Selective molecular transport through the protein shell of a bacterial microcompartment organelle

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Bacterial microcompartments are widespread prokaryotic organelles that have important and diverse roles ranging from carbon fixation to enteric pathogenesis. Current models for microcompartment function propose that their outer protein shell is selectively permeable to small molecules, but whether a protein shell can mediate selective permeability and how this occurs are unresolved questions. Here, biochemical and physiological studies of structure-guided mutants are used to show that the hexameric PduA shell protein of the 1,2-propanediol utilization (Pdu) microcompartment forms a selectively permeable pore tailored for the influx of 1,2-propanediol (the substrate of the Pdu microcompartment) while restricting the efflux of propionaldehyde, a toxic intermediate of 1,2-propanediol catabolism. Crystal structures of various PduA mutants provide a foundation for interpreting the observed biochemical and phenotypic data in terms of molecular diffusion across the shell. Overall, these studies provide a basis for understanding a class of selectively permeable channels formed by nonmembrane proteins.

microcompartment | protein channel | carboxysome | Salmonella | B₁₂

The complex behavior of biological systems depends fundamentally on the controlled movement of molecules between cellular compartments. Such processes occur in a wide range of biological contexts through the movement of ions and small molecules across lipid bilayers via proteins—channels and pumps—embedded in the bilayer. Achievements in understanding molecular transport in transmembrane systems have contributed to scientific disciplines from cell biology and physiology to membrane biophysics (1, 2). Interestingly, there exists a second type of system for molecular transport through proteins that is fundamentally different and much less understood. Hundreds of species of bacteria produce large subcellular organelles known as microcompartments (MCPs), which consist of metabolic enzymes encapsulated within proteinaceous shells reminiscent of viral capsids (reviewed in ref. 3). For MCPs to function, substrates and products must move across their outer protein shell, which lacks any lipid-based membrane. In the last several years, 3D structures of the proteins that comprise MCP shells have revealed narrow pores through their centers that have been hypothesized to be the routes by which substrates enter (and products escape from) MCPs (4; reviewed in ref. 5). However, experimental evidence to support this key hypothesis and the molecular principles involved is lacking.

The overarching function of MCPs is to optimize metabolic pathways that have toxic or volatile intermediates. MCPs are present across at least 11 different bacterial phyla, where they carry out diverse metabolic processes (6–12). The carboxysome MCP is used to enhance CO₂ fixation in nearly all bacteria that use the Calvin cycle, and it has been estimated that 25% of the carbon fixation on Earth occurs within this proteinaceous bacterial organelle (9). The 1,2-propanediol utilization (Pdu) and ethanolamine utilization (Eut) MCPs are used to optimize 1,2-propanediol (1,2-PD) and ethanolamine catabolism, respectively

(13–15), and the degradation of these compounds is thought to promote enteric pathogenesis (12, 16, 17). Although the Pdu and Eut MCPs, the carboxysome, and other metabolically diverse MCPs of unknown function encapsulate distinct sets of enzymes, all have shells built from homologous proteins suggesting they operate by conserved functional principles. Most models of MCP function propose that the protein shell acts as a diffusion barrier that allows passage of substrates (and products) while limiting the escape of a toxic or volatile metabolic intermediate such as CO₂ or toxic aldehyde (9, 18), but selective permeability by MCP shells has not been established experimentally.

The shells of MCPs are assembled primarily from a family of small proteins that have so-called bacterial microcompartment (BMC) domains (5). Many BMC domain proteins form flat, hexagonally shaped oligomers that tile into extended sheets that form the basis of the MCP shell (4, 19, 20) (Fig. 1). In most cases, MCP shells are composed of four to eight different types of functionally diversified BMC domain proteins, some of which have pores proposed to mediate the selective movement of metabolites across the shell (4, 7, 8, 18). For example, the PduA shell protein from the Pdu MCP has a small central pore (~6 Å) that is lined with numerous hydrogen-bond donors and acceptors, leading to a suggested role in the preferential movement of 1,2-PD over the less polar propionaldehyde (a toxic intermediate) (21). In

Significance

Here, we describe a type of selective channel formed by a nonmembrane protein that is used to control metabolite movement between cellular compartments. In this case, the channel controls the movement of metabolites between the bacterial cytoplasm and the lumen of a bacterial microcompartment, a primitive organelle that is bounded by a protein shell and lacks any lipid bilayer. These studies are the first to our knowledge to prove selective molecular transport through a protein-based barrier. These findings broaden our understanding of how cells control the movement of molecules between cellular compartments, which is fundamental to biological systems. It also solves a key question about the function of bacterial microcompartments, which themselves impact many diverse and important biological processes.

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Table 1. Pore diameter and hydrophobicity

PduA mutant	Pore diameter, Å	Pore segment	Solvation free energy difference in the pore, kcal·mol ⁻¹
Wild-type	5.6	I38-L42	—
PduA-S40A	5.9	I38-L42	-2.1 ± 0.5
PduA-S40L	5.5	I38-L42	4.7 ± 0.7
PduA-S40GSG	1.9	I38-L44	-1.3 ± 0.9
PduA-S40Q	0.5	I38-L42	-0.2 ± 0.1
PduA-S40H	4.3	I38-L42	1.5 ± 0.2

In cases where multiple instances of a hexamer were present in a crystal structure, the reported pore diameters and solvation-free energies are average values.

Table S2). The pore of PduA-S40L is considerably more hydrophobic, but not more occluded than the wild type. The S40H mutant has a pore whose natural sixfold symmetry is broken, but which is likewise not occluded. The S40C mutant shows polymorphic behavior; three distinct instances of the hexamer occur in the crystal form, and the pore can be open or occluded depending on the cysteine side chain orientations and whether disulfide bonds are formed between them. The S40GSG insertion mutation has a fully occluded pore (which was the intended outcome) in two of three distinct hexamers of the crystal structure. The S40Q mutant also has an occluded pore in both distinct hexamers of the crystal structure (*SI Appendix, Table S2*). Three other mutants (S40A, S40M, and S40T) formed essentially normal-appearing MCPs but were not characterized by crystallography. Models of those mutants were generated by conservative computer modeling. In particular, the S40A mutant, whose physiological behavior was notable, was modeled simply by removing the serine hydroxyl group. The PduA-S40A model has a central pore that is not significantly larger than wild type (despite loss of the hydroxyl groups), but which is less polar (Table 1 and *SI Appendix, Table S2 and Fig. S6*).

Effects of PduA Pore Mutations on Shell Function. Prior studies showed that mutational impairment of the shell of the Pdu MCP results in faster growth of *Salmonella* on 1,2-PD at limiting B₁₂ concentrations, whereas shell defects lead to propionaldehyde toxicity at saturating B₁₂ (27, 28). When B₁₂ is saturating, diol dehydratase rapidly converts 1,2-PD to propionaldehyde, which leaks from defective MCPs causing toxicity and growth inhibition (27–29). At limiting B₁₂, a broken or damaged shell results in faster growth due to the increased availability of enzyme substrates and cofactors to the 1,2-PD degradative enzymes encased within the MCP (27, 29). No aldehyde toxicity occurs at limiting B₁₂ due to the lower rate of propionaldehyde formation by diol dehydratase. Hence, growth rate at saturating B₁₂ provides a means to measure the extent of propionaldehyde leakage from the MCP, whereas growth rate at limiting B₁₂ allows an estimate of the permeability of the shell to substrates (27, 29).

In our initial tests, we looked at the effects of the eight PduA pore mutants (described above) on shell permeability by measuring growth of *Salmonella* on 1,2-PD at limiting B₁₂. PduA-S40T, PduA-S40A, PduA-S40H, and PduA-S40M mutants grew similarly to wild type (*SI Appendix, Fig. S3*), indicating no significant change in shell permeability. In contrast, the PduA-S40L, PduA-S40C, PduA-S40Q, and PduA-S40GSG mutants grew more slowly than wild type on 1,2-PD (*SI Appendix, Fig. S3*). This finding suggested that the PduA-S40L, PduA-S40C, PduA-S40Q, and PduA-S40GSG pore mutations resulted in a shell that is less permeable to substrates. Notably, three of these slow-growing mutants (PduA-S40GSG, PduA-S40Q, and PduA-S40C) had occluded pores in the crystal structures. Moreover, additional tests with the PduA-S40L, PduA-S40GSG, PduA-S40C, and PduA-S40Q mutants showed that increasing the

1,2-PD concentration from 0.4 to 2.4% corrected their slow-growth phenotype, consistent with an impaired diffusion of 1,2-PD into the MCP that could be offset by a much higher concentration gradient (*SI Appendix, Fig. S4*).

To more directly test whether PduA pore mutations impaired the movement of 1,2-PD across the shell of the Pdu MCP, we measured the coenzyme B₁₂-dependent diol dehydratase (DDH) activity in purified MCPs. DDH is an MCP lumen enzyme that catalyzes the first step of 1,2-PD degradation (the conversion of 1,2-PD to propionaldehyde); hence, its activity depends on the diffusion of 1,2-PD across the MCP shell (13). MCPs purified from PduA-S40L, PduA-S40GSG, and PduA-S40Q mutants had ~50%, 62%, and 75% DDH activity compared with the wild-type level, whereas MCPs from PduA-S40T, PduA-S40C, PduA-S40A, PduA-S40H, and PduA-S40M mutants had activities similar to wild type (Table 2). The markedly reduced DDH activity of the PduA-S40L, PduA-S40Q, and PduA-S40GSG mutants are most simply explained by altered structural features that block the influx of 1,2-PD and/or that impair the egress of propionaldehyde (which might lead to product inhibition). The former explanation is most consistent with the experiments noted above in which increased 1,2-PD concentration mitigated the growth defect.

It was notable that overall the DDH activities of MCPs purified from the PduA pore mutants correlated well with the growth rates of those mutants on 1,2-PD, providing both in vivo and in vitro evidence that certain PduA pore variants limit substrate influx. The PduA-S40C mutant, however, provided one interesting exception. It showed normal DDH activity in vitro for purified MCPs, although it showed a reduced growth rate on 1,2-PD (Table 2 and *SI Appendix, Fig. S3*). To test whether the in vitro activity of this mutant might be lowered by environmental conditions likely to occlude the pore, we added iron (FeCl₂) under reducing conditions (adding DTT) with the expectation that the multiple proximal cysteine residues might bind iron at the center of the pore. Indeed, under these conditions DDH activity for the S40C mutant dropped to 66% (similar treatment lowered the activity in the wild type case to 87%) (*SI Appendix, Table S3*). This finding provided further evidence that occluding the pore of the PduA shell protein limits flux into the

Table 2. DDH activity of purified mutant MCPs

Type of purified MCPs	Specific activity, μmol·min ⁻¹ ·mg ⁻¹
Wild-type (LT2)	28.4 ± 0.6
PduA-S40L	14.8 ± 0.8
PduA-S40GSG	17.5 ± 0.5
PduA-S40Q	21.2 ± 0.7
PduA-S40H	28.5 ± 0.4
PduA-S40T	29.4 ± 0.4
PduA-S40M	29.2 ± 0.2
PduA-S40N	27.8 ± 0.8
PduA-S40A	26.1 ± 0.8
PduA-S40C	27.1 ± 0.2

MCP and also suggested that the inconsistency between growth test and DDH assays (noted above) might be due to differences between in vivo and in vitro conditions.

Control experiments were performed to rule out ancillary effects of pore mutations. If the PduA-S40L and PduA-S40GSG mutants reduced DDH activity by impeding the movement of 1,2-PD across the MCP shell (rather than by some other unanticipated effect), then breaking the MCP should return the DDH activity to wild-type levels. The measured activities of wild type, PduA-S40L, and PduA-S40GSG MCPs following disruption using standard protocols were all very similar (*SI Appendix, Table S4*).

Movement of Propionaldehyde Through the PduA Pore. The hypothesized role of the Pdu MCP shell is to retain propionaldehyde while allowing 1,2-PD entry. We therefore tested the effects of selected PduA pore mutants on the efflux of propionaldehyde from the Pdu MCP by measuring the amount of propionaldehyde that diffused out of the MCP, through the bacterial cell membrane, and into the culture medium during growth of *Salmonella* on 1,2-PD as described (28) (Fig. 3). Simultaneously, we measured growth rate and 1,2-PD consumption. The PduA-S40L and PduA-S40GSG mutants (whose pores were shown to diminish 1,2-PD uptake) consumed 1,2-PD more slowly than wild

Fig. 3. Consumption of 1,2-PD and propionaldehyde release by select PduA pore mutants. 1,2-PD consumption and propionaldehyde release was monitored during growth of wild-type and various PduA pore mutants on 1,2-PD minimal medium. Wild-type *Salmonella* (WT) compared with a PduA-S40L mutant (A and B), a PduA-S40GSG mutant (C and D), and a PduA-S40A mutant (E and F). Left column, red and black lines are OD₆₀₀ and green and blue lines are 1,2-PD. Right column, red and black lines are OD₆₀₀ and green and blue lines are propionaldehyde.

type, but excreted propionaldehyde in similar amounts. Most notably, the PduA-S40A mutant consumed 1,2-PD at a rate similar to wild type, but excreted approximately 2.5-fold more propionaldehyde. Increased excretion led to a marked inhibition of growth (from 12 to 30 h) as a result of propionaldehyde toxicity as was previously observed for deletion mutations that disrupt the MCP shell (27) (Fig. 3). These results indicated that the wild-type PduA pore provides a key route for inward diffusion of 1,2-PD, and that its detailed structural features are important for restricting the egress of propionaldehyde to mitigate aldehyde toxicity.

A second test used to assess the permeability of PduA pore variants to propionaldehyde was to grow *Salmonella* on 1-propanol under conditions where growth was limited by diffusion of propionaldehyde, produced by oxidation of 1-propanol in the cytoplasm, into the Pdu MCP (*SI Appendix, SI Materials and Methods*). These tests indicated that the relative rate of propionaldehyde movement into the Pdu MCP was PduAS40A > PduS40L > wild type ~ PduAS40T (*SI Appendix, Fig. S5*). The modest increase in the permeability of S40L compared with wild type for propionaldehyde influx (*SI Appendix, Fig. S5*) can be reconciled with the unaffected rate of aldehyde efflux during growth on 1,2-PD (Fig. 3) by a reduced rate of internal aldehyde production (by diol dehydratase) in this mutant (Table 2). Most importantly, however, this finding was the second experiment that indicated the S40A mutant has an increased permeability to propionaldehyde compared with wild type.

Effects of Polarity of the PduA Pore on Small Molecule Diffusion.

Some of the observed data on transport through mutant PduA pores (summarized in *SI Appendix, Table S6*) are easily interpreted, such as the blockage of 1,2-PD influx in the fully occluded S40GSG mutant and in the pore-constricted S40Q mutant, whereas the behavior in other cases calls for a more detailed analysis. Two observations in particular are worth examining. First, the S40L mutant did not produce a pore with a narrower diameter, yet influx of 1,2-PD was diminished. Second, the S40A mutation did not significantly increase the minimal radius of the pore, yet propionaldehyde efflux was enhanced. We examined the physicochemical properties of the mutant pores to shed light on these phenomena. We evaluated the polarity of the pore in the different structures according to a calculated estimate of the difference in solvation free energy of the pore (30). The trend in solvation free energies sheds light on the transport of small molecules through the pore. Not unexpectedly, the S40L pore is much more hydrophobic (i.e., higher solvation energy) than the wild type (Table 1 and *SI Appendix, Fig. S6*). In addition, the longer leucine side chain produces a pore with an extended length compared with the wild-type structure, wherein the pore narrows at a more focused point. Although the pore is not occluded, the hydrophobic nature of the S40L pore likely creates an energetic barrier for the 1,2-PD substrate. The 1,2-PD is presumably strongly hydrated and hydrogen bonded to water in the bulk phase, and would need to be desolvated during its passage along the length of the hydrophobic pore in the S40L mutant. In the case of the S40A mutant, removal of the hydroxyl group (and its hydrogen bonding capacity) did not hinder 1,2-PD influx under the conditions tested. It did, however, substantially enhance escape of the propionaldehyde intermediate. This finding suggests that the polar character, and perhaps the hydrogen bonding capacity, of the native serine hydroxyl at residue 40 creates a relatively lower energy barrier for passage of 1,2-PD compared with propionaldehyde, which is less polar than 1,2-PD. The substantial effects on transport properties that are caused by subtle changes in the structure of the PduA pore attest to its finely tuned role in the Pdu MCP.

The Movement of Large Cofactors into Purified Mutant and Wild-Type MCPs. Prior studies indicated that PduP, which catalyzes the conversion of propionaldehyde + HS-CoA + NAD⁺ →

propionyl-CoA and NADH, localizes to the lumen of the Pdu MCP (31). Therefore, measurement of PduP activity provides a way to assess the movement of the large cofactors, HS-CoA and NAD⁺, across the MCP shell. Enzyme assays on purified MCPs (both broken and intact) showed that wild-type and mutant MCPs all had similar levels of PduP activity (approximately 2.8 μmol·min⁻¹·mg⁻¹) when assayed under conditions identical to those used to show that the PduA-S40L and PduA-S40GSG mutations restricted the influx of 1,2-PD (*SI Appendix, Table S4*). This finding suggests that large cofactors can enter mutant Pdu MCPs capable of restricting the movement of 1,2-PD and propionaldehyde. The absence of effect with mutants in PduA supports earlier hypotheses that large cofactors cross the MCP through other specialized shell proteins that have gated pores that open and close when triggered for specific transport (22, 23, 32).

Selectivity of the PduA Pore for 1,2-PD and Glycerol. A multiple sequence alignment showed that the serine at position 40 of PduA is widely conserved among diverse genera of bacteria except for the *Lactobacilli*, where all species examined had a histidine at that position (*SI Appendix, Fig. S7*). Interestingly, previous studies showed that *Lactobacillus* uses the Pdu MCP for the catabolism of both 1,2-PD and glycerol (33, 34). In contrast, *Salmonella* uses the Pdu MCP for 1,2-PD degradation but catabolizes glycerol by alternative pathways. To test whether the H40 amino acid of the *Lactobacillus* PduA protein might be an adaptation that allows movement of both glycerol and 1,2-PD into the *Lactobacillus* Pdu MCP, we constructed a chromosomal PduA-S40H mutation in *Salmonella*, and measured its effects on the influx of 1,2-PD and glycerol into the Pdu MCP. The PduA-S40H mutation had no significant effect on growth of *Salmonella* on 1,2-PD or on the DDH activity of purified MCPs (*SI Appendix, Fig. S3* and Table 2), indicating that H40 allowed normal entry of 1,2-PD into the Pdu MCP under the conditions used. To examine molecular transport in the PduA S40H mutant in vitro, we looked at glycerol inhibition of DDH in purified Pdu MCPs; glycerol is both a substrate and an inhibitor of DDH (35). The DDH activity of purified PduA-S40H MCPs was inhibited by glycerol at a five times lower concentration compared with wild-type MCPs, indicating substantially greater permeability toward glycerol (Table 3). Conversely, DDH inhibition in the PduA-S40L and PduA-S40GSG mutants required five times higher concentrations of glycerol. This finding indicated a change in the relative permeability of the pore to 1,2-PD and glycerol, which compete for the DDH active site. We also found that purified recombinant DDH (*SI Appendix, Fig. S2*) was particularly sensitive to glycerol inhibition, further indicating the selectivity of the MCP shell in general (Table 3). Collectively, these results emphasize the role of the MCP shell and the PduA in dictating transport selectivity in the Pdu MCP.

Discussion

A fundamental question about bacterial MCPs is how their protein shells restrict the efflux of volatile/toxic pathway intermediates while allowing enzyme substrates, cofactors, and products to pass. In this report, we show that the PduA protein forms selectively permeable pores across the shell of the Pdu MCP that allow the retention of the propionaldehyde intermediate while

Table 3. DDH inhibition by glycerol in MCPs purified from different pore mutants

Type of purified MCP	IC ₅₀ of glycerol, mM
Wild-type (LT2)	291.7 ± 2.2
PduA-S40L	1,560 ± 50.1
PduA-S40GSG	1,410 ± 35.9
PduA-S40H	55.3 ± 1.7
Purified DDH	1.2 ± 0.1

allowing influx of the 1,2-PD substrate. These studies demonstrate that the pores of MCP shell proteins are selectively permeable to small molecules. In the work presented here, crystal structures of several of the PduA pore mutants provided a framework for interpreting the phenotypic data in terms of physical properties of the pore. Mutants unable to take up the 1,2-PD substrate could be correlated with pores that were fully occluded or substantially more hydrophobic. The behavior and structure of the S40A mutant shed light on the importance of the detailed properties of the pore for aldehyde retention. In this case, the removal of (six copies of) a hydroxyl group in the pore does not have a significant effect on 1,2-PD entrance, but propionaldehyde escape is allowed. The hydroxyl groups at this constricted point in the pore provide a highly polar environment that evidently disfavors occupancy by propionaldehyde (which is less polar than 1,2-PD). The S40H mutant showed altered transport specificity, allowing influx of glycerol, consistent with the metabolic capabilities of bacterial species that have this particular amino acid at the key residue 40 in their PduA protein sequence. Together, these results provide substantial evidence that MCP shell proteins have evolved to meet specific transport roles and that a selectively permeable protein shell is important to the function of diverse MCPs.

In summary, these studies highlight the existence of a family of proteins—entirely distinct from transmembrane protein channels and pumps—that have evolved diverse selective transport functions in bacteria. The distinct features of these protein-based transport systems, particularly the potential advantage of their

nonlipidic environments, should motivate vigorous investigations of their mechanisms. Important future goals include understanding the dynamic mechanisms that underlie molecular transport in MCP shell proteins, and intelligently modifying MCP proteins to create selectively permeable molecular chambers for synthetic biology applications (36).

Methods and Materials

MCP purification, enzyme assays, and growth studies were performed as described (27, 31, 37). Site-directed mutants were made by using standard protocols (26). Crystals were prepared by the hanging drop method. Some minor modifications to prior protocols are described in *SI Appendix, SI Methods and Materials*.

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