National Institutes of Health (NIH), USA; and by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek 714.018.003 and 711.017.012 (GT and DJS). Computational resources were in part provided by the NIH HPC facility Biowulf.
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