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Micelles Protect Membrane Complexes from Solution to Vacuum

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The ability to maintain interactions between soluble protein subunits in the gas phase of a mass spectrometer gives critical insight into the stoichiometry and interaction networks of protein complexes. Conversely, for membrane protein complexes in micelles, the transition into the gas phase usually leads to the disruption of interactions, particularly between cytoplasmic and membrane subunits, and a mass spectrum dominated by large aggregates of detergent molecules. We show that by applying nanoelectrospray to a micellar solution of a membrane protein complex, the heteromeric adenosine 5′-triphosphate (ATP)–binding cassette transporter $BtuC_2D_2$, we can maintain the complex intact in the gas phase of a mass spectrometer. Dissociation of either transmembrane (BtuC) or cytoplasmic (BtuD) subunits uncovers modifications to the transmembrane subunits and cooperative binding of ATP. By protecting a membrane protein complex within a *n*-dodecyl-β-D-maltoside micelle, we demonstrated a powerful strategy that will enable the subunit stoichiometry and ligand-binding properties of membrane complexes to be determined directly, by precise determination of the masses of intact complexes and dissociated subunits.

Mass spectrometry of noncovalent protein complexes has made substantial contributions to structure elucidation, revealing the overall stoichiometry of protein subunits (*1*–*3*) as well as their topology (*4*) and interaction networks (*5*). Despite its undoubted power, mass spectrometry has not yet been applied in earnest to intact membrane protein complexes. This is due in part to their inherent insolubility in buffers that are compatible with electrospray (*6*), as well as the ready dissociation of subunit interactions, either between transmembrane subunits (*7*) or between transmembrane and cytoplasmic subunits (*8*). The vast excess of detergent aggregates that are present in the electrospray droplet (*7*, *9*–*12*) can also lead to the suppression of ionization (*13*). As a result of these difficulties, progress has been limited, with relatively few examples of intact membrane protein complexes having been reported. These include electrospray mass spectrometry of an intact homotrimer (*14*) and of the homomeric transmembrane complex of c-ring subunits of an adenosine 5′-triphosphate (ATP) synthase after separation from the soluble subunits and analysis with a novel ionization method (*15*). To date, however, there have been no reports of nanoelectrospray mass spectrometry of intact heteromeric membrane protein complexes containing both cytoplasmic and transmembrane domains, primarily because most previous protocols have involved the extensive removal of detergent molecules (*7*, *8*). Here, however, we show that by maintaining detergent micelles in solutions, well above the critical micelle concentration (CMC), it is possible to protect interactions between cytoplasmic and transmembrane subunits and, through gas-phase activation, release intact membrane complexes.

We selected five membrane protein complexes for our study, including some with well-defined structures and others with unknown subunit stoichiometry. We obtained mass spectra of all five complexes and chose to illustrate our approach with the well-characterized heteromeric transmembrane complex $BtuC_2D_2$, a vitamin B_{12} importer from *Escherichia coli* and a member of the ATP-binding cassette (ABC) transporter superfamily. These ABC transporters are ubiquitous membrane proteins, with representatives in organisms ranging from prokaryotes to humans, and they couple ATP hydrolysis to the transport of a diverse range of substrates across membranes (*16*). The overall structure of the $BtuC_2D_2$ assembly has been established from x-ray analysis of the complex in its ATP-free form (*17*). Two transmembrane subunits (BtuC), with a total of 20 transmembrane helices per subunit, form the translocation channel and bind to two cytoplasmic nucleotide-binding subunits (BtuD). Each subunit contacts its two immediate neighbors but has no interface with the remaining diagonally positioned subunit. A prominent cytoplasmic loop of BtuC forms two short helices that contact BtuD. The interactions between BtuC and BtuD transmit the conformational changes thought to occur upon nucleotide binding, opening the transmembrane channel for the import of vitamin B_{12}. Because this tetrameric complex consists of two different subunits and three types of intersubunit contacts, we can take advantage of its modular composition to explore interactions between subunits and different nucleotides, as well as compare gas-phase dissociation pathways and solution-phase unfolding reactions.

We used *n*-dodecyl-β-D-maltoside (DDM) to maintain the solubility of $BtuC_2D_2$, because this detergent is often preferred for retaining the native state of membrane proteins in solution (*18*) and stabilizes $BtuC_2D_2$ in an active state. Moreover, high concentrations of nonionic detergents can be tolerated more readily in electrospray than can ionic detergents (*6*), and DDM has previously been used with nanoelectrospray (*7*) and other ionization techniques (*15*, *19*). A 2-μl aliquot of a solution containing DDM above the CMC (fig. S1) (*20*) and ~5 μM membrane protein complex (*21*) was introduced from a gold-coated nanoflow capillary, and a spectrum was recorded on a mass spectrometer optimized for maintaining and focusing large macromolecular ions (*22*). Subjecting the protein-detergent assembly to maximum acceleration voltages, both within the electrospray source and across the collision cell of the mass spectrometer, gave rise to a spectrum in which the dominant peaks at low mass/charge ratios (*m*/*z*) were assigned to DDM clusters (fig. S2). At higher *m*/*z*, a broad distribution of species was observed, consistent with our previous ob-

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servations for micelles transported into the gas phase (*12*). We assigned this diffuse peak to a heterogeneous assembly containing ≥100 DDM molecules that remain associated with the complex (compare this with the solution-phase aggregation number of 130 in these buffer conditions, estimated from gel filtration). Within the same spectrum, the dissociated subunit BtuD was observed (Fig. 1A). Under these mass spectrometry conditions, all soluble protein complexes that we studied underwent substantial gas-phase dissociation (*23*), implying that this complex is protected by the detergent assembly. As the pressure inside the collision cell was increased, DDM molecules dissociated from the assembly, revealing a distinct series of peaks assigned to the intact protein tetramer.

Increasing the number of collisions and maintaining the high acceleration conditions gave rise to charge states above and below the peaks assigned to the intact tetramer. In common with mechanisms proposed for soluble complexes (*24*–*26*), these peaks were assigned to the unfolding and stripping of monomeric subunits, a phenomenon governed by the surface accessibility of the subunits as well as their size and ease of unfolding (*27*, *28*). The ejection of unfolded BtuD from the complex coincided with observations of large numbers of DDM molecules adhering to the complex. In contrast, when the complex was devoid of DDM in the gas phase, unfolded BtuC emerged as the dominant dissociation product (fig. S3). If we compare gas-phase dissociation pathways with solution-phase experiments, in which we used chemical denaturants to probe the unfolding of the soluble and transmembrane subunits, BtuD loses adenosine triphosphatase activity, consistent with some unfolding in 3 M urea (*29*). In contrast, circular dichroism studies of BtuC subunits in aqueous DDM micelles, at the same urea concentration, have shown little change in the secondary structure, implying that BtuD unfolds more readily in solution than BtuC. This is analogous to our gas-phase experiments, in which unfolding and dissociation of BtuD occurred predominantly in the presence of the gas-phase micelle. Unfolding and dissociation of BtuC, however, was observed only in the gas phase once the stabilizing effects of the detergent assembly had been disrupted.

Further support for the protective effects of the detergent assembly comes from a comparison of the average charge state of the intact tetramer with the relationship deduced for the surface area and charge of globular proteins and their complexes (*26*, *30*). We found that $BtuC_2D_2$ has ~16% lower average charge than a soluble protein complex of the same accessible surface area (Fig. 1B). The most likely explanation for this significant difference is that when charging takes place, during the droplet phase of electrospray, the complex is shielded by the DDM micelle. A similar effect to that observed for $BtuC_2D_2$ is also seen for a second ABC transporter, MacB. In this case, the average charge is ~10% lower than for a soluble complex with the same surface area. If we now subtract from the total surface area the membrane-embedded regions (*29*), we achieve closer agreement with the relationship deduced

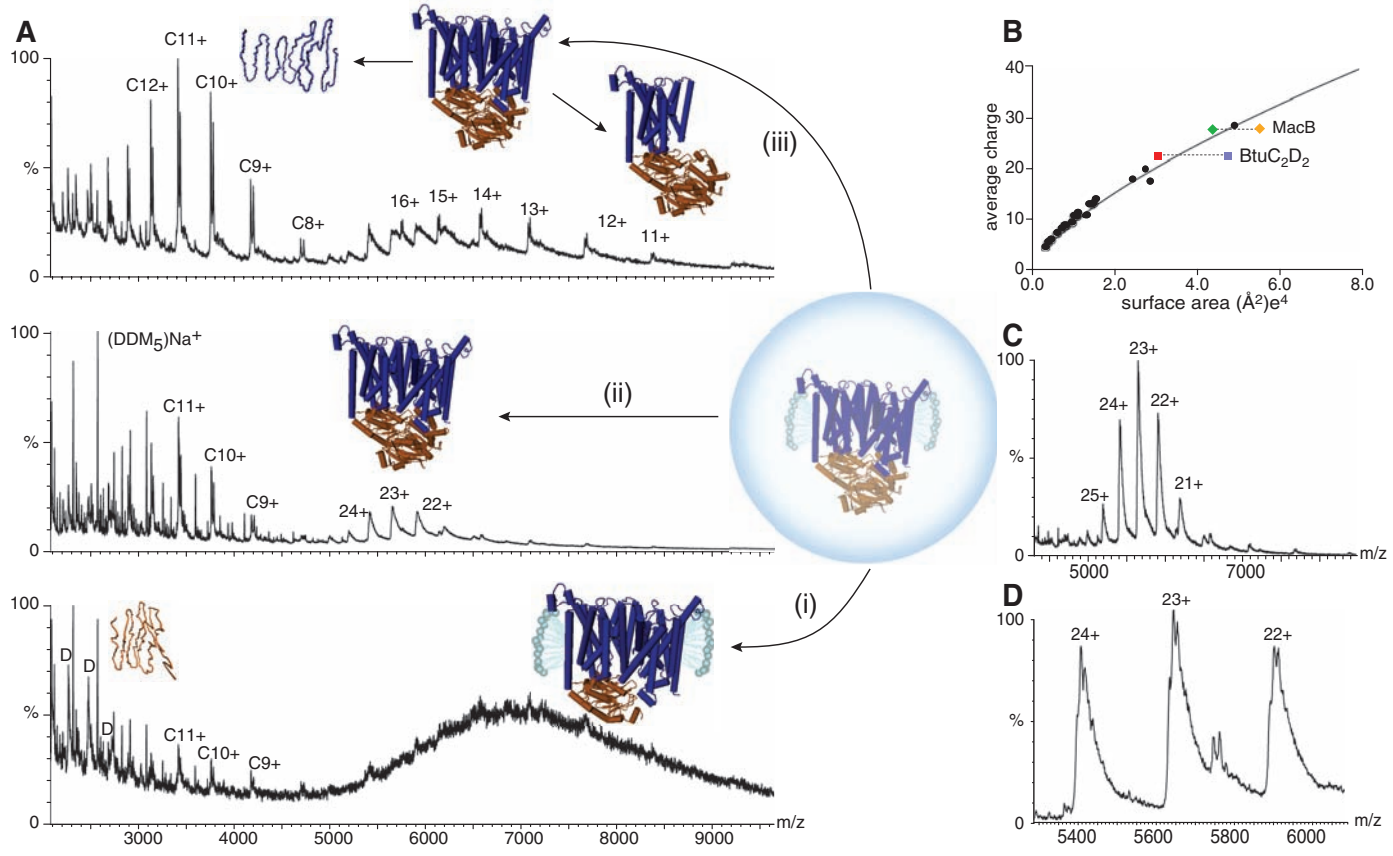

Fig. 1. Schematic representation of the emergence of the intact membrane complex from a micelle contained within an electrospray droplet and subsequent gas-phase dissociation pathways. (**A**) Populations of ions corresponding to the protein complex associated with aggregates of DDM molecules are observed above *m/z* 5000. At low *m/z*, the dominant dissociation product is the unfolded BtuD subunit [pathway (i)]. Increasing the number of collisions leads to the release of the intact tetramer [pathway (ii)]. Further increases in the number of collisions lead to the dissociation of BtuC and formation of a trimer [pathway (iii)]. (**B**) Plot of the average charge state of a series of globular and membrane protein complexes against their surface area ($Å^2 \times 10^4$) (*26*). Globular proteins are shown in black and membrane complexes are shown in blue and yellow ($BtuC_2D_2$ and MacB, respectively). The exposed surface area (that which is not surrounded by the micelle) was calculated either from x-ray structure coordinates ($BtuC_2D_2$, red) (*30*) or estimated based on secondary-structure prediction (MacB, green) (*29*, *37*). (**C** and **D**) Two different expansions across the 22+ to 24+ charge states reveal splitting assigned to a posttranslational modification. Structures were prepared from the x-ray coordinates (the Protein Data Bank file for $BtuC_2D_2$ is 1L7V). The transmembrane BtuC subunits (blue), cytoplasmic BtuD subunits (orange), and the presence of the micelle are illustrated.

for the surface area and charge (Fig. 1B). This implies that the DDM micelles are protecting the complexes, not only against the dissociation and unfolding of the transmembrane subunits during the phase transition but also from charging in the electrospray droplet.

Under the appropriate activation conditions, when extensive interactions with the DDM aggregates are disrupted, it is possible to observe the intact tetrameric protein complex in the gas phase with minimal dissociation (Fig. 1C). Close agreement between the measured and calculated masses (129,642 and 129,520 daltons, respectively) confirms the overall stoichiometry of the complex as being $BtuC_2D_2$, the majority of DDM molecules having dissociated under these conditions. Further expansion of the charge states revealed splitting of the peaks that were assigned to the intact tetramer, consistent with either binding to small molecules or posttranslational modification of the transmembrane or cytoplasmic subunits (Fig. 1D and table S1). To determine the origin of this splitting, we activated the tetramer to induce its gas-phase dissociation (Fig. 2A). All four of the anticipated dissociation products were formed, corresponding to unfolding and dissociation of either subunit and formation of the corresponding "stripped" trimers at higher *m/z*. For the $BtuCD_2$ trimer, split peaks were observed that were consistent with a ~1:1 ratio. Conversely, a 1:2:1 statistical distribution of modified forms was observed when interactions between the transmembrane subunits were maintained ($BtuC_2D$) (Fig. 2A, inset). This observation, together with the splitting observed for the unfolded BtuC monomer at low *m/z*, is consistent with a posttranslational modification of BtuC. Mass spectra recorded for the denatured protein subunits (fig. S4) confirm this modification to be an α-*N*-D-gluconyl-His tag, an established posttranslational modification for histidine-tagged proteins in *E. coli* (*31*).

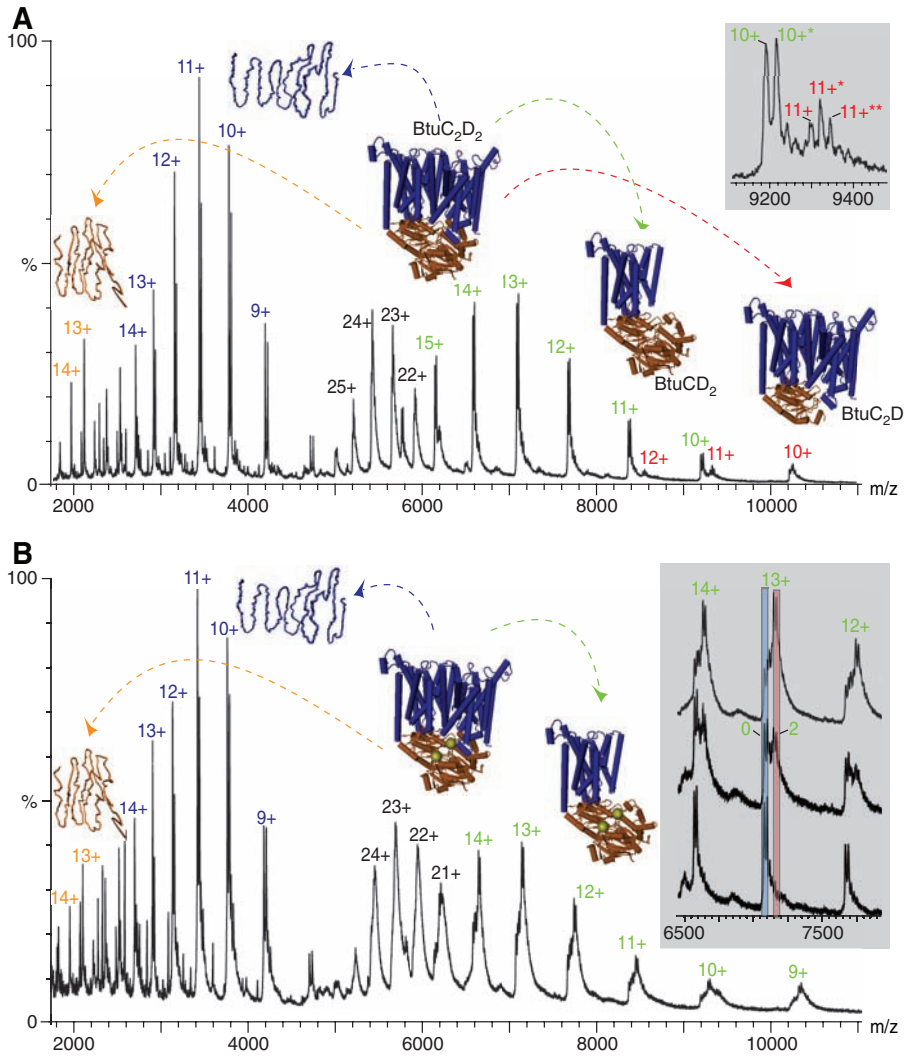

Fig. 2. Gas-phase dissociation of $BtuC_2D_2$ with and without ATP/Mg^{2+} reveals cooperative binding of ATP. (**A**) Intact tetramer, charge states 22+ to 25+ (black), undergoes asymmetric dissociation, expelling unfolded BtuD (orange) or BtuC (blue). The respective stripped trimers are observed at higher *m/z* $BtuCD_2$ (green) and $BtuC_2D$ (red). The inset shows the expansion of the 10+ and 11+ charge states of the trimeric dissociation products, showing a 1:2:1 statistical distribution of posttranslationally modified subunits in $BtuC_2D$, whereas the doublet assigned to $BtuCD_2$ corresponds to 0- and 1-modified subunits. (**B**) Dissociation of the complex in the presence of substoichiometric quantities of ATP/Mg^{2+} reveals the predominant dissociation pathway involving loss of transmembrane subunits and a distinct mass shift corresponding to binding of two molecules of ATP, represented by yellow spheres placed in the cyclotetravanadate-binding sites located crystallographically (*17*). The inset shows the expansion of the 13+ charge states of the stripped trimer $BtuCD_2$ in the absence of ATP (bottom) and in the presence of ATP 12 μM and 20 μM (middle and top, respectively). The multiplicity of peaks arises from the modified forms of BtuC as well as the 0 and 2 ATP-bound states (shaded blue and pink, respectively).

Using a similar approach to that described above, we investigated the effects of nucleotide binding by comparing the gas-phase dissociation pathways of apo $BtuC_2D_2$ with spectra recorded in the presence of ATP/Mg^{2+} (Fig. 2B). A clear increase in the average mass (1016.3 ± 9.7 daltons) is observed for the trimeric dissociation product $BtuCD_2$. This is consistent with the binding of two molecules of ATP (1022 daltons) together with Mg^{2+} adducts; however, the latter are not resolved at this high *m/z* value. The dissociation product BtuD shows no evidence of ATP binding. Moreover, it was not possible to discern a series of peaks assigned to $BtuC_2D$. This is consistent with the requirement for two BtuD subunits to form each ATP-binding site, predicted from the x-ray analysis of the cyclotetravanadate-binding sites (*17*). The low-intensity BtuD series presumably arises from dissociation of the apo form at substoichiometric ATP concentrations. Similarly, addition of adenosine 5′-diphosphate and an ATP analog, α,β-methyleneadenosine 5′-triphosphate, resulted in the binding of two nucleotides to the complex in each case. We also found that the collision energy required for gas-phase dissociation of protein subunits from all nucleotide-bound forms was higher than for the apo complex (fig. S5). Comparison with our solution-phase studies in the presence of ATP showed an increased ability of BtuD subunits to hydrolyze ATP and remain associated with BtuC when urea concentrations were raised (*29*). This shows that BtuD subunits are more resistant to unfolding when ATP is bound than when the complex is in its apo form. Together, these results imply an increase in the overall stability of the complex upon nucleotide binding, both in solution and gas phases, through increased interactions between the nucleotide-binding domains.

To investigate the cooperativity of ATP binding, proposed for all ABC transporters (*32*), we titrated increasing amounts of ATP/Mg^{2+} into solutions of $BtuC_2D_2$ (Fig. 2, inset, and fig. S6). The resulting mass spectra of the activated complex reveal that for the $BtuCD_2$-dissociation product, the population of the one ATP-bound state does not exceed either that of the apo or the two ATP-bound states, even when substoichio-

metric equivalents of ATP are added. This shows the cooperativity of ATP binding. This is evidence that ligand binding can be observed within the complex, without dissociation, even when the transmembrane subunits are stripped.

We have shown that we can maintain the seemingly unfavorable hydrophobic interactions within a heteromeric membrane protein complex by encapsulating it in a solution-phase detergent micelle and transferring this protected complex into the gas phase. Although the formation of micelles in solution has been shown to be facilitated by evaporation (*9*, *33*) and could be considered akin to the early stages of electrospray (*7*), the overall structure of gas-phase detergent assemblies is difficult to establish. Previous studies have shown preservation of micellar structure and protein encapsulation within reverse micelles (*12*). However, the fact that we have been able to employ mass spectrometry acceleration conditions far in excess of those used previously to preserve noncovalent complexes (*23*) implicates the stabilizing effects of the gas-phase detergent assembly regardless of its structure. Consequently, this enables us to release the complex intact and with ligand binding maintained. The observation that BtuD subunits are released preferentially, when large numbers of detergent molecules are present, while dissociated BtuC subunits predominate in their absence, is further evidence of the protection of the transmembrane subunits within gas-phase detergent assemblies. The low dielectric interior of lipid bilayers, which closely mimics the vacuum conditions of a mass spectrometer, may also contribute to the stability of the membrane complex once it is released from the gas-phase detergent assembly (*34*). It is established that solution-phase micelles exert pressure (*35*) sufficient to maintain the packing of the BtuC helices and subunit interfaces. We speculate that a similar effect may also be operative here, enabling us to preserve the deleterious effects of the phase transfer and to retain hydrophobic interactions between the transmembrane subunit interfaces. Irrespective of the origin of this unexpected gas-phase stability, and given the long-standing difficulties encountered in studying membrane protein complexes by mass spectrometry, as well as the ambiguities experienced in determining the stoichiometry of subunits within micelles using classical approaches, this represents a substantial advance, not only in methodology but also for assessing the effects of small-molecule drug candidates. More generally, the ability to release membrane protein complexes from detergent aggregates in the gas phase, devoid of detergent and with ligand binding intact, has great potential, not only for structural genomics but also for the many imaging techniques that are rapidly coming to the fore (*36*).

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Supporting Online Material

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Structural Basis of Trans-Inhibition in a Molybdate/Tungstate ABC Transporter

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Transport across cellular membranes is an essential process that is catalyzed by diverse membrane transport proteins. The turnover rates of certain transporters are inhibited by their substrates in a process termed trans-inhibition, whose structural basis is poorly understood. We present the crystal structure of a molybdate/tungstate ABC transporter (ModBC) from *Methanosarcina acetivorans* in a trans-inhibited state. The regulatory domains of the nucleotide-binding subunits are in close contact and provide two oxyanion binding pockets at the shared interface. By specifically binding to these pockets, molybdate or tungstate prevent adenosine triphosphatase activity and lock the transporter in an inward-facing conformation, with the catalytic motifs of the nucleotide-binding domains separated. This allosteric effect prevents the transporter from switching between the inward-facing and the outward-facing states, thus interfering with the alternating access and release mechanism.

Active transport proteins consume cellular energy to move substrates across biological membranes against their (electro)chemical gradients. Transport processes are regulated at various stages; this includes genetic regulation of the expression levels or control of the transporters by inhibitory cellular signals. Another mechanism is trans-inhibition, which occurs when substrates exert a concentration-dependent, inhibitory effect on the transporter after the translocation has occurred, that is, on the target side (trans side) of the membrane (*1*). This type of inhibition results in the decrease of the transport rates as the concentration of substrate increases (*2*). It is therefore a functional equivalent to product inhibition of soluble enzymes. Trans-inhibition has been reported for ion transporters (*3*) and various amino acid transporters (*4*–*8*), including glutamate transporters in astrocytes (*9*). It has also been described for binding protein–dependent ATP-binding cassette (ABC) transporters, such as those specific for