Transmembrane allosteric energetics characterization for strong coupling between proton and potassium ion binding in the KcsA channel

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Contributed by Ann E. McDermott, June 2, 2017 (sent for review September 26, 2016; reviewed by Patrick Loria and Ichio Shimada)

The slow spontaneous inactivation of potassium channels exhibits classic signatures of transmembrane allosteroy. A variety of data support a model in which the loss of K+ ions from the selectivity filter is a major factor in promoting inactivation, which defeats transmission, and is allosterically coupled to protonation of the intracellular selectivity filter, with its strongly conserved signature sequence (TVGYG) (11, 12). Ion flux is controlled by molecular conformations and ensembles of conformational states (1, 2). In systems that function in signaling to metabolic regulation and other contexts (3, 4), ligand binding in distal pockets modulates protein function via long-range molecular rearrangements. A canonical expression of allosteroy is the effect of binding of one ligand on the affinity of another in a distant pocket. Detailed models such as Koshland–Nemethy–Filmer (KNF) (6), and the extended population shift theory (7) provide theoretical frameworks to understand allosteric coupling. These models are most meaningful when detailed thermodynamic, kinetic, and structural data can be obtained under functionally relevant conditions, which is generally a challenging objective. NMR has the potential to provide quantitative probes of the thermodynamic and kinetic details of allosteric behaviors in a site-specific and structurally detailed fashion.

Potassium channels show classic transmembrane allosteric regulation, controlling their mean open time. This ubiquitous family of membrane proteins controls the flow of the potassium ions through membranes, a fundamental step for many physiological functions (8, 9). High selectivity and conduction rate (1010 ions per s−1) (10) have been attributed to the selectivity filter, with its strongly conserved signature sequence (TVGYG) (11, 12). Ion flux is controlled by channel inactivation (8, 13), which causes the spontaneous cessation of ion flow after the channels are activated. KcsA is a model system for potassium channels and shares functional similarities with several voltage-gated potassium channels (8, 14).

A kinetic hypothesis for channel activation and C-type inactivation is shown in Fig. 1A. The deactivated resting state has maintenance of ion flow at the intracellular gate, but has its transmembrane helices in a compact closed state, occluding flow at the intracellular gate. Protons are known to bind to the pH sensor in the deactivated state (15), causing the helical bundle to open (16) and a concomitant surge in current through the channel. This transiently populated conductive form is called the activated state. Thereafter, a spontaneous structural change inactivates the channel, and the current gradually drops to reach a plateau with very low channel open probability. The formation of the inactivated state is reversible and in slow equilibrium with other states (17). Inactivation is distinct from deactivation, which is the reverse of activation. In contrast to activation and deactivation, mutations that perturb inactivation in KcsA (and in Shaker channels) (18, 19) are clustered in region near the selectivity filter, distinct from the activation domain and presumably sensitive to K+ ion binding (20, 21). The kinetics of inactivation are dependent on permeant ions; higher concentrations of K+, or of other permeant ions like Rb+, can significantly slow the rate of inactivation (22, 23), whereas there is little dependence on the activating stimulus (pH for KcsA as it activates at low pH; e.g., pH 3−4) (22, 24).

The formation of the inactivated state is assumed to be an allosteric response to activation (25) because of its spontaneous kinetics following activation. Analogous regulatory mechanisms are known to be important in a range of biological contexts, such as regulating the frequency and duration of firing in excitable cells (26) associated with QT syndrome in cardiac tissue, and other pathologies (9, 27).

Motivated by its significance, efforts have been made to illustrate the mechanism of inactivation, and the structure of the inactivated state (23, 28−30). The inactivated state is believed to have a distinct structure from the deactivated or activated state (22, 24). Kinetic signatures and mutation profiles suggest that inactivation involves ion release in the selectivity filter, due to a large allosteric change in affinity as indicated in the thermodynamic cycle, shown in Fig. 1B; we probe this hypothesis for the nature of inactivation in this work.

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**Significance**

Although C-type inactivation in potassium channels is crucial in many biological signaling processes, its mechanism is debated. We address a hypothesis that K+ affinity and ion loss due to allosterically coupled activation is a major factor in promoting inactivation. We show that in KcsA, activation by proton binding at the intracellular pH sensor can significantly reduce K+ affinity at the extracellular selectivity filter, which would be expected to contribute to current cessation (inactivation). This allosteric coupling is substantially reduced by mutating a residue that connects the inner transmembrane helix hinge (involved in activation) to the base of the selectivity filter, thus providing clear evidence for the allosteric coupling network and explaining why this mutant is inactivationless.
Cast in this way, the inactivation process is a classic allosteric effect of one binding event on the affinity for a second ligand in a distant pocket. A number of studies have already suggested that the inactivated state is similar to a low-K⁺ state (28, 31, 32). The putative loss of K⁺ ions and associated structural change at the selectivity filter would logically lead to cessation of ion flow: in the "knock-on" mechanism (33), steady-state K⁺ binding and facile ion binding is conductive, but has a closed intracellular gate, with a deprotonated pH sensor and the TM2 bundle crossed and occluded. This stable species is observed in numerous X-rays structures of KcsA. Following a drop in pH, the activated state slowly and spontaneously decays to an inactivated state (PDB ID code 3F5W). Our working hypothesis for inactivation is that the inactivated state differs from the activated state by loss of the K⁺ ions in the selectivity filter and associated conformational and hydration changes. Electro-physiology studies show that protein can recover from the inactivated state to the deactivate state at high pH, here represented by the dashed lines through a putative state inactivated* or I* that is "closed at both gates," meaning both deprotonated at the pH sensor and K⁺ deplete or possibly by way of the activated state. (8) A thermodynamic cycle for H⁺ and K⁺ ion binding in the coupling network of KcsA. The blue dashed and angled arrow represents the activation followed by C-type inactivation as observed in pH jump electrophysiology experiments; the red dashed and angled arrow represents our observations of the result of lowering [K⁺] at constant neutral or slightly elevated pH (35). The allosteric coupling factor defines the strength of the allosteric coupling and is calculated as: a = \frac{K_{apparent}}{K_w} at constant neutral or slightly elevated pH (35). The allosteric coupling factor is a measure of the strength of the transmembrane allosteric coupling in KcsA using solid-state NMR (SSNMR), contrasting wild-type and site-specific mutants. These site-specific binding measurements, carried out in an authentic bilayer environment, are an important step toward gaining a comprehensive understanding of the molecular network that mediates this allosteric coupling and will be a vital benchmark for further investigations of coupling. The NMR shifts provide rich structural information for the underlying states. This study is a unique quantitative and site-specific analysis of allosteric coupling in a membrane system by NMR.

**Results**

**SSNMR Shifts Probe KcsA Structures in Its Apo and Ion Bound States.**

NMR chemical shift measurements provide highly specific fingerprints for the structures of proteins, probing conformation, and binding. We used chemical shifts to probe KcsA, with particular focus on the selectivity filter. We contrasted spectra at near neutral (pH 7.5) vs. low (3.5) pH, at a broad range of [K⁺], to achieve the four limiting states in Fig. 1A, in functionally relevant lipid and buffer conditions. Consistent with the fact that these channels function at great interest.

To confirm participation of specific residues, we probed the strength of the transmembrane allosteric coupling in KcsA using solid-state NMR (SSNMR), contrasting wild-type and site-specific mutants. These site-specific binding measurements, carried out in an authentic bilayer environment, are an important step toward gaining a comprehensive understanding of the molecular network that mediates this allosteric coupling and will be a vital benchmark for further investigations of coupling. The NMR shifts provide rich structural information for the underlying states. This study is a unique quantitative and site-specific analysis of allosteric coupling in a membrane system by NMR.
those at neutral pH (Fig. 1C), indicating that KcsA is folded at pH 3.5.

**Similar selectivity filter structural transitions are observed at neutral and acidic pH.** Previously, we characterized K$^+$-dependent changes in the selectivity filter at neutral pH (38) that correspond to the high K$^+$ (conductive) and low K$^+$ (collapsed) states described by crystallography (39). Several NMR reporters were identified, including T74 (CA, CB, CG), T75 (CA, CB, CG), and V76 (CB, CG1, CG2). Here, we observe a similar transition between high and low K$^+$ states at low pH, each of which shows highly similar structures to its corresponding counterpart at neutral pH (Fig. 1C); this means that the large difference in pH does not perturb the structure of selectivity filter directly, and that its capability to bind ions is intact. Moreover, the NMR markers for K$^+$ binding are valid also at low pH.

**Ph sensor residues E118 and E120 are protonated at pH 3.5 across a broad range of K$^+$ concentrations.** The pH sensor binds protons (15, 36) and activates channel preparation. Previous studies show that the effective pK$_a$ value for activation is 5 or 5.5 (15). Accordingly, the E118 and E120 CG-CG NMR cross-peaks show diagnostic chemical shift changes associated with significant protonation at pH 3.5 (32, 35); at neutral pH, the peaks are characteristic of a deprotonated anionic state (Fig. S1), unless the ambient [K$^+$] is significantly lowered to cause a coupled protonation process (35).

**Potassium Ion Affinity at Acidic pH: Quantifying the Strength of Allosteric Coupling.** To confirm strong allosteric coupling of the two binding events, and the participation of specific residues in the allosteric process, we developed a SSNMR approach to analysis of binding strength and applied it to this system. Although the low K$^+$ and high K$^+$ structures (and NMR shifts) of the selectivity filter show little sensitivity to pH, we demonstrate here that the energetics of the transition, as characterized by the affinity of the selectivity filter for K$^+$ ions, are dramatically dependent on pH. Such a pH dependence was suggested by previous studies but has not been studied systematically or quantified in a membrane environment (28, 40). Our four-state thermodynamic formulation allows the magnitude of the allosteric coupling to be quantitatively recast as the difference in the equilibrium K$^+$ binding affinity at high vs. low pH. We are able to measure the K$^+$ affinity in a site-specific manner and in a native bilayer environment using solid-state NMR. Because the K$^+$ binding event is coupled with structural changes, the apparent potassium affinity (pK$_a$) is measured. This titration study is rich with structural information because it is based on NMR shifts.

We measured pK$_{apparent}$, contrasting acidic pH (3.5) vs. neutral pH (7.5) by quantifying the relative populations of K$^+$ bound vs. apo states using the “marker” cross-peaks identified above, including T74 (CB-CA), T75 (CB-CA, CA-CG), and V76 (CA-CG1, CG2) (Fig. 2A and Fig. S2). These residues were selected because of their robust signal and good resolution with respect to other signals in 2D planes. Fitting the titration data (using a fixed Hill coefficient of 1) yielded a pK$_{apparent}$ of 14 ± 1 mM at pH 3.5 at 0 °C; in contrast, the pK$_{apparent}$ at neutral pH is 4 ± 1 μM, under otherwise comparable conditions (Fig. 2B). The temperature control for these measurements is improved relative to prior measurements (38) due to the use of an Efree probe with reduced sample heating (41). We calculated the allosteric coupling factor alpha, defined as the ratio of the potassium affinities contrasting open vs. closed activation gate (i.e., at low vs. neutral pH); there is a remarkable shift of the pK$_{apparent}$ by more than three orders of magnitude (α = 3.500) over the pH range where the channel converts from the deactivated to the inactivated state (7.5-3.5).

The thermodynamic cycle (Fig. 1B) also dictates that if the ambient pH affects the equilibrium K$^+$ affinity, then the ambient K$^+$ level must also alter the apparent pK$_a$ of the coupled pH sensor. Prior work showed that pH gate residues E118 and E120 bind protons as the [K$^+$] is lowered to about 0.2 μM at pH 7.5, which indicates a large pK$_a$ shift in the pH sensor as a function of K$^+$ (35), and confirms the identity of the pH sensor as the coupled protonation sample (whereas in contrast, the proximal E71 remains protonated in all [K$^+$]) (28). The K$^+$-dependent pH$_{shift}$ in E118 and E120 has not been fully quantified, but it is clear that the pK$_a$ is altered by at least 3 pH units comparing low and high potassium conditions, because at low [K$^+$] the pK$_a$ must be 8.0 or higher, and at high [K$^+$] the pK$_a$ value is known to be ~4.5 and is consistent with our experimental results (22, 36).

The free energy of allosteric coupling between K$^+$ and H$^+$ binding was estimated from the pH dependence of the K$^+$ pK$_{apparent}$ values: ∆ΔG = −RTln[K$_{apparent}$(pH 7.5)/K$_{apparent}$(pH 3.5)] = −19 ± 2 kJ-mol$^{-1}$. A tentative value estimated from the pK$_{shift}$ with [K$^+$] would similarly be ∆ΔG = −2.3RTln[pK$_a$] = −16 kJ-mol$^{-1}$. We regard these values as estimates; they were calculated using a simplified binding model that treats both K$^+$ and H$^+$ binding as single-ligand, noncooperative events. The stoichiometry and cooperativity of the binding events is certainly more complex and the details remain debated (42-44).

Due to the relatively large pH range used, the phenomenological shift in binding affinity can include a number of effects. We argue that a large portion of it is allosteric coupling. Guoy–Chapman analysis suggests that the differences with respect to previous micelle measurements are too large to be explained by K$^+$ ions concentratating at a negatively charged lipid interface (an ~10-fold effect) (37). Nonspecific effects of pH on the lipid bilayer and protein structure and thermodynamics could certainly also contribute to the altered affinity, although our data show that KcsA remains folded throughout this range. Finally, a large and specific effect of site-specific mutation involving the nonionizable F103 (discussed below) provides strong support that the altered affinity is dominated by contacts in the protein that result in nonadditive energies of binding (19, 23).

**Kinetics of Ion Release Are Slow Across All Conditions Studied.** Inactivation has a clear kinetic signature, and is a remarkably slow process (on the seconds timescale) (22). The origin of the kinetic barrier is debated; molecular dynamics simulations suggest that changes in solvation associated with the conformational change in the filter present a kinetic bottleneck (17). We observe clear evidence for slow conformational exchange between the K$^+$ bound and depleted states at both acidic and neutral pH. At both conditions, intermediate K$^+$ concentrations result in sharply resolved NMR lines corresponding to two states of the filter and indicating slow exchange (100 ms or slower, which is well below estimates of
Confirming Candidates in the Allosteric Coupling Pathway: Studies of the Inactivationless F103A. F103 has been previously suggested as a possibly important allosteric residue. Prior evidence from crystallography and molecular simulations suggested that steric interactions between T74 at the base of the selectivity filter, F103 in the TM2 “hinge” (second transmembrane helix hinge), and I100 from a neighboring monomer are involved in the allosteric network (19, 23, 30). Experimental support for this role consisted in changes in inactivation. In contrast, here we provide a direct experimental test of its contribution to allosteric coupling. The quantitative contribution of F103 to the allosteric coupling was probed by using a mutant, F103A. We speculated that if the F103A mutant “loosens” this network, the energetic coupling between the pH gate and the selectivity filter will become much weaker, rendering K$^+$ release much slower, and steric interactions are expected to be important in the coupling network (19, 46). We speculate that the dramatic chemical shift perturbation is caused by the change in environment of I100 in that there is a strong interaction between I100 and F103 in wild-type when the channel gets activated that is absent in F103A (Fig. 3 B–H). The side chain CG of T74 is also sensitive to the mutation F103A: in WT-KcsA, CG shows a large chemical shift change in the transition from bound to apo state; in contrast, in F103A, the CG chemical shift does not change even though the backbone shows the same structural transition (Fig. 3 C–H). These observations are consistent with the hypothesis that steric contacts occur between the aromatic ring of F103 and the T74 sidechain for WT-KcsA during inactivation, as suggested by the recently published mechanism for allosteric coupling in KcsA developed from crystallography and molecular dynamics simulations (30).

More importantly, consistent with our predictions, the mutant channel F103A indeed exhibits much weaker allosteric coupling. Fig. S3 contrasts $^{12}$C-$^{13}$C correlation spectra of key residues at the selectivity filter for WT vs. F103A samples (under inactivating, i.e., low pH and moderately high $[K^+]$). Compared with the WT-KcsA channel F103A, which significantly populates the $K^+$-depleted state under these conditions, the F103A mutant remains bound to $K^+$. We conducted a similar titration study on this F103A mutant and, as the results show in Fig. S4, the $K^+$ affinity of the F103A ($K_{apparent} = 2 \pm 1 \mu M$) is comparable to that of WT-KcsA at neutral pH, whereas at acidic pH, the affinity is much tighter ($K_{apparent} = 0.32 \pm 0.10 \mu M$), which indicates a significantly reduced allosteric coupling strength (Fig. 3B) ($\alpha = 160$; contrasted with 3,500 for WT-KcsA under the same conditions). These results are interesting in connection with electrophysiology studies on mutant E71H-F103A, where the mutant channel shows significant conductance despite the presence of the strongly inactivating mutation E71H (30). We note that these data provide strong additional support for the hypothesis that ion loss is a key step of the inactivation process: F103A is expected to be virtually inactivationless from prior studies, whereas our own NMR results on F103A indicate it does not release $K^+$ ions upon activation.
Discussion

We offer insights into the slow allosteric transition underlying C-type inactivation, a vital gating mechanism in potassium channels that partly controls open time. This titration study, carried out in an authentic lipid environment, allowed us to prepare and stabilize functionally critical states, presumably closely related to the intermediates of electrophysiology experiments. Detailed information from SSNMR, such as chemical shifts, therefore helps to form a more direct connection between structure and function. Our results involving the role of F103 directly disprove a long-standing hypothesis that C-type inactivation is associated with protonation effects proximal to the selectivity filter, and provide strong support for the allosteric hypothesis wherein large-scale conformational events during intracellular activation are responsible for the affinity change at the extracellular selectivity filter.

We quantitatively probed the allosteric coupling strength for wild-type channel in membranes using these pH-dependent ion titrations. We observe a difference of three orders of magnitude in K+ affinity over the typically used pH range, which indicates substantial transmembrane coupling. Quantification of the potassium affinities of KcsA under various conditions has been the subject for several previous studies, using different methods. Functional methods suggest that the affinity of the selectivity filter for K+ at pH 7.5 (deactivated channel) is ~29 μM (47), and inactivation at pH 3.5 is associated with ion loss with an affinity of ~0.9 mM (32).

A different picture emerged in isothermal titration calorimetry experiments in detergents, which showed the potassium affinity to be 0.41 mM at pH 7.5 (43). Shimada and colleagues (36) reported elegant solution NMR experiments with detergent-stabilized KcsA showing that the potassium affinity changes from 6 to 50 mM between pH 3.2 and 6.7 at 45 °C; the pH dependence of potassium affinity in these studies is much weaker than estimates based on functional studies. Our experiments show a pH dependence that is much larger than these previously reported, and are more compatible with the available functional data and with the hypothesis that ion loss is the essential step of inactivation. This allosteric effect of pH on protein function is analogous to other systems, for example the case of Yeast pyruvate kinase, in which pHI modulates protonation state of various ionizable residues to ultimately allosterically affect the reactivation rates (48). We presume the difference between the solution NMR or thermoccalorimetric affinity measurements for KcsA and these SSNMR studies is due to our use of membrane bilayers, and conclude that dramatically stronger allosteric coupling occurs in the bilayer. The importance of the membrane environment for membrane protein integrity and function has been reported in numerous studies (49–52).

We used analogous measurement of allosteric coupling strength to test the involvement of an important residue in the allosteric network. These results show that replacement of the bulky aromatic sidechain of F103 with the smaller sidechain of alanine greatly reduces the allosteric coupling presumably by diminishing the energy penalty caused the clash between F103 sidechain to I100 that destabilizes the activated state. In contrast to functional studies, the thermodynamic characterization of the coupling energy leaves us with a clear picture for why F103A does not inactivate: loss of K+ ion is not expected for F103A after channel activation.

Through this study we also stabilized the activated state of a wild-type functional KcsA at low pH and high [K+], with both the activation gate open (protonated pH sensor) and inactivation gate open (K+ bound in the selectivity filter). The activated state is normally an evanescent or metastable intermediate in functional studies of KcsA, and has been difficult to stabilize for structural characterization, requiring use of multiple mutations and other alterations in the system. Stabilizing this species enables structural and dynamics studies by NMR in native bilayers.

NMR has been particularly powerful for studying allosteric coupling, because of the rich structural and dynamic information it can provide (53–57). Chemical shift analysis has been used to identify residues that participate in long-range response to ligand binding (54). The ability of NMR to probe the slightly populated or “invisible” states in coupled systems (55) also holds great potential for detecting minor population involved in allostery and monitoring its population shift to facilitate biological functions (56). NMR studies of allostery have highlighted the possibility of dynamically driven allostery, as in the case of the catabolite activator protein to dissect the entropy and enthalpy contributions to an allosteric DNA binding in a WT and mutation (57). These prior studies feature proteins in solution, but it is still a formidable task to study transmembrane allostery, which is ubiquitous in critical biological systems, including G protein-coupled receptor, kinases, EGFR, ion channels, and many other signaling systems, and has been resistant to quantitative mechanistic investigations (53–58). The present study helps to pave the way for similarly exciting studies of allosteric membrane proteins by solid-state NMR. We envision that approaches demonstrated in this study will be helpful for understanding other systems of relevance to human health.

Materials and Methods

NMR Sample Preparation. 13C-15N uniformly enriched WT-KcsA and the mutant F103A-KcsA were prepared by overexpression in Escherichia coli JM83 cells in M9 minimal media using a tetracycline-regulated expression vector pask90, as described previously (31, 59). Both constructs have an N-terminal hexahistidine tag. The KcsA-F103A mutant was prepared using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) and verified by gene sequencing (Genevac). F103A-KcsA is less stable as a tetramer compared with WT-KcsA, as indicated by SDS gels, but stays as a tetramer revealed by size-exclusion chromatography. KcsA was reconstituted into liposomes by reducing the detergent concentration through dialysis; the liposomes contain 9:1 ratio of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine by mass, and 1:1 ratio of protein/lipid by mass. To prepare samples at acidic pH values, proteoliposomes were first dialyzed at room temperature against a 50 mM Tris buffer at pH 7.5 with the desired potassium concentrations and then further dialyzed at 4 °C against 10 mM sodium citrate buffer at pH 3.5 with the appropriate potassium concentration. NaCl was added to achieve a consistent total concentration [K+] + [Na+] of 50 mM to keep the ionic strength constant in the titration series (except the sample with 80 mM [K+]). After dialysis, liposomes were centrifuged using a Sorvall benchtop centrifuge at 4,125 rpm (3,697 × g, 4 min) to concentrate the liposomes and then left at room temperature for 40 min. The pH values of the supernatants after centrifugation were verified to be within 0.1 unit of the desired pH value. The proteoliposomes were then subjected to three freeze–thaw cycles to remove excess water. Finally, samples were packed into 3.2-mm Bruker rotors for NMR measurement.

NMR Data Collection and Processing. The details of data collection can be found in SI Materials and Methods. All spectra were measured at 0 ± 2 °C if the temperature is not otherwise specifically indicated. All spectra were processed in NMRpipe (60) with –30 Hz Lorentzian and 90 Hz Gaussian apodization; Sparky (61) was used for spectral visualization and integration of cross-peaks. The populations of the bound states for each residue were quantified by integrating the bound and apo cross-peaks of T74 (CB-CA), T75 (CB-CA, CA-CG), and V76 (CB- CG, CGGZ) on a 13C-15N 2D correlation (Fig. S2). The normalized population ratios of protein were calculated by averaging the population ratios of reporter residues, T74, T75, and V76. The bound-state normalized populations, defined as integral(bound)/[integral(bound) + integral(apo)] × 100% for each residue. Normalized populations were fit to the binding expression below with Hill coefficient, n, equal to 1 (noncooperative), where i is the normalized population:

\[ \theta = \frac{\text{bound}}{\text{bound} + \text{apo}} \]

The reported errors are dominated by the fitting error (K_0), which is roughly on the same scale of errors from peak integration and averaging.

ACKNOWLEDGMENTS. We thank members of A.E.M.’s group: Dr. Wylie and Rikvah Rogowskie for helpful discussions, and Dr. Ivan Sergeyev and Dr. Wenbo Li for NMR techniques. We also thank the laboratory of Crina Nimigean for the PASK90 plasmid; Dr. Dorothy Kim and Dr. David Pason for the help of electrophysiology measurement; and Dr. Boris Rin at the New York Structural Biology Center (NYSBC) for support with NMR instrumentation. A.E.M. is a member of NYSBC, a Strategically Targeted Academic Research center supported by the New York State Office of Science, Technology, and Academic Research. This work was supported by NIH Grant NIH R01 GM088724 (to A.E.M.).