Journal of Structural Biology: X 6 (2022) 100062

Contents lists available at ScienceDirect

Journal of Structural Biology: X

journal homepage: www.sciencedirect.com/journal/journal-of-structural-biology-x

Recent advances in the structural biology of encapsulin bacterial nanocompartments

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ARTICLE INFO

Keywords:
Encapsulin
Nanocompartments
Compartmentalization
X-ray crystallography
Cryo-electron microscopy
Structural biology

ABSTRACT

Large capsid-like nanocompartments called encapsulins are common in bacteria and archaea and contain cargo proteins with diverse functions. Advances in cryo-electron microscopy have enabled structure determination of many encapsulins in recent years. Here we summarize findings from recent encapsulin structures that have significant implications for their biological roles. We also compare important features such as the E-loop, cargo-peptide binding site, and the fivefold axis channel in different structures. In addition, we describe the discovery of a flavin-binding pocket within the encapsulin shell that may reveal a role for this nanocompartment in iron metabolism.

Introduction

Large bacterial nanocompartments called encapsulins are widespread in bacteria and archaea (Andreas and Giessen, 2021; Giessen and Silver, 2017). They are generally homo-oligomers in which the subunits assemble into large capsid-like structures (Sutter et al., 2008). Encapsulin genes are normally located on an operon with one or more cargo protein genes and encapsulin monomers assemble around and encapsulate cargo proteins as they are translated (Giessen and Silver, 2017). The sequestration of enzymes inside an encapsulin nanocompartment confers several advantages to the cell. The catalytic rate of encapsulated enzymes may increase due to higher local concentrations of substrates or stabilizing the cargo protein (Nichols et al., 2020; Rahmanpour and Bugg, 2013). Furthermore, toxic metabolites and reactions are segregated from the rest of the cell, which could prevent damaging side reactions (Giessen and Silver, 2017; McHugh et al., 2014).

Nanocompartment proteins can be categorized into four major families based on phylogeny and cargo protein function (Radford, 2014; Andreas and Giessen, 2021). Encapsulins in Family 1 are called classical encapsulins because they are the most widespread and best characterized. They contain either dye-decolorizing peroxidases, ferritin-like proteins, or proteins associated with anammox processes (Giessen and Silver, 2017; Sutter et al., 2008). Family 2 is the most abundant class and these encapsulins contain sulfur metabolism or natural product synthesis cargo proteins (Nichols et al., 2020). They can be divided into two classes based on the absence (2A) or presence (2B) of a C-terminal cyclic nucleotide (cNMP) binding domain (Andreas and Giessen, 2021). Family 3 encapsulins contain cargo proteins related to natural product biosynthesis. Family 4 diverges the most from other encapsulins and consists of highly truncated encapsulins found mostly in hyperthermophilic archaea. At present, most of the experimental biochemical and structural work is on encapsulins from family 1 (Fig. 1) (Akita et al., 2007; Giessen et al., 2019; He et al., 2019, 2016; LaFrance et al., 2021; Lončar et al., 2020; McHugh et al., 2014; Sutter et al., 2008; Tang et al., 2021; Wiryaman and Toor, 2021; Xiong et al., 2020). Nevertheless, some researchers have also explored encapsulins from families 2, 3, and 4 (Clancy Kelley et al., 2007; Gorges et al., 2018; Nichols et al., 2020). This graphical review will summarize the structural studies of encapsulin nanocompartments, with an emphasis on recent structures released in the last two years.

The first X-ray crystal structures of the *P. furiosus* and *T. maritima* encapsulins were published in 2007 and 2008, respectively, followed by a cryo-electron microscopy (cryo-EM) structure of the *M. xanthus* encapsulin in 2014 (Fig. 1A) (Akita et al., 2007; McHugh et al., 2014; Sutter et al., 2008). The advent of cryo-EM in structural biology over the past decade has been a boon to the field of bacterial nanocompartments because large symmetrical assemblies such as encapsulins are ideal for cryo-EM (Wiryaman and Toor, 2021). In the past two years alone, six more articles with experimental encapsulin structures have been published (Giessen et al., 2019; Lončar et al., 2020; Nichols et al., 2020;

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https://doi.org/10.1016/j.yjsbx.2022.100062
Received 8 October 2021; Received in revised form 14 January 2022; Accepted 20 January 2022
Available online 23 January 2022

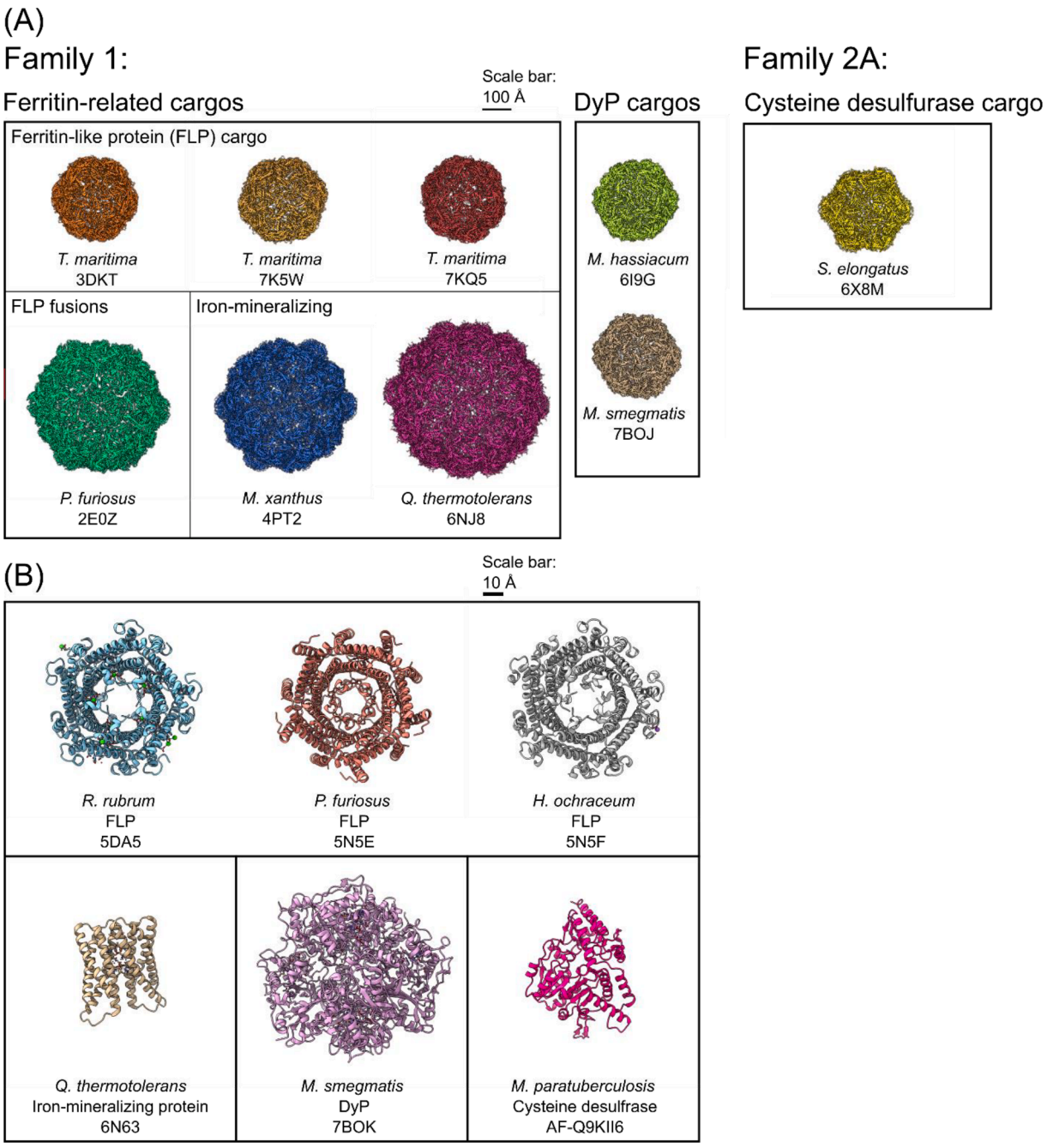

Fig. 1. (A) Experimentally determined encapsulin structures to date, sorted by phylogenetic families and cargo types. Four letter/number codes correspond to the PDB ID. (B) Experimentally determined cargo protein structures to date. Four letter/number codes correspond to the PDB ID, except AF-Q9KII6 which is the closest match to the *S. elongatus* SufS cysteine desulfurase cargo protein in the Alphafold database.

Tang et al., 2021; Wiryaman and Toor, 2021; Xiong et al., 2020). There are also several preprints with exciting encapsulin structures that will likely be released to the Protein Data Bank in the near future (LaFrance et al., 2021; Ross et al., 2021). Several structures of cargo proteins are also available (Fig. 1B) (Giessen et al., 2019; He et al., 2019; Piergentili et al., 2020; Tang et al., 2021; Xiong et al., 2020).

Encapsulin nanocompartments are typically composed of a single subunit assembling into large 60-mer ($T = 1$), 180-mer ($T = 3$), or 240-mer ($T = 4$) assemblies (Akita et al., 2007; Giessen et al., 2019; Sutter et al., 2008). The encapsulin monomers have a similar structure to phage capsids and are categorized as HK97 gp5 folds with a mixed α-helical and β-sheet structure (Fig. 2A) (Sutter et al., 2008). The monomer can be divided into three major domains. The P-domain is the largest and contains several α-helices and β-strands, along with the *N*-terminus. The A-domain also has several α-helices and β-strands and most notably forms the relatively disordered fivefold symmetry interface. Finally, the E-loop is important for assembly and determines the symmetry of the complex. In $T = 1$ encapsulins, the E-loop forms an intermolecular β-sheet with the E-loop from another subunit to form a tight dimer (Sutter et al., 2008). In $T = 3$ or 4 encapsulins, the E-loop is rotated about 80° relative to the E-loop of $T = 1$ encapsulins (Fig. 2B) (Akita et al., 2007; Giessen et al., 2019; McHugh et al., 2014). They also tend to be more disordered and adopt different conformations depending on their position in the capsomere. Although encapsulins assemble differently, it is difficult to trace the *T*-number of the assembly to differences in the primary sequences of encapsulins. More research is needed in the

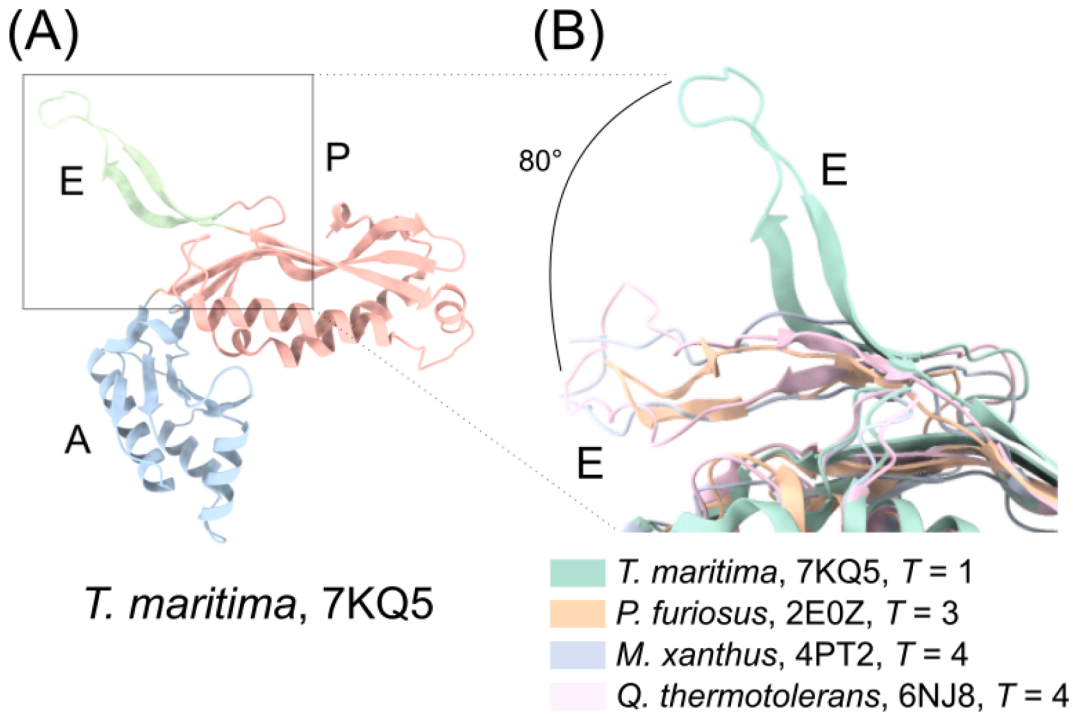

Fig. 2. (A) General domain architecture of an encapsulin monomer, which consists of the P- and A-domains and the E-loop. (B) Inset showing the E-loops of encapsulins with different *T*-numbers. $T = 3$ or 4 encapsulins have E-loops that are rotated about 80° compared to $T = 1$ encapsulins.

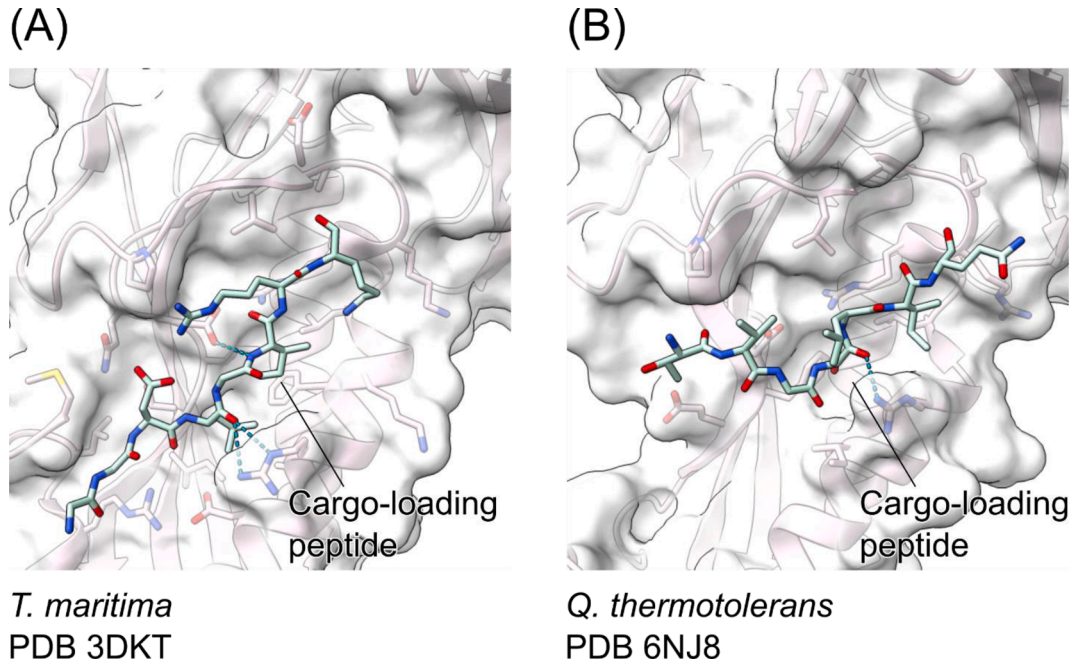

Fig. 3. Binding sites for cargo-loading peptides in encapsulins. (A) The cargo-loading peptide binding site in *T. maritima* encapsulin (PDB 3DKT). The cargo-loading peptide is colored in light blue. (B) The cargo-loading peptide binding site in *Q. thermotolerans* encapsulin (PDB 6NJ8). The cargo-loading peptide is colored in light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assembly pathway of encapsulins to clarify the important structural factors that determine symmetry of an encapsulin.

One of the most remarkable features of encapsulin nanocompartments is that they have an interior binding site for a cargo-loading peptide (CLP) (Fig. 3) (Sutter et al., 2008). The CLP is an extension of the cargo protein that targets the cargo to the interior of the encapsulin nanocompartment. The crystal structure of *T. maritima* encapsulin and the cryo-EM structure of *Q. thermotolerans* encapsulin have densities corresponding to a cargo-loading peptide in the binding site (Giessen et al., 2019; Sutter et al., 2008). The binding site is a mostly hydrophobic pocket that forms one or two ionic interactions with the CLP (Fig. 3). Despite these two structures, density corresponding to the CLP is often absent from experimental encapsulin structures even if they are loaded with cargo proteins (Giessen et al., 2019; Lončar et al., 2020; Nichols et al., 2020; Tang et al., 2021; Xiong et al., 2020). The lack of CLP density may be due to low occupancy and imposing symmetry during X-

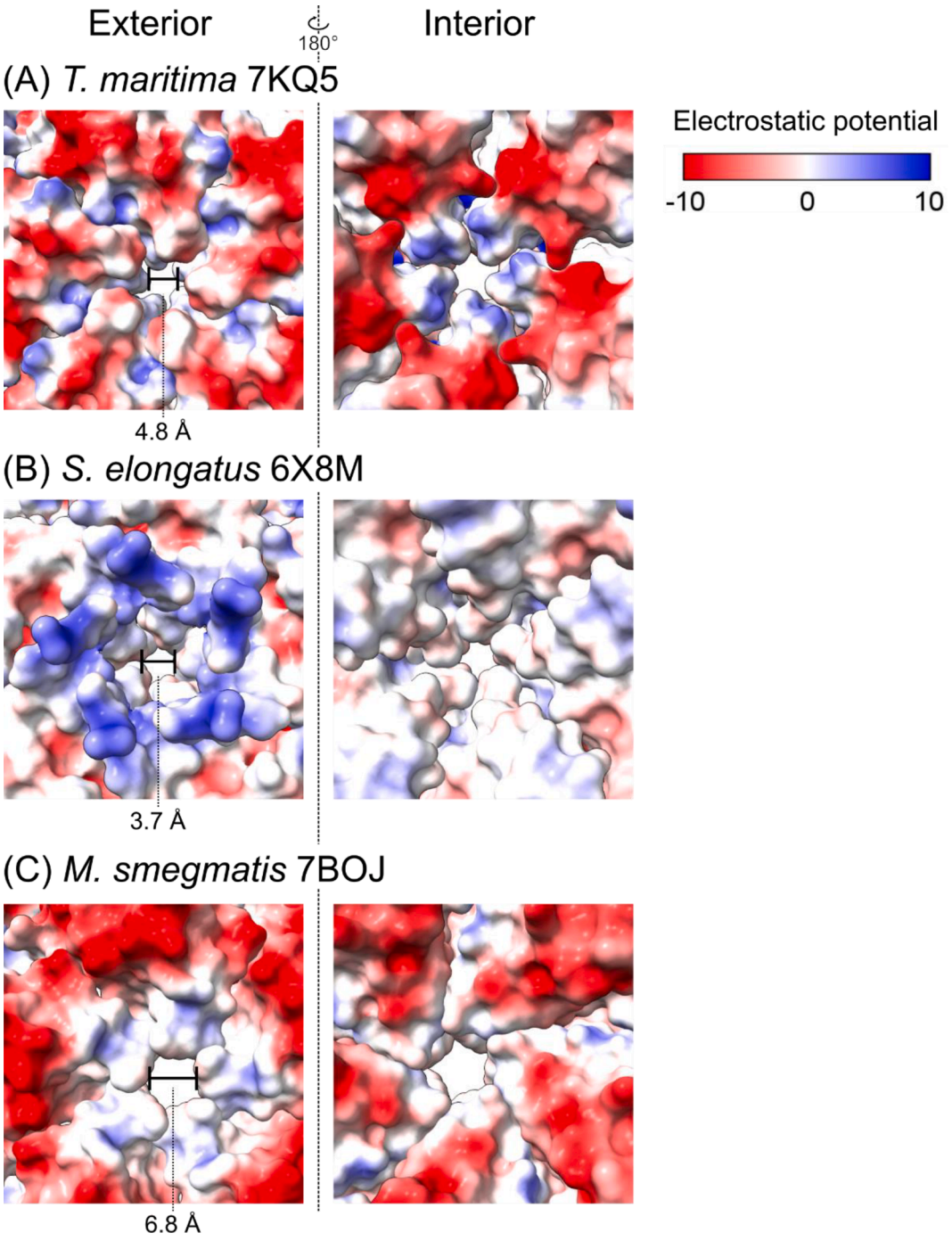

Fig. 4. Pores at the fivefold axes of (A) *T. maritima*, (B) *S. elongatus*, and (C) *M. smegmatis* encapsulins. The figures in the left column are the views from the exterior of the encapsulins and the ones in the right column are views from the interior of the encapsulins. The surface representation is colored by electrostatic potential where blue is positive and red is negative. The measurements in the center of the figures indicate the diameter of the pores in Å. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ray crystallography or cryo-EM reconstructions, which would average out the signal for the CLP. This result also belies the challenges of modeling the cargo proteins inside encapsulin nanocompartments for the same reasons. At present, experimental densities for cargo protein assemblies in encapsulins are absent or low resolution, which is an obstacle to understanding the interactions between encapsulins and cargo proteins that lead to enhanced catalytic rates (Giessen et al., 2019; LaFrance et al., 2021; Lončar et al., 2020; Sutter et al., 2008; Xiong et al., 2020). New software, algorithms, and strategies for 3D reconstruction of cryo-electron density maps that can resolve flexible regions may be necessary to resolve cargo protein densities in encapsulins. Nevertheless, the cryo-EM structure of *M. smegmatis* DyP in an encapsulin nanocompartment shows that 3D classification, particle subtraction, and local refinement strategies can resolve density maps of some nanocompartment-cargo protein complexes (Tang et al., 2021).

The presence of catalytic cargo proteins inside encapsulin nanocompartments suggests a mechanism for selectively transporting substrates in and products out of encapsulins (Nichols et al., 2017; Sutter et al., 2008). Because encapsulins contain different types of enzymes, this mechanism might depend on the nature of the substrates (Nichols

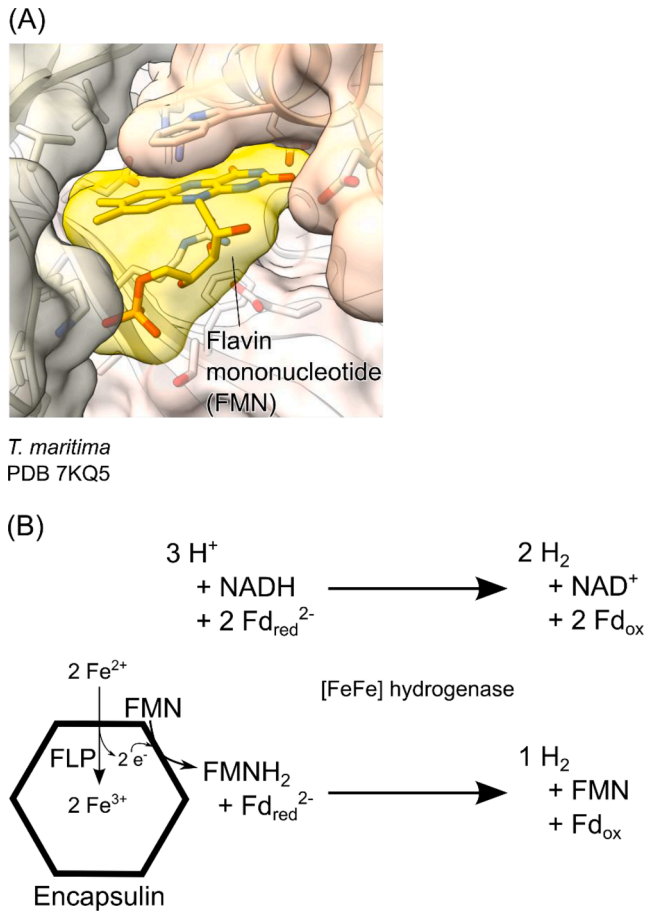

Fig. 5. (A) The flavin binding site in *T. maritima* encapsulin (PDB 7KQ5). Flavin mononucleotide (FMN) is highlighted in yellow. (B) Proposed mechanism of encapsulin/ferritin-like protein (Enc/FLP) complexes involved in anaerobic redox metabolism. The top reaction catalyzed by a [FeFe] hydrogenase consumes NADH and reduced ferrodoxin from glycolysis to reduce H^+ to H_2 and recycle the redox cofactors. The bottom reaction is a proposed mechanism where an encapsulin/ferritin-like protein complex couples oxidation of Fe^{2+} to the reduction of FMN. We hypothesize that [FeFe] hydrogenases can use reduced FMNH or FMH_2 to reduce H^+ to H_2, which may be an oxidative stress response mechanism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2020). The most likely transport channels are located at the fivefold symmetry axis because these channels are the largest pores in the encapsulin and can accommodate the size of different substrates (Giessen et al., 2019; Sutter et al., 2008; Wiryaman and Toor, 2021). In the *T. maritima* encapsulin that contains a ferritin-like protein, the channel is mostly neutrally charged and is relatively hydrophobic (Fig. 4A) (Sutter et al., 2008; Wiryaman and Toor, 2021). Nevertheless, the entrance on the exterior side has five histidines, which could have a high affinity for divalent metal ions such as Fe^{2+} (Fig. 4A, left). Also, the interior side has many negatively charged residues, which indicates that there may be a defined pathway to the ferritin-like protein for iron ions after they enter the encapsulin (Fig. 4A, right). The fivefold channel of the *S. elongatus* encapsulin is markedly different from *T. maritima* and it is characterized by positively charged residues on the exterior that protrude as spikes (Fig. 4B, left) (Nichols et al., 2020). These features appear to be adaptations for a negatively charged, deprotonated cysteine substrate due to the high pH environment of *S. elongatus*. *M. smegmatis* encapsulin also has positively charged histidines around the fivefold channel (Fig. 4C), suggesting that this encapsulin selects for negatively charged aromatic compounds for its dye-decolorizing peroxidase cargo protein (Tang et al., 2021). In contrast to this model of substrate selectivity, a recent article has found open and closed conformations of the fivefold channels in *H. ochraceum*, suggesting that the fivefold channel could act as a gate that responds to external stimuli (Ross et al., 2021).

In two recent cryo-EM structures of the *T. maritima* encapsulin, density corresponding to a flavin ligand was identified in a flavin-binding pocket on the exterior of the nanocompartment (Fig. 5A) (LaFrance et al., 2021; Wiryaman and Toor, 2021). The role of this bound flavin ligand is not precisely known. Because flavins are redox cofactors, this result suggests encapsulin/ferritin-like protein complexes may play a role in the redox metabolism of anaerobic bacteria. In *T. maritima*, an [FeFe] hydrogenase has been shown to catalyze the reduction of H^+ to H_2 using glycolytic products NADH and reduced ferredoxin (Fd) as electron donors (Fig. 5B) (Schut and Adams, 2009). The activity of this enzyme depends on FMN, and this enzyme is thought to have two FMN binding sites, but there is only experimental evidence for one (Buckel and Thauer, 2013). This finding raises the possibility the second binding site may accommodate free reduced FMNs, which can also act as a substrate for [FeFe] hydrogenases. The presence of a flavin binding site in *T. maritima* encapsulin suggests that anaerobic bacteria with encapsulin/ferritin-like protein complexes can couple iron oxidation to FMN reduction, which may then be used to produce H_2 by [FeFe] hydrogenases (Fig. 5B). This pathway would represent a mechanism through which anaerobic bacteria can respond to oxidative stress and deal with excess Fe^{2+} ions that lead to dangerous Fenton reactions.

Clues to the biological roles of some encapsulin nanocompartment systems have begun to emerge from structures of cargo proteins. Several crystal structures of encapsulated ferritin-like proteins (FLPs) have been determined that show a decameric ring assembly (Fig. 1B) (He et al., 2016; He et al., 2019). The *R. rubrum* FLP decamer docks nicely into the $T = 1$ encpasulin pentamer, suggesting that the fivefold axis may serve as an entrance point for iron (He et al., 2016). Nevertheless, further structures and biochemical experiments of the *P. furiosus and H. ochraceum* FLPs suggest that the decameric complex may dominate in the crystallization conditions, but the FLPs are more conformationally dynamic in solution (He et al., 2019). These proteins have ferroxidase activity, but interestingly, it is hypothesized that they do not store iron and iron mineralization is accomplished by the encapsulin nanocompartment (He et al., 2016). Another class of ferritin-like protein cargos are the iron-mineralizing encapsulin-associated Firmicute (IMEF) system (Giessen et al., 2019). The crystal structure of the IMEF cargo shows that two IMEF monomers form a dimer with the ferroxidase active site in the center (Fig. 1B). Nevertheless, the exact oligomeric assembly of the IMEF cargo inside encapsulins is unknown. Further studies are needed to clarify how the FLPs and nanocompartments work together to oxidize and store iron.

Besides ferritin-like proteins, the other type of cargo that is well-characterized is the dye-decolorizing peroxidase (DyP) family, particularly in the human pathogen *Mycobacterium tuberculosis*. It encodes a nanocompartment that is released from the cell and induces an immune response in animal models of tuberculosis (Rosenkrands et al., 1998). Several studies have shown it encapsulates a DyP cargo protein (Contreras et al., 2014; Lien et al., 2021). *M. tuberculosis* is resistant to reactive oxygen species produced by activated macrophages, suggesting that the nanocompartment-DyP complex could have antioxidant properties (Contreras et al., 2014). Indeed, the nanocompartment-DyP complex is necessary to resist oxidative stress at low pH and growth in the presence of macrophages and the antibiotic pyrazinamide (Lien et al., 2021). A cryo-EM structure of the *M. tuberculosis* nanocompartment DyP complex illustrates that DyP forms a dodecameric assembly inside the nanocompartment (Fig. 1) (Tang et al., 2021). The size and presence of positively charged residues on the fivefold pore suggest that it is the entry point for negatively charged aromatic substrates, which are shuttled to the DyP active sites. These results demonstrate that nanocompartment-DyP is a critical component of *M. tuberculosis* pathogenesis and may represent a drug target for this resilient bacteria.

The diverse physiological roles of nanocompartment-cargo protein complexes demonstrates the biological utility of encapsulated nanoreactor or nanostorage systems. Nevertheless, nanocompartments and their cargos appear to play diverse roles and many interesting systems have not been characterized. The *in vitro* structural and functional studies along with the *in vivo* work have been fruitful for understanding nanocompartment biology and this kind of work will continue to yield insights when applied to other nanocompartment complexes of unknown function. These studies will also lead to new insights into applications of bacterial nanocompartments in therapeutics and research (Gabashvili et al., 2020; Rodríguez et al., 2021).

CRediT authorship contribution statement

Timothy Wiryaman: Conceptualization, Writing – original draft, Writing – review & editing. **Navtej Toor:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by NIH grant 1R35GM141706 awarded to N.T. T.W. was supported by the Cellular and Molecular Genetics Training Grant at UC San Diego (NIH Program 2T32GM007240).

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