Optically functional isoxanthopterin crystals in the mirrored eyes of decapod crustaceans

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The eyes of some aquatic animals form images through reflective optics. Shrimp, lobsters, crayfish, and prawns possess reflecting superposition compound eyes, composed of thousands of square-faceted eye units (ommatidia). Mirrors in the upper part of the eye (the distal mirror) reflect light collected from many ommatidia onto the photosensitive elements of the retina, the rhabdoms. A second reflector, the tapetum, underlying the retina, back-scatters dispersed light onto the rhabdoms. Using microCT and cryo-SEM imaging accompanied by *in situ* micro-X-ray diffraction and micro-Raman spectroscopy, we investigated the hierarchical organization and materials properties of the reflective systems at high resolution and under close-to-physiological conditions. We show that the distal mirror consists of three or four layers of plate-like nanocrystals. The tapetum is a diffuse reflector composed of hollow nanoparticles constructed from concentric lamellae of crystals. Isoxanthopterin, a pteridine analog of guanine, forms both the reflectors in the distal mirror and in the tapetum. The crystal structure of isoxanthopterin was determined from crystal-structure prediction calculations and verified by comparison with experimental X-ray diffraction. The extended hydrogen-bonded layers of the molecules result in an extremely high calculated refractive index in the H-bonded plane, $n = 1.96$, which makes isoxanthopterin crystals an ideal reflecting material. The crystal structure of isoxanthopterin, together with a detailed knowledge of the reflector superstructures, provide a rationalization of the reflective optics of the crustacean eye.

isoxanthopterin | eyes | crystal | reflection | mirror

Many spectacular optical phenomena exhibited by animals are produced by the reflection of light from organic (1, 2) or inorganic crystals (3). A fascinating manifestation of such biological reflectors is in vision (4). Certain animals use mirrors instead of lenses to form images. This strategy is particularly useful in aquatic environments, where the reduced refractive index contrast in water makes conventional lens-based eyes less effective. Mirror-containing eyes are often extremely efficient light-collectors and are found in nocturnal animals or animals inhabiting dim-light environments (5). Two types of image-forming reflective eyes are known: concave mirrored eyes and reflection superposition compound eyes. The former is epitomized by the scallop eyes, which produce well-resolved images by reflecting light from a concave, guanine crystal-based mirror located at the back of the eye onto the retina above it (6, 7). Similar eyes are also found in deep-sea fish (8, 9) and crustaceans (10). The latter type of eye, the reflecting superposition compound eye, is the focus of this study.

The reflecting superposition compound eye is found in decapod crustaceans (11, 12). Each compound eye is composed of thousands of square-faceted eye units called ommatidia. An image is formed when light is reflected from the top of each ommatidium onto the retina, comprising the photosensitive “rhabdoms” (Fig. 1) (13). In contrast to apposition compound eyes, where each ommatidium acts as an isolated optical unit, in the reflecting compound eye, light collected from many ommatidia is reflected across

a “clear zone” and is brought to focus as a single upright image on the convex-shaped retina (Fig. 1C). The superposition of light rays from multiple eye units effectively increases the pupil size, making this an extremely light-sensitive device, which is well adapted for dim-light habitats. Reflection of light from the ommatidia operates in two regimes: Grazing incidence light, with angles of incidence less than $\sim 15^\circ$ relative to the ommatidial axis, is internally reflected from the walls of the ommatidia (12, 14). This is made possible by the refractive index contrast in the upper parts of the ommatidium ($n = 1.41$ inside and $n = 1.34$ outside the ommatidium in the region of the “crystalline cone”; Fig. 1C) (12, 14). At larger angles of incidence, this index contrast is too small to lead to significant reflectivity. Light impinging at higher angles is thus reflected by a high-refractive-index square mirror (“distal mirror”; Fig. 1C), which surrounds each eye unit. Vogt postulated (11, 15) that the distal mirror of crustacean eyes is a multilayer reflector, but using conventional electron-microscopy methods, he was unable to retain the *in vivo* structure. The square arrangement of the mirror acts like a corner reflector, whereby light incident from oblique planes will be reflected from two orthogonal mirrors by a total of 180° and will return parallel to its original direction and be brought to focus on the retina (12, 14). A second reflector, the tapetum, lies

Significance

Some aquatic animals use reflectors in their eyes either to form images or to increase photon capture. Guanine is the most widespread molecular component of these reflectors. Here, we show that crystals of isoxanthopterin, a pteridine analog of guanine, form both the image-forming “distal” mirror and the intensity-enhancing tapetum reflector in the compound eyes of some decapod crustaceans. The crystal structure of isoxanthopterin was determined, providing an explanation for why these crystals are so well suited for efficient reflection. Pteridines were previously known only as pigments, and our discovery raises the question of which other organic molecules may be used to form crystals with superior reflective properties either in organisms or in artificial optical devices.

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To ascertain if the tapetum material of *C. quadricarinatus* and *M. rosenbergii* is crystalline, we performed in situ X-ray diffraction (XRD) experiments using a microspot beam at the BESSY synchrotron. XRD patterns obtained from the region of the rhabdoms (green spot, Fig. 3A) exhibited no Bragg scattering, but did display a weak anisotropic scattering pattern at small angles, consistent with the orthogonal ordering of the microvilli in the rhabdoms (25). In contrast, highly intense powder XRD patterns were obtained from the tapetum in regions surrounding the bottom parts and immediately below the retina/rhabdom layer (red spot, Fig. 3A). This confirmed that the tapetum contained crystalline material and that the crystals had no preferred orientation.

To determine the ultrastructural arrangement of the tapetum at the nanoscale to microscale level, we performed cryogenic scanning electron microscopy (cryo-SEM) imaging on high-pressure-frozen, freeze-fractured eyes of *C. quadricarinatus* and *M. rosenbergii*. Cryo-SEM images of longitudinal eye-sections showed membrane-bound extensions of the tapetal cells lining the lower parts of each rhabdom (Fig. 3C), matching the light-microscopy observations of the birefringent tapetum material (Fig. S2). These elongated protrusions were typically 5- μm wide and were densely packed with nanoparticles with an average diameter of 390 nm (± 33 nm, $n = 44$) (Fig. 3D and E). The outer parts of these hollow nanoparticles were composed of small segments, which were arranged in an “onion-skin” lamellae with an average shell thickness of 80 nm (± 11.5 nm, $n = 35$) (Fig. 2E). The same nanoparticles were also observed in *M. rosenbergii* (Fig. S4). These nanoparticles appeared to be the principal component of the tapetal cells, and based on spatially resolved XRD and Raman measurements (Fig. 3A and B), we concluded that they were composed of crystalline isoxanthopterin. The tapetum nanoparticles were comparable in dimensions to the wavelengths of visible light and will therefore scatter light by Mie scattering. We calculated the ratio of back- to forward-scattered light using Mie theory for a 390-nm-diameter nanoparticle, with a shell thickness of 80 nm, assuming an effective refractive index of $n = 1.78$ (Fig. S5 and SI Materials

Fig. 4. Ultrastructure and chemical properties of the multilayer mirror in *C. quadricarinatus* eyes. (A) Cryo-SEM image of the boundary between two ommatidia in the upper part of the eye viewed along the eye axis. (A, *Inset*) Lower-magnification cryo-SEM image of the transverse section through four ommatidia. (B) Cryo-SEM image of the multilayer lining of an ommatidium from a longitudinal section through the eye. In A and B, crystals are pseudocolored blue, and holes (produced by crystals being lost during freeze-fracture) are colored green. (C) UV-Vis absorption (*Upper*) and emission (*Lower*) spectra of the reflecting material extracted from the multilayer mirror (solid blue trace) and the tapetum (dotted blue trace) alongside the spectra obtained from isoxanthopterin crystals (black trace).

and Methods). We found two peaks in the back-scattering of 10% and 25% at wavelengths of 470 and 700 nm, respectively.

Cryo-SEM images in the area of the distal mirror in the upper part of the eye (Figs. 1C and 2A–C) revealed that each ommatidium was bound by a multilayer comprising three or four rows of solid objects interspersed with cytoplasm (Fig. 4A and B). Polarized light microscopy strongly suggested that the objects were crystalline (Fig. 2C). Each row of the multilayer contained numerous crystals housed inside a membrane-delimited compartment surrounding the entire perimeter of an ommatidium. In the innermost rows, the crystals were plate-like (typically 200 nm wide and 40 nm thick), although there was no well-defined or conserved crystal morphology. The crystals were not closely packed, and we estimated that in a single row, there was ~50% coverage of crystals. A dense packing of larger, ~1-μm absorbing pigment granules (27) occupied the central region between two ommatidia. These granules had a similar texture to the absorbing pigment granules found below the tapetum (Fig. 3E). The reflecting material extracted from both the multilayer and tapetum reflectors was highly fluorescent and exhibited excitation and emission peaks in the UV-Vis spectra characteristic of isoxanthopterin (19) (Fig. 4C). The simulated reflectance spectra derived from the crystal thicknesses and spacings measured by cryo-SEM (Fig. S6) showed that the mirror exhibited broadband reflectivity and was 50–100% reflective for light impinging at angles of incidence between 0° and 30° (with respect to the ommatidial axis), but was only 20% reflective at normal incidence.

XRD patterns obtained from the crystals in *C. quadricarinatus* and *M. rosenbergii* tapeta exhibited sharp reflections indicative of well-ordered crystals (Fig. 5A). These experimental data formed the basis for verifying the crystal structure of isoxanthopterin predicted independently from first-principles calculations. To determine the crystal structure of isoxanthopterin, we utilized a similar approach to that reported for the crystal structure prediction of biogenic guanine (28). Both guanine and isoxanthopterin are planar-conjugated molecules with numerous H-bond donor and acceptor

sites. Our calculations on isoxanthopterin relied on the assumption that the crystal structure is dominated by intermolecular H-bonding and close-packing interactions. This assumption was supported by the presence of a very intense reflection with a d -spacing of ~ 3.2 Å in the XRD patterns, suggestive of a close-packed intermolecular layer arrangement. In the guanine XRD pattern, the 3.2-Å reflection corresponds to the distance between H-bonded layers of guanine molecules.

The first step in our crystal structure prediction was to construct a probable H-bonding motif for the isoxanthopterin molecules. The planar molecule (Fig. 5*B*, *Left*) contained four NH and one CH proton donor and six lone-pair electron lobes. We first formed a cyclic centrosymmetric dimer containing four H-bonds as the basic building block of the crystal structure (Fig. 5*B*, brown circle). We then took advantage of the H-bonding complementarity between the two adjacent sides of the isoxanthopterin molecule (Fig. 5*B*, *Right*) to form a 2D H-bonding array by twofold screw (2_1) and c-glide symmetry (Fig. 5*B*). By such means, we generated a unique layer of H-bonded molecules of symmetry $2_1/c$. After generating different interlayer packing motifs via monoclinic and orthorhombic symmetries, we optimized these crystal structures using dispersion-inclusive density functional theory (DFT) (*SI Materials and Methods*) and compared the calculated and observed XRD patterns.

The best fit between the observed and computed diffraction data was found for an orthorhombic structure, where the interlayer packing motif was generated from the planar H-bonded

Fig. 5. Crystal structure of isoxanthopterin. (A) X-ray powder diffraction profiles; calculated (Calc.) isoxanthopterin structure (black trace) alongside experimental data obtained from the tapetum of *M. rosenbergii* and *C. quadricarinatus*. The intensity of the strongest reflection (200) was truncated to make all of the reflections more visible. Additional details are in [SI Materials and Methods](#) and [Fig. S8](#). (B) Molecular structure of isoxanthopterin and the crystal H-bonded layer. The brown contour indicates a centrosymmetric H-bonded dimer. (C) Calculated crystal structure of isoxanthopterin.

layer by an additional *b*-glide at $x = 1/4$ (parallel to the *bc* layer), yielding a new molecular layer at $x = 1/2$, in the *Pbca* space group (Fig. S7). DFT-based geometry optimization of this structure yielded a higher-symmetry structure, possessing the rarely observed space group *Cmce*, which corresponds to a perfectly planar H-bonded layer of isoxanthopterin molecules (Fig. 5C and *SI Materials and Methods*). The XRD conditions for the *Cmce* space group fit well with the unusual experimentally observed XRD pattern of the biogenic samples, insofar as there was a region in reciprocal space ($0.1 < d^* < 0.2 \text{ \AA}^{-1}$) lacking any reflections (Fig. 5A and Fig. S8). Agreement between the computed and observed XRD data were excellent. A first-principles calculation of the refractive index of isoxanthopterin crystals, based on density functional perturbation theory, yielded a high value of $n = 1.96$ for light incident along the *a* axis (*SI Materials and Methods*).

Discussion

In situ chemical and structural analyses of the eyes of *C. quadricarinatus* and *M. rosenbergii* showed unequivocally that the tapetum reflector is constructed from crystalline isoxanthopterin. UV-Vis analysis of the reflecting material extracted from the upper distal reflector showed that this is also composed of isoxanthopterin, and polarized light microscopy showed that the material is birefringent, and thus most probably crystalline. These results are consistent with earlier chemical analyses that isoxanthopterin is present in crustacean eyes (19). The crystal structure of isoxanthopterin is characterized by planar arrays of H-bonded isoxanthopterin molecules, reminiscent of the crystal structure of biogenic guanine. The extremely high calculated refractive index in the H-bonded plane, $n = 1.96$, makes isoxanthopterin an ideal reflecting material. In contrast to inorganic biominerals, very few types of functional organic crystals have been identified in animals. Crystalline guanine is found widely in the reflective systems of animals (2), and the purines xanthine and uric acid have been identified as the reflective materials in insect's eyes (29) and cuticles (30), respectively. Pirie reported that the eye-shine of lemurs was produced by crystals of riboflavin (31). A key question is how many other reflecting organic crystals remain to be discovered in nature.

Isoxanthopterin belongs to the pteridine family of molecules—heterocycles composed of fused pyrimidine and pyrazine rings. Historically, it was thought that pteridines functioned exclusively as light-absorbing pigments, intrinsically associated with yellow/red xanthophore pigment cells (32). Pteridine molecules are indeed used as pigments in a wide variety of animals and especially in insects, but not in a crystalline form. Here, we present conclusive evidence of a crystalline pteridine in nature challenging the conventional functional distinction between the light-absorbing pteridines and light-reflecting (crystalline) purines. The discovery of crystalline isoxanthopterin in two distantly related species of decapod [*C. quadricarinatus* (Astacidea) and *M. rosenbergii* (Caridea)] (33) suggests that this material is widespread in the decapod crustaceans. There is indirect evidence that other animal tissues, including the avian iris and the iridophores of amphibians, contain reflecting pteridine granules (32).

High-resolution cryo-SEM images of the eyes of *C. quadricarinatus* and *M. rosenbergii* showed that the isoxanthopterin crystals of the tapetum are located inside ~400-nm nanoparticles comprising concentric crystalline lamellae. Other ultrastructural studies found similar 400-nm “reflecting granules” in both the tapeta (22, 27) and epidermis (34) of decapod crustaceans, and Matsumoto (35) described pteridine-containing granules with a “concentric lamellar” structure in fish. It remains to be seen whether this texture, which seems to be a characteristic feature of pteridine granules in nature, is always indicative of the presence of crystalline particles (Fig. 3E). The tapetum nanoparticles we observed are comparable in dimensions to the wavelengths of visible light and will therefore scatter light by Mie scattering. The relatively high scattering efficiency of these particles is due to the high refractive index of isoxanthopterin

crystals. Both *C. quadricarinatus* and *M. rosenbergii* live in turbid, shallow freshwater, and ~10–12% of the yellow-green light which penetrates this environment will be back-scattered by an individual nanoparticle (Fig. S5) (36). The scattering nanoparticles are densely packed inside ~5-μm-thick tapetal cell extensions that closely encapsulate the base of the rhabdom (Fig. 3C and D). Photons impinging on the tapetum will thus undergo multiple scattering events from numerous individual particles in these extensions resulting in a significant amount of light being back-scattered to the rhabdoms.

The principal function of the tapetum is to direct photons which are not initially absorbed by the retina back to the retina, providing a second opportunity for photon capture. The ultrastructural arrangement of the tapetum, which forms a reflective sheath around the base of each rhabdom, also suggests a second function, namely, to prevent optical cross-talk between rhabdoms (23, 24). In the dark-adapted reflection superposition eye, light is collected from a wide field of view. This could result in loss of resolution, since light incident from the peripheral field of view with steep angles of incidence might not remain within a single rhabdom. The protective sheath of the tapetum prevents such light from being transmitted between adjacent rhabdoms. The tapetum thus serves to simultaneously increase the sensitivity and preserve the resolution of the eye. The fact that the tapetum sheath only partially encapsulates (22, 23) the rhabdom (Fig. 3C) may also be functional, since a fully encapsulated rhabdom may prevent sufficient light from reaching the target, lowering the eyes' sensitivity (23). In light-adapted eyes, proximal absorbing pigments, housed within the retinal cells, migrate from below the basal membrane and completely envelope the tapetum sheath (22, 37), preventing the rhabdoms from being damaged by overexposure to photons (37).

Diffusely scattering tapeta have also been observed in fish (38), crocodiles, and the opossum (39) and are constructed from a range of crystalline and noncrystalline materials (40). The light-scattering submicrometer-sized guanine crystals found in the eyes of the elephant nose fish (41) increase the amount of light absorbed by colocalized melanin pigment granules, enabling simultaneous activation of both rod and cone cells in the same ambient light conditions. Wilts et al. (42) reported that the bright colors of *Pieridae* butterfly wings are produced by Mie-scattering from ellipsoid granules of pteridines with an extremely high refractive index. The high refractive index is due to a high-density of pteridines, which they assume must approach a “close-packing” assembly in the granules.

The multilayer reflector in the upper part of *C. quadricarinatus* and *M. rosenbergii* eyes is formed from three or four rows of sparsely populated plate-like bodies, which we assume are nanocrystals of isoxanthopterin. Since grazing incidence light ($< \sim 15^\circ$) is reflected from the walls of the ommatidia by total internal reflection (12, 14), the role of the distal mirror is to reflect light entering at higher incidence angles, effectively increasing the pupil size and sensitivity of the dark-adapted eye. Reflectivity simulations assuming their crystalline nature show that the mirror is 50–70% reflective for light entering the ommatida at 15–30°, but drops to ~20% for light impinging at normal incidence due to the low packing-density of the crystals and the small number of rows in the multilayer. The pigments lying between ommatidia will absorb any light not reflected by the mirror at such angles. We note that our calculation of broadband reflection in the *C. quadricarinatus* mirror is inconsistent with Land's observations of the distal mirror in an oceanic shrimp. Land (12) observed that it had a “green-specular” appearance when viewed at near normal incidence. This difference might be accounted for by the different light conditions in the habitats of these two species.

The poor performance of the mirror at very high angles of incidence may actually be functional in the dark-adapted eye. Light entering the eye at extreme angles of incidence will be particularly affected by spherical aberrations and, as such, will contribute disproportionately to image blurring (24). A mirror

that becomes progressively less efficient at very high angles will contribute relatively less light from the peripheral field of view, thus mitigating against image blurring—a process known as apodization in optics. The poor performance of the mirror at high angles would also prevent light undergoing multiple reflections in a single ommatidium, which would lead to light being scattered stochastically across the clear zone onto different parts of the retina. Much like the tapetum, then, the ultrastructural organization of the distal mirror seems to be tuned to optimize the balance between increasing the sensitivity of the eye on the one hand and maintaining resolution on the other (23, 24).

Conclusions

Surprisingly little is known about functional organic crystals in nature, and few of these materials have been identified. Our discovery that crystalline isoxanthopterin is the highly reflective optical material in the eyes of decapod crustaceans paves the way for further research in the emerging field of “organic biomineralization” and suggests that other such materials, together with their optical properties, may remain to be discovered. Our results also call for pteridines found more widely in the animal kingdom to be investigated and their possible role as biological reflectors to be reexamined. Eyes based on reflective optics are often extremely light-sensitive and are found in animals inhabiting environments where light is at a premium. However, in such wide-aperture eyes, there is often a trade-off between sensitivity (e.g., increasing the field of view) and resolution (e.g., due to spherical aberration). The ultrastructural organization of the reflectors in the dark-adapted eyes of decapod crustaceans appear to be tuned to optimize the balance between increasing light sensitivity and maintaining image resolution.

Materials and Methods

Specimen Preparation and Experimental Methods. Eyestalks of *M. rosenbergii* and *C. quadricarinatus* were extracted in the dark-adapted state. For cryo-SEM, SEM, light microscopy, in situ XRD, and Raman spectroscopy, the eyes were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer before vibratome sectioning. For the X-ray microCT experiments, whole, fixed eyes were measured. For the UV-Vis experiments, crystals were extracted from the relevant eye locations after fixing in 70% ethanol. Further details are in *SI Materials and Methods*.

DFT, Crystallographic Structure, and Simulated Diffraction Patterns. The electronic structure, total energy, refractive index, and geometry of the material structures were calculated by solving the Kohn–Sham equations of DFT within the generalized gradient approximation, by using the Perdew–Burke–Ernzerhof exchange–correlation functional, augmented by Tkatchenko–Scheffler pairwise dispersive interactions. The process of building the crystal structure from a single molecule up to a 3D entity was performed by using the Materials Studio visualization software (Version 6.1). Further details are in *SI Materials and Methods*.

Calculation of Mie-Scattering and Reflectivity Simulations. The Mie-scattering efficiency of the tapetum nanoparticles was calculated by using a multilayer Mie solver. The simulated reflectivity spectrum of the distal mirror was simulated by using a Monte Carlo transfer matrix calculation. Further details are in *SI Materials and Methods*.

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