Molecular mechanism of reflectin's tunable biophotonic control: Opportunities and limitations for new optoelectronics

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(Received 30 May 2017; accepted 6 August 2017; published online 24 August 2017)

Discovery that reflectin proteins fill the dynamically tunable Bragg lamellae in the reflective skin cells of certain squids has prompted efforts to design new reflectin-inspired systems for dynamic photonics. But new insights into the actual role and mechanism of action of the reflectins constrain and better define the opportunities and limitations for rationally designing optical systems with reflectin-based components. We and our colleagues have discovered that the reflectins function as a signal-controlled molecular machine, regulating an osmotic motor that tunes the thickness, spacing, and refractive index of the tunable, membrane-bound Bragg lamellae in the iridocytes of the loliginid squids. The tunable reflectin proteins, characterized by a variable number of highly conserved peptide domains interspersed with positively charged linker segments, are restricted in intra- and inter-chain contacts by Coulombic repulsion. Physiologically, this inhibition is progressively overcome by charge-neutralization resulting from acetylcholine (neurotransmitter)-induced, site-specific phosphorylation, triggering the simultaneous activation and progressive tuning of reflectance from red to blue. Details of this process have been resolved through *in vitro* analyses of purified recombinant reflectins, controlling charge-neutralization by pH-titration or mutation as surrogates for the *in vivo* phosphorylation. Results of these analyses have shown that neutralization overcoming the Coulombic inhibition reversibly and cyclably triggers condensation and secondary folding of the reflectins, with the emergence of previously cryptic, phase-segregated hydrophobic domains enabling hierarchical assembly. This tunable, reversible, and cyclable assembly regulates the Gibbs-Donnan mediated osmotic shrinking or swelling of the Bragg lamellae that tunes the brightness and color of reflected light. Our most recent studies have revealed a direct relationship between the extent of charge neutralization and the size of the reflectin assemblies, further explaining the synergistic effects on the intensity and wavelength of reflected light. Mutational analyses show that the "switch" controlling reflectins' structural transitions is distributed along the protein, while detailed comparisons of the sequences and structures of the recently evolved tunable reflectins to those of their ancestral, non-tunable homologs are helping to identify the specific structural determinants governing tunability. [http://dx.doi.org/10.1063/1.4985758]

Cephalopods, which make up a class of invertebrates that includes cuttlefish, squid, and octopus, are well known for their ability to adaptively manipulate light.[1,2] These abilities are utilized in diverse ways, ranging from intra-species communication to camouflage to both catch and avoid becoming prey. Researchers have therefore sought to understand the physical and chemical mechanisms of the diverse variety of biophotonic systems by which cephalopods control their appearance, in hopes of

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building biomimetic next-generation systems that may mimic and expand upon the dynamic abilities of these organisms.[3,4] Cephalopods' complex dynamic coloration is generated from both structural and pigmentary mechanisms, operating through the interplay of several specialized photonic organs and cells types located at different depths within the skin.[3] At the outermost level are reversibly expandable chromatophores, pigment-filled neurally actuated cells that generate color through selective light absorption.[2–4] Beneath the chromatophores are two classes of reflective cells, iridocytes and leucophores, that generate "structural color" by light scattering.[2–4] Iridocytes produce iridescence, or angle-dependent color, whereas leucophores are broadband white reflectors. Iridocytes sometimes have been called iridophores; we use the name iridocyte according to the convention of molecular and cell biology indicating an "iridescent cell," with no mechanism implied. Chromatophores, iridocytes, and leucophores function cooperatively to produce a broad palette of patterns and colors that enable cephalopods to thrive in a variety of conditions.[2]

Structural color emerges from the coherent scattering of light by structures of comparable sizes to the wavelength of light itself. Thus, all highly reflective animal cells (iridocytes and leucophores, as well as other reflective structures such as bird feathers, butterfly wings, insect cuticle, and fish scales) contain arrays of nanoscale structures of high refractive index material. These structures often are composed of hard materials such as keratin, purine crystals, or chitin.[5,6] In contrast, the photonically active nanostructures of the cephalopod reflective cells contain a soft proteinacious material, the reflectin proteins.[7,8] The iridescent colors of the iridocytes are produced through the constructive interference of light reflected from an ordered array of reflectin-filled intracellular lamellae that are stacked within folds of the periodically invaginated cellular membrane to form an intracellular Bragg reflector.[9] Incident light passing through these aligned invaginations encounters high refractive index condensations of reflectin protein, alternating with lower refractive index extracellular space. The color and intensity of the reflected light is a function of multiple factors, including the thickness, spacing and number of Bragg lamellae in the stack, and the refractive index contrast between the intra- and extra-lamellar spaces.[10,11]

In contrast to the chromatophores, which are capable of rapid expansion and contraction mediated by their attached muscle fibers, the iridocytes and leucophores in most species are static reflectors, with the notable exception of certain cells in squids of the family Loliginidae. Members of this family, including the closely related Atlantic (*Doryteuthis pealeii*) and Pacific (*Doryteuthis opalescens*) squids, possess tunable iridocytes, which can switch from a non-reflective to reflective state, and progressively tune the color of the reflected light in response to activation by the neurotransmitter, acetylcholine (ACh).[8] Examination of both the static and activated tunable iridocytes in these species by transmission electron microscopy revealed ~100 nm thick[8,10] darkly staining homogenous Bragg lamellae, subsequently confirmed to contain reflectin by gold conjugated anti-reflectin antibody staining.[10] In contrast, the lamellae of the tunable iridocytes prior to activation were seen by TEM to contain heterogenous nanoparticles and fibrils of protein that subsequently condensed in response to ACh.[8,10,12] While switchable iridocytes in the Loliginidae have been known for several decades, switchable leucophores were only recently discovered.[13] In these switchable leucophores, ACh triggers the similar condensation of reflectin in intracellular vesicles, increasing their refractive index and activating broadband reflectance.[13] The physiological switch activating these tunable iridocytes and leucophores, ACh, is released from nerve cells innervating the iridocyte layer;[14] innervation of the switchable leucophores has not yet been traced. Binding of acetylcholine to receptors on the tunable iridocyte membrane activates a signal transduction pathway that culminates in enzymatic phosphorylation of the reflectins, neutralizing excess net positive charge on these proteins and driving their assembly.[9,10,12,15,16] This assembly of the reflectins drives expulsion of water from the membrane-bound Bragg lamellae through Gibbs-Donnan re-equilibration, further increasing reflectin concentration and reflectivity, while simultaneously shrinking the thickness and spacing of the lamellae to progressively tune the color of the reflected light.[9,10]

In light of the impressive biophotonic abilities of cephalopods, there has been great interest in understanding the mechanism of reflectin assembly as a model of a tunably controlled, self-assembling optically active material, and its potential use for biophotonic and other applications. Purified recombinant reflectin shows unusual self-assembling properties, forming oligomeric and multimeric nanospheres and nanoribbons, and can be processed into thin films, fibers, and diffraction

gratings.[17–21] Reflectin thin films can exhibit colors ranging across the entire visible spectrum, with the reflected color depending upon the thickness of the cast protein layer.[17,18] Coloration is a product of thin film interference and not due to special optical properties of the reflectins themselves. The thickness and resultant color of these reflectin films are extremely sensitive to both hydration and pH. Acidification of thin films by exposure to acetic acid vapor in particular drives significant swelling, causing an increase in film thickness of almost two fold and driving the wavelength of the reflectance peak into the infrared, an intriguing result that has been explored for possible applications for infrared camouflage.[19,20] Reflectin in thin films also has been discovered to act as an efficient proton conductor and has been harnessed to form protonic transistors.[22,23] Reflectin also can serve as a substrate for neural stem cell growth,[24] an activity we suspect may reside largely in its cationic charge balance.

Since their original identification in the bioluminescent light organ of the Hawaiian bobtail squid (*Euprymna scolopes*), reflectin protein sequences have been identified in a diverse assortment of cephalopods including the California two-spotted octopus (*Octopus bimaculoides*),[25] common cuttlefish (*Sepia officinalis*),[26,27] and the loliginids *Loligo forbesii*,[28] *D. pealeii*[15] and *D. opalescens* (see Fig. 1 and caption for more information). No introns have been found within reflectin genes. Some molluscs such as giant clams (*Tridacna gigas*) also possess proteinacious platelet-filled iridescent cells, although the proteins have not been confirmed as reflectins at the sequence level.[29,30] Most reflectins (see below for exceptions) resemble block copolymers, being composed of positively charged polyelectrolyte linker regions interspersed with highly conserved polyampholyte segments. Protein-based block copolymers, composed of linearly linked segments of distinct physicochemical composition, have attracted interest as versatile building blocks for self-assembling materials.[29] The polyelectrolytic linkers are particularly rich in aromatic and arginine residues and almost entirely devoid of negatively charged residues, while the polyampholytic subdomains ("reflectin motifs") have a conserved sequence particularly rich in methionine residues. Two types of patterned ~25 amino acid methionine-rich motifs are observed: the N-terminal motif, and following, variable number of repeating domains. The single very highly conserved N-terminal motif is found in most reflectin proteins and differs in its N-terminal half from the repeating motif. The conserved reflectin motif has been conventionally designated as $M/FD(X)_5MD(X)_5MDX_{3/4}$, although this notation underrepresents the degree of conservation since almost all "X" sites are populated largely by one specific residue, with minor alternative residues usually represented in only one or a few reflectin motifs in the entire known library. Additionally, most variability in the motifs (as well as the linkers) is found towards the C-terminal half of the protein, indicating a weaker selective drive to sequence conservation in this region of the protein. This suggests to us that the N-terminal half of the protein likely plays a more fundamental role in determining the assembly and biophotonic-controlling properties of the proteins.

A few *S. officinalis* and *O. bimaculoides* reflectins contain deletions of the N-terminal reflectin motifs and assorted other segments that are easily recognized by comparisons in the multiple sequence alignment of all the known reflectins. The possible physiological relevance of these apparent deletion mutants, and even whether they are expressed and co-assemble with the reflectins *in vivo*, is unknown. As mentioned above, reflectin proteins differ in the number of linker/motif repeats, varying from 4 to 8 motif/linker diblocks (including the N-terminal motif), resulting in proteins of lengths from 208 to 483 residues (27-62 kDa). Differing degrees of diversity in reflectin sizes are observed within different organisms, with the greatest diversity seen in *O. bimaculoides* and *D. pealei/D. opalescens*, although it is critical to note that this may be an artifact of the non-systematic collection of currently known sequences. A recombinant protein composed of a single linker/motif diblock from *E. scolopes* 1a, called refCBA, forms structures similar to the full-length protein, indicating that the drive for reflectin self-assembly is retained even in very short segments.[18] Almost all reflectins have flanking linker sequences at both the N-terminal and C-terminal ends, with the residue composition of these flanks differing from other linkers, being generally more positively charged at the N-terminus and more negatively charged at the C-terminus. Residue conservation of both linkers and motifs is greater towards the N-terminal half of the protein, indicating that this portion may play the greater role in determining the assembly and biophotonic properties of the proteins.

In addition to the "A-type" block copolymer-like reflectins described above, an additional "B-type" reflectin type has been identified in the loliginids and cuttlefish (we have been unable to find

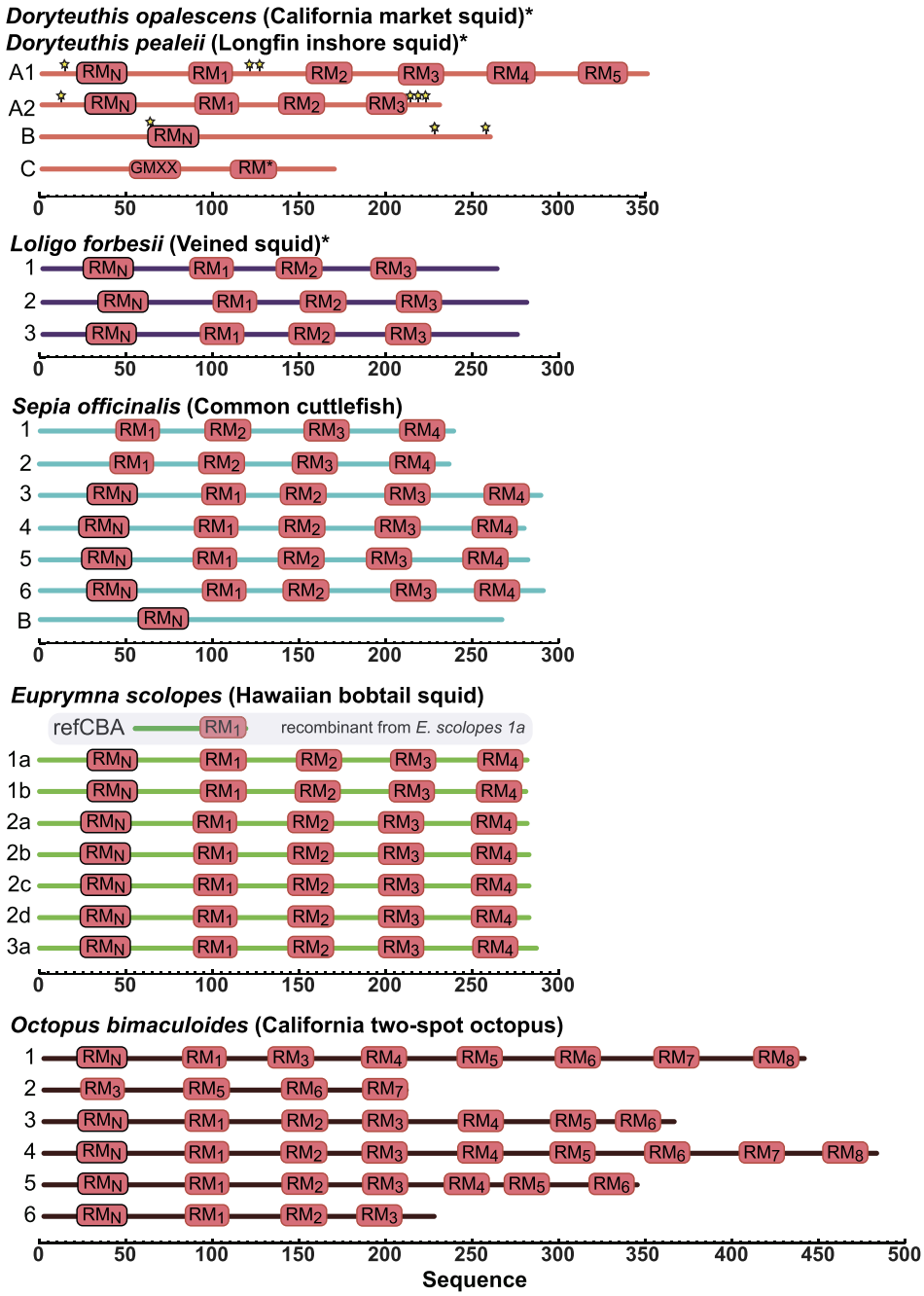

FIG. 1. Schematics of most known reflectin sequences. Species with tunable iridescence (of family Loliginidae) are marked with asterisks. Conserved Reflectin Motifs (RMs) are designated by bolded (N-terminal motif, RM_N) and unbolded (regular repeats, $RM_{1\text{-}8}$) boxes, while linkers are lines. Repeat motifs of proteins that include deletions are numbered according to their implied order in the common ancestor reflectin, as determined by multiple sequence alignment.[30] Previously identified phosphorylation sites in the *D. opalescens* reflectins are marked with stars.[12,15] *O. bimaculoides* reflectin sequences are numbered in the order presented previously.[25] Schematics of *S. offinalis* reflectins show genomic sequences from embryos, except the *S. offinalis* B-type reflectin is an expressed sequence tag from an earlier publication.[26,27] Schematics of *D. opalescens* and *D. pealeii* are combined, since they differ by only a few residues overall.

any B-type reflectins within the genome of *O. bimaculoides*, the only cephalopod for which a full genome sequence has been published).[15,26] One type of reflectin (called reflectin B in *D. opalescens*) possesses the single N-terminal motif but does not have any of the reflectin repeat motifs. The remainder of the sequence, excluding the N-terminal motif, has a composition roughly similar to A-type

reflectin linkers and has been found to colocalize with the other reflectins in the Bragg lamellae.[12] Interestingly, active tunability in *D. opalescens* dorsal iridocytes is correlated with increased relative concentrations of reflectin B, indicating that this molecule may play a role in conferring tunability to these cells, perhaps by altering the stability (or metastability) of reflectin oligomers or assemblies (see below).[12] In addition to reflectin B, an additional reflectin (reflectin C) has also been identified in loliginids. This protein, whose linker composition is similar to reflectin B, lacks the N-terminal motif and contains only one poorly conserved repeat motif and a unique GMXX (where G is glycine, M is methionine, and X represents variable residues) tetrarepeat.[12] This tetrarepeat has an overall higher hydropathy than the rest of the protein and has been found to be present in greater concentrations near the membranes bounding the Bragg lamellae, suggesting that this protein might play a role in anchoring the reflectin assemblies to the intracellular Bragg reflector.[12]

Reflectins have unique amino acid compositions, greatly enriched overall in aromatic (particularly tyrosine), methionine, arginine, and asparagine residues, while almost entirely deficient in the aliphatic residues typically found in folded proteins. Residue compositions are similar across all cephalopods, with only subtle possible differences between the tunable and non-tunable reflectins. Proline and glycine levels are consistent with those of an average protein and are substantially less than those found in proteins that form elastin-like coacervates.[31] Motif and linker subdomains differ in residue compositions, with the motifs being relatively enriched in methionine, glutamine and negatively charged aspartic acid and glutamic acid, residues, while the linkers are relatively enriched in aromatic residues—particularly tyrosine, comprising 24% of all linker residues across the reflectins shown in Fig. 1—histidine, arginine, and asparagine residues. The high concentration of aromatic and methionine residues likely plays two complementary roles, providing a driving force for reflectin assembly through π-π stacking interactions, especially important in the absence of other hydrophobic residues, while simultaneously giving the reflectins one of the highest predicted incremental refractive indices (*dn*/*dc*: the change in solution refractive index as a function of solute concentration) for a known protein family.[32–34] Interestingly, the low absorptivity of the reflectins in the visible wavelengths contributes to their effectiveness in the Bragg reflectors operating in this range, whereas their high absorption in the UV would make them inefficient at such shorter wavelengths. The reflectins' resulting large *dn*/*dc* value in the visible helps give the densely packed reflectin assemblies a high refractive index in the Bragg lamellae (ca. 1.44-1.56) relative to that of the intra-lamellar (extracellular) space (ca. 1.33), as determined by microspectrophotometry of the activated *D. opalescens* Bragg reflectors, enhancing their reflectivity.[11,35] The high proportions of aromatic, methionine, and arginine residue also indicate a high likelihood of synergistic sulfur-pi and cation-pi interactions, both of which are often overlooked but common and substantial sources of protein structural stability.[34,36,37] Proximity to aromatic residues may also inhibit methionine oxidation, enhancing reflector stability.[38]

Reflectins generally have overall pI values in the range of ~8.2-9.0, although some deviations, as in two of the *L. forbesii* reflectins, exist (these specific reflectins were characterized from the static eye reflector). These pI values mean that the proteins have slight net positive charges at typical physiological pH (~7.2). *In vivo*, ACh-activated, multi-site phosphorylation of the linkers in the reflectins is correlated with the emergence of iridescence, and inhibition of the activity of protein kinase was shown to block the ACh-activation of this phosphorylation and iridescence.[15] Because the specific kinase(s) involved in reflectin phosphorylation have not yet been identified, we devised a simple system using pH titration as an *in vitro* surrogate for phosphorylation to study the charge neutralization-driven assembly behavior of purified recombinant *D. opalescens* reflectins.[16] Changes in reflectin net charge in this pH range are thought to be driven predominantly by deprotonation of histidine side chains, located largely in the linker regions, similar to the sites of *in vivo* phosphorylation. To perform this assay, we dilute water solubilized reflectin into low ionic strength buffer of varying pH, ranging from pH 4.5 to 8.0. Analyzing the molecules by dynamic light scattering and transmission electron microscopy, we found that the tunable reflectins form monodisperse spherical nanoparticles of progressively larger sizes as a function of increasing pH, indicating an exquisitely sensitive relationship between net charge and assembly. Using *D. opalesens* reflectin A1 in this assay, assembly sizes can range from roughly oligomeric particles with radii of hydration (R_h) values of ~5 nm at pH 4.5, estimated to contain approximately a dozen reflectin monomers (as determined from the calculated partial specific volume), to larger multimeric particles of R_h ~ 40 nm at pH 8.0, estimated to contain

thousands of monomers each.[16,39] The sizes determined by dynamic light scattering showed excellent agreement with visualization by electron microscopy.[16] Acidification restores positive charge on the reflectins, driving disassembly of the multimeric assemblies back to oligomers. The presence of oligomers as intermediates in multimer formation is consistent with previous analyses of reflectin assembly and resembles the self-assembly behavior of amelogenin, a protein involved in tooth enamel formation.[17,40,41] The overall assembly pathway is shown schematically in Fig. 2. This *in vitro* assembly and disassembly was reproducibly cyclable multiple times before being crowded out by the formation of off-pathway aggregates. Reflectin assemblies in this assay are highly sensitive to the presence of additives and ionic strength. Increasing the ionic strength, through increased buffer concentration or addition of NaCl, leads to increased assembly sizes at all pH values, although a positive relationship between size and pH is still observed. It is important to note that the *in vitro* assembly of recombinant reflectins at increased pH described here does not produce iridescence, since that effect depends on the formation of the physiological, membrane-bounded Bragg reflector; rather, it produces a milky white suspension.

Interestingly, although *D. opalescens* reflectin A2 has a >70% sequence identity with A1, an identical pI, and forms multimeric assemblies of similar sizes at higher pH conditions, it forms larger assemblies than A1 at low pH and does not disassemble to $R_h \sim 5$ nm oligomers upon addition of acetic acid. Recent work from our laboratory offers an explanation for this incongruity. Examination of the assembly behavior of an assortment of diverse *D. opalescens* reflectin A1 mutants indicates that distributed net charge density, particularly of the linker regions, yields the best prediction for reflectin assembly size (Levenson *et al.*, *manuscript in preparation*). The discovery of this relationship also explains the distinct behavior of A2: with its substantially lower histidine content than A1, the A2 molecule thus has lower net charge density than A1 at pH values approaching the pK_a of the imidazole side chain (~6.0-6.5). Thus, despite their differences in sequence and assembly, it seems that the reflectins in these conditions behave as simpler polymers whose assembly is described by basic electrostatic parameters.

Analysis of the conserved reflectin motifs indicates that in contrast to the linkers, they possess a high potential for bifacially phase-segregated amphilicity upon folding into α-helical or β-sheet secondary structure, in agreement with x-ray scattering data showing the emergence of β-sheets upon addition of salt to reflectin A1.[10,16] This initially cryptic drive for structure emerges despite the only slightly greater overall hydropathy of the motifs in comparison to that of the linkers. Folding and consequent hierarchical assembly are initially prevented by the positively charged linker domains, which exert strong Coulombic inhibition of intra- and inter-chain interactions. This inhibition is overcome by progressive charge neutralization (resulting from acetylcholine-triggered, enzymatic phosphorylation of the tunable reflectins *in vivo*, and by titration with increasing pH used as a surrogate of phosphorylation *in vitro*). This neutralization drives the emergence of phase-segregated hydrophobic surfaces on the secondary structured conserved domains that, as we previously suggested,[16] may act like molecular Velcro™, facilitating the formation of oligomers which then hierarchically assemble to form multimers. Alternatively, it remains possible that residues in the linker peptides may provide docking sites for this assembly. In the living iridocytes, a combination of water exclusion and desolvation from the assembling reflectins, via Gibbs-Donnan re-equilibration and colligative changes in osmotic pressure, drive the reversible dehydration of the membrane-enclosed, reflectin-containing Bragg lamellae, simultaneously increasing their refractive index contrast while shrinking

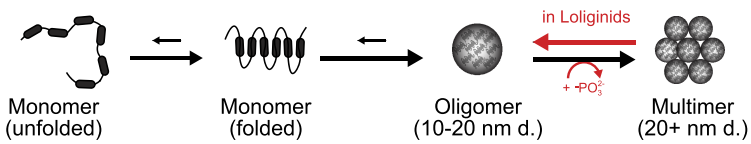

FIG. 2. Progression of neutralization-induced reflectin condensation and hierarchical assembly. Intra- and inter-strand Columbic repulsion competes with attractive hydrophobic and electrostatic interactions to control reflectin assembly state. Phosphorylation (*in vivo*) or pH-titration (*in vitro*) neutralizes net charge and drives progressive hierarchical assembly. The process is reversible and repeatedly cyclable.[16]

their thickness and spacing to simultaneously activate reflectance and progressively tune the color of the reflected light from red to blue.

Intrigued by the fact that tunability of reflectin-mediated reflectance has been observed only in the loliginid squids and motivated by the observation of the great geneticist, Dobzhansky, that "nothing in biology makes sense except when viewed in the light of evolution,"[42] we endeavored to understand the evolutionary advent of tunability in the reflectin system. A broad survey of cephalopod species reveals that nearly all cephalopods have iridescent structures, but only the loliginids possess tunable iridescence (as stimulated by ACh) (Fig. 3). Dermal iridocytes of many other species were examined and displayed no sign of modulation when exposed to ACh or other potential neurotransmitters (glutamate, serotonin, dopamine).[43] Interestingly, cuttlefish have been shown to actively control the polarization of reflectance from their dermis, an ability that may be used for intraspecific communication.[44,45] However, the mechanism of this effect is not yet known, and our observations with both

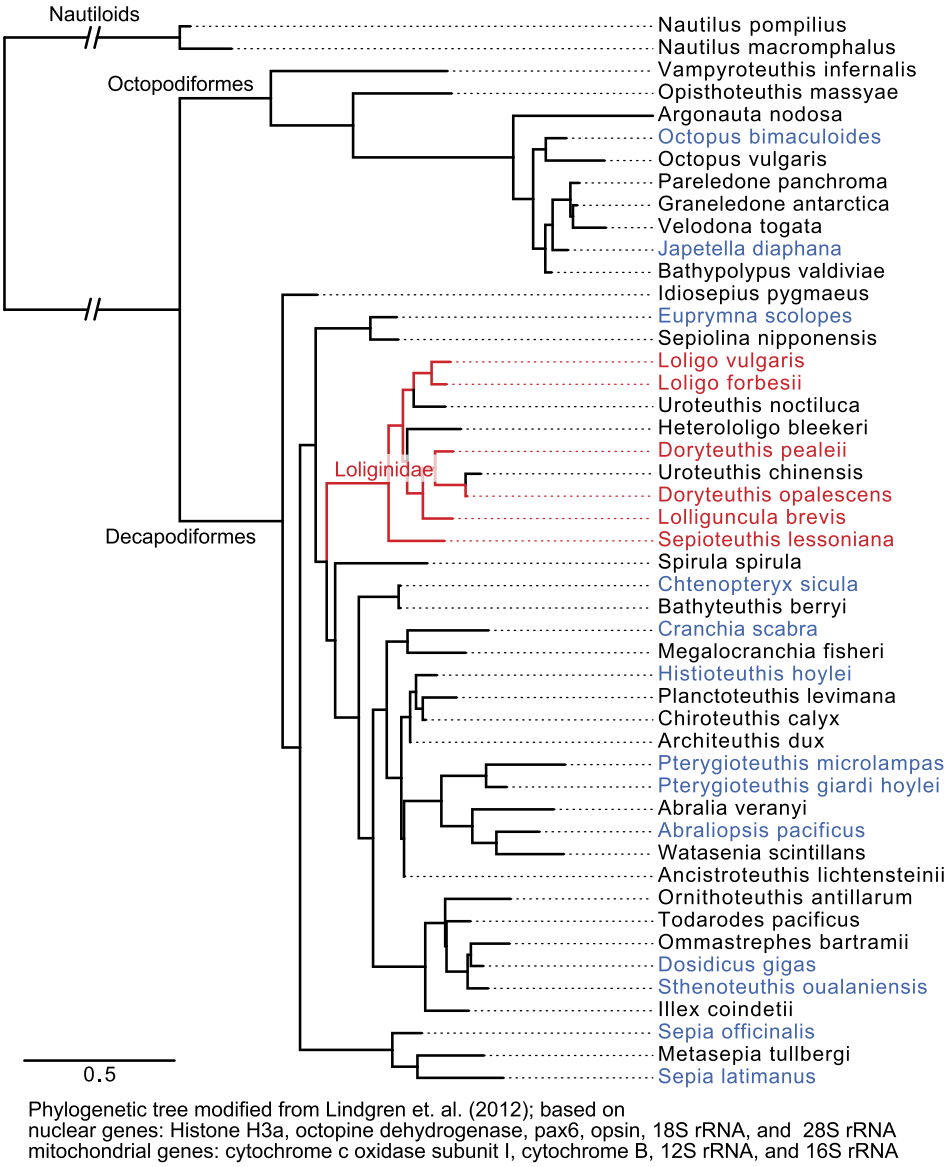

FIG. 3. Phylogenetic tree showing distribution of adaptive iridescence among cephalopods. Only members of the family Loliginidae exhibit adaptive iridescence (red), all other species we tested have static dermal iridocytes (blue). Other species not surveyed are included for reference (black).

Sepia officinalis and *S. latimanus* have shown no indication of wavelength tunable iridocytes, either *in vivo* or in response to exogenous neurotransmitters (Fig. 3).

Ancestral cephalopods dominated the oceans during the early Jurassic period, but the rapid emergence of the more mobile teleost fishes into the Cretaceous period are thought to have driven the slow cephalopods to lose their cumbersome shells in order to adapt competitive mobility, thus giving rise to modern cephalopod diversity.[46] As cephalopods spread to nearly every habitat in the ocean, loliginids (estimated to have diverged approximately $\sim 150 \times 10^6$ years ago) populated the water column in optically dynamic near shore environments, possibly enabled by the evolution of tunable dermal iridescence.[46] The information in Fig. 3 allows us to conclude that modifications in the reflectin gene stock conferring tunability arose from the ancestral non-tunable precursor.

Reflectins in the lamellae of both static (non-tunable) and acetylcholine-activated, tunable iridocytes appear homogenous and densely staining, while the unactivated tunable iridocytes are heterogeneously filled with 5-10 nm radius nanoparticles and nanofibrils.[8] Assuming that these smaller particles in the unactivated iridocytes are analogous to the reflectin oligomers we observe at lower pH values *in vitro*, one possibility is that tunable loliginid reflectins have different stabilities of the oligomeric and multimeric forms relative to those of the non-tunable reflectins, pushing the equilibrium towards oligomers when the iridocyte resides in the unactivated state. For these tunable cells, further charge neutralization of the reflectins, triggered by phosphorylation, is required to complete multimer assembly. The molecular-level differences in the tunable vs. non-tunable reflectins responsible for this difference in behavior are not yet clear, but we consider a few possibilities below. One possibility is that mutational/evolutionary differences in sequence or composition might enhance the stability of the oligomers (and/or destabilize the multimers) of the tunable reflectins relative to their ancestral precursors. This effect may be reflected in a difference in the entropic drive for phase-segregated secondary structure, as discussed below. Another possibility is the destabilization of multimers by increased levels of reflectin B, as observed in the correlation between tunability and reflectin B concentration in *D. opalescens*.[12] Increased reflectin B incorporation into oligomers may alter the oligomeric structure, destabilizing oligomer-oligomer interfaces and hindering subsequent multimer formation. Lastly, changes in lamellae membrane surfaces or small molecule composition may alter the relative stability of oligomers and multimers, as demonstrated by the sensitivity of reflectin assembly *in vitro* to buffer conditions.

To help assess these possibilities, we conducted computational structural analyses of a prototypical tunable reflectin and selected examples of its ancestral non-tunable precursors using hydrophobic moment plots (Fig. 4). These show calculations of the net amphiphilicity of a given protein segment as a function of the angle between successive residue side chains.[47,49,50] This calculation provides a measure of the entropic drive to intramolecular phase segregation and folding, with darker red indicating combinations of reflectin sequence and angle with higher hydrophobic moment, and darker blue indicating lower hydrophobic moment. Because the segregation of hydrophobic side chains is generally the largest driver of self-assembly and folding of proteins in water,[51] we can interpret the hydrophobic moment at varying successive side chain angles as a measure of the entropic drive of the resulting secondary structures. Figures 4(a) and 4(b) shows plots of hydropathic moments for the tunable reflectin A1 from the loliginid squid, *D. opalescens*, and the homologous (46% sequence identity), archetypal non-tunable reflectin 3 from the static iridocytes of the octopus, *O. bimaculoides*. Maximal moments are observed within the reflectin repeat motifs, at successive side chain angles near those found in α-helical (100°) and β-sheet (160°) domains. Figure 4(c) shows the average hydrophobic moment profiles across all or designated portions of these reflectins and includes the non-tunable *E. scolopes* reflectin 1A as well. Although all three proteins have similar hydrophobic moments when averaged across the entire sequence, *D. opalescens* A1 shows slightly larger moments within the repeat motifs segments compared to the others. In contrast, none of the proteins show clear patterns of preference for α-helical and β-sheet structure within the linker domains. While this analysis cannot be used for the reliable prediction of precise secondary structure elements, the difference in hydrophobic moment profiles between linkers and motifs illuminates the differential roles these two sets of segments play in reflectin assembly, particularly underscoring the structural role of the motifs as a consequence of their potential entropic drive to form amphiphilic secondary structure.

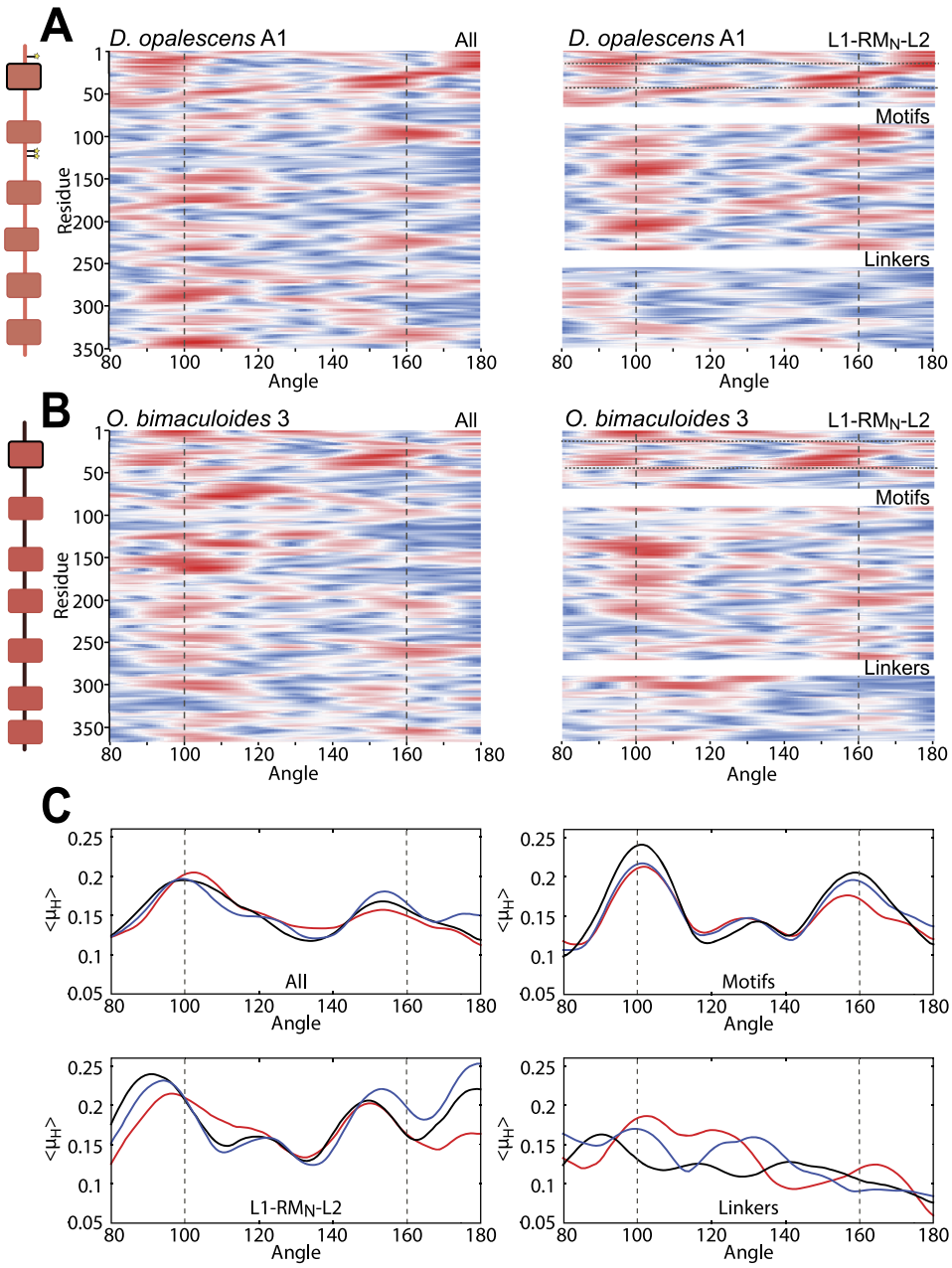

FIG. 4. Calculated hydrophobic moments (μ_H) plots of *D. opalescens* reflectin A1 (GenBank accession number KF661517.1), *O. bimaculoides* reflectin 3 (GenBank accession number KOF78298.1), and *E. scolopes* reflectin 1A (GenBank accession number AY294649.1). (a) *D. opalescens* reflectin A1 and (b) *O. bimaculoides* reflectin 3 plots showing (in red) areas of potentially high phase-segregation of hydrophobic and hydrophilic residues as a function of position along the reflectin molecule (vertical axis) and degrees between successive residue side chains (x-axis). Calculations were performed in single degree increments using a centered moving 18 residue window across the protein sequence (windows centered less than 9 residues to the N- and C- ends are excluded), using the method and hydrophobicity scale of Eisenberg *et al.*[47,48] Left plots show the entire calculations of the entire protein sequence, while right plots show calculations for concatenated designated portions of the reflectin with other segments deleted. Bond angles corresponding to prototypical α-helical and β-sheet secondary structures are marked with dotted lines.[47] Plots are colored according to calculated hydrophobic moment intensities, ranging from blue (smallest μ_H) to white to red (greatest μ_H), scaled consistently across all plots. Values were calculated and plotted by spreadsheet. (c) Mean hydrophobic moments ($\langle\mu_H\rangle$) as a function of degrees per residue for all or designated portions of *D. opalescens* reflectin A1 (black), *O. bimaculoides* reflectin 3 (red), and *E. scolopes* reflectin 1A (blue). Bond angles corresponding to α-helical and β-sheet secondary structure are marked with dotted lines.

We note again that sequence alignments show that the motif segments have generally higher conservation overall (varying between 60% and 80% pair-wise identity for the three reflectins analyzed here), while the linkers show less conservation (45%-50% pair-wise identity), suggesting less selective pressure for conservation of the precise linker sequences. Interestingly, the maximal moments of the N-terminal motifs are all shifted ∼10°smaller relative to the downstream repeated motifs, indicating that these N-terminal regions may be biased to form a distinct structure that may be important in assembly.

As described above, comparison of Figs. 4(a) and 4(b) shows that when the linkers are computationally removed from the tunable reflectin sequence, the entropic drive for phase-segregation and secondary folding of the conserved motifs is enhanced, but this effect is not seen upon comparable treatment of the non-tunable reflectin. These results are consistent with the hypothesis that evolutionary acquisition of "the switch" to make the *Doryteuthis* reflectins tunable resides in sequences at least partially within the linkers, and that these reduce the drive of the conserved motifs to condense and form phase segregated secondary structures. As the phase segregation of the conserved domains highlighted in Fig. 4 represents the folding-dependent emergence of previously cryptic hydrophobic patches that enable oligomer formation and subsequent hierarchical assembly,[40] we can interpret the results of these computational analyses to suggest that the polycationic linkers sufficiently inhibit secondary folding and assembly to require charge-neutralization to trigger these processes in the tunable reflectins but not in their non-tunable counterparts.

While these limited computational analyses need to be viewed with caution and require experimental analysis for verification or falsification, they may suggest—computationally, at least—why the *D. opalescens* reflectins reside in an "off," oligomeric resting state physiologically, enabling assembly to be tunable by linker charge-neutralization, in contrast to the ancestral, non-tunable reflectins that are permanently folded and fully assembled under physiological conditions. The differences seen in Fig. 4 suggest that subtle evolutionary changes in both the conserved domain sequences and the linker sequences may play a role in the evolutionary appearance of tunability. We are currently in the process of testing these possibilities.

As mentioned above, purified reflectins and reflectin-based peptides have been used to form an impressive array of thin films, fibers, and diffraction gratings, with reflectance tunable by swelling or shrinking in response to changes in humidity or other factors.[17–21] While these responses have generally been attributed to the unique structure and biological function of the reflectins, they can in fact be observed with many other biopolymers that have nothing to do with photonic systems, with the attribution of their uniqueness frequently based on an error that recurs in the literature: the mistaken assumption that the reflectins are the biologically active photonic structures themselves. However, micro-spectrophotometric analyses show unequivocally that it is the membrane-bound, reflectin-filled Bragg lamellae of the squid iridocytes that have the tunable dimensions and refractive indices responsible for the observed signal-induced changes in color and brightness of reflectivity and not the reflectins themselves.[11] Given that the reflectins act as a molecular machine that drives an osmotic motor to cyclably control the reversible dehydration and rehydration of the Bragg lamellae, thus simultaneously tuning the refractive index, thickness and spacing of the lamellae that govern the brightness and color of their reflectance, how might the structure and complex function of the reflectins best inspire advantageous new approaches to photonic control? While faithful translation of the reflectin's mechanism of action might require complex gel- or fluid-based components, we suggest that the most powerful direct advantage of reflectin-inspired devices will be lie in the synergy of their simultaneous tuning of dimension and refractive index—in contrast to the conventional control of one or the other of these parameters.

Reflectins of type A are complex block copolymers whose 3-dimensional and hierarchical structures are controlled by phase segregation. Unlike their simpler, synthetic counterparts, however, the capacity for phase segregation in the tunable reflectins of this class is cryptically encoded in the sequences of their canonical and polycationic linker domains, with secondary folding to reveal amphiphilicity emerging only after charge neutralization. This neutralization and the consequent condensation and assembly are reversibly controlled by neurotransmitter-mediated phosphorylation *in vivo* and by pH titration or genetic mutation *in vitro*. New details of these processes have been revealed by experimental, computational, and evolutionary analyses.

The reflectins thus represent a highly unique region of protein sequence space enabling spectacular tunable optical activity *in vivo* and diverse hierarchical assemblies *in vitro*. Elucidation of the design principles underlying reflectin assemblies and their tunability offers potential inspiration for the development of new tunable optically active materials. The more deeply we continue to understand the tunable control of reflectin condensation, structural rearrangement and assembly, the better we may be able to rationally design photonic systems controlled by reflectin-inspired components.

We gratefully acknowledge support for this research from: the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Materials Sciences and Engineering under Award No. DE-SC0015472; the Institute for Collaborative Biotechnologies through Grant No. W911NF-09-0001 from the U.S. Army Research Office; and the U.S. Army Office of Research under Award No. W911NF-17-1-0160. The content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

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