First investigation of the collagen *D*-band ultrastructure in fossilized vertebrate integument

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The ultrastructure of dermal fibres of a 200 Myr thunniform ichthyosaur, *Ichthyosaurus*, specifically the 67 nm axial repeat *D*-banding of the fibrils, which characterizes collagen, is presented for the first time by means of scanning electron microscopy (SEM) analysis. The fragment of material investigated is part of previously described fossilized skin comprising an architecture of layers of oppositely oriented fibre bundles. The wider implication, as indicated by the extraordinary quality of preservation, is the robustness of the collagen molecule at the ultrastructural level, which presumably contributed to its survival during the initial processes of decomposition prior to mineralization. Investigation of the elemental composition of the sample by SEM–energy dispersive X-ray spectroscopy indicates that calcite and phosphate played important roles in the rapid mineralization and fine replication of the collagen fibres and fibrils. The exceedingly small sample used in the investigation and high level of information achieved indicate the potential for minimal damage to prized museum specimens; for example, ultrastructural investigations by SEM may be used to help resolve highly contentious questions, for example, ‘protofeathers’ in the Chinese dinosaurs.

Keywords: ichthyosaur; fibres; collagen *D*-band ultrastructure

1. INTRODUCTION

Jurassic ichthyosaurs are considered the fastest sustained swimmers of the Mesozoic seas based on calculations of a deep, streamlined body shape and high-aspect ratio tail (Massare 1988). They were included along with the extant dolphins, lamnid sharks and tuna in the small exclusive group of fast thunniform swimmers (Lighthill 1975; McGowan 1992). Subsequent studies revealed a complex architecture of dermal fibres, arranged in multiple layers (Lingham-Soliar 1999, 2001; Lingham-Soliar & Plodowski 2007), ostensibly similar to high tensile collagenous dermal fibre structures found in tuna, dolphins and sharks in which they are considered to play a significant role in high-speed swimming hydrodynamics (Wainwright *et al.* 1978; Hebrank & Hebrank 1986; Pabst 1996; Lingham-Soliar 2005a,b). However, owing to the rarity of the soft tissue material in ichthyosaurs, it was impractical to confirm by chemistry or ultrastructure the molecular nature of even a single dermal fibre in ichthyosaurs to date.

Collagen is distinct from other proteins in that the molecule comprises three polypeptide chains (α -chains), which form a unique triple helical structure. For the three chains to wind into a triple helix, they must have the smallest amino acid, glycine, at every third residue along each chain. Each of the three chains, therefore, has the repeating structure Gly–Xaa–Yaa, in which Xaa and Yaa can be any amino acid but frequently the amino acids proline and hydroxyproline. This triple helical structure of

the amino acids plays a major role in the molecular conformation of collagen (Kadler *et al.* 1996).

Determining the nature of the ichthyosaur fibres by chemical analysis of their rare and scanty fossilized soft tissue may be reasonably ruled out because ‘molecular and chemical analytical methods are expensive and destructive to rare fossil material’ (Schweitzer 2003, p. 1). However, the molecules of collagen types I, II, III, V and XI are packed into *D*-periodic cross-striated fibrils (*D*-bands), typically *D*-67 nm, the characteristic axial periodicity of collagen, sometimes referred to as the quarter-stagger structure because adjacent molecules are transposed just under one-quarter of their length in the axial direction (Smith 1968).

We conduct the first investigation by scanning electron microscopy (SEM) of the ultrastructure of the dermal fibrils of an ichthyosaur, *Ichthyosaurus* GLAHM V1180a (figure 1a). It allows a rare chance to attempt to determine whether or not there is evidence for the fibril axial band periodicity that characterizes collagen. We also investigate the elemental composition of our material in an effort to shed light on conditions surrounding the preservation of the volatile fibre structures in GLAHM V1180a (Lingham-Soliar 1999).

2. GEOLOGY

Ichthyosaurus GLAHM V1180a came from the 200-Myr-old Lower Lias beds of the Severn Valley in Gloucestershire, England (Delair 1966; Lingham-Soliar 1999). The Lias sediments were deposited in three principal marine basins, the Cleveland, Midlands-Severn and Wessex Basins (Floyd *et al.* 2003), separated by shallow shelf seas or islands (Floyd *et al.* 2003). The Lias Group of the Midlands-Severn Basin forms the Vale of Gloucester

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Figure 1. Specimen of *Ichthyosaurus* GLAHM V1180a. (a) Fibre bundles preserved on the lower jaw. (b) Well-preserved fibre bundles demarcated by circle in (a). (c) Part of a small fragment, investigated here, that had chipped of the edge of the preserved soft tissue. The whitened area, predominantly calcite (see electronic supplementary material, table 1), represents the poorest preserved soft tissue of the specimen with the arrows pointing to traces of fibre bundles (buff-coloured). Scale bars, (a) 2 cm and (b,c) 2.5 mm.

and Vale of Berkeley at an elevation of 20–60 m above ordnance datum between the River Severn floodplain to the west and the Cotswold limestone escarpment to the east. Here, there is a complete stratigraphic succession of dark blue-grey to black massive and fissile mudstones with marls, argillaceous limestone beds and carbonate nodular horizons (Floyd *et al.* 2003; Simms *et al.* 2004).

(a) *Palaeontology: fauna*

Unfortunately, *Ichthyosaurus* GLAHM V1180a lacks precise locality data other than the above-mentioned one. However, the Lower Lias of the Severn Valley is exposed at two well-known points that show a rich diverse fauna, Blockley Station Quarry, grid ref. SP180370 (Simms *et al.* 2004) and Robin's Wood Hill Quarry, grid ref. SO835148 (Simms *et al.* 2004). The fauna includes ammonites, abundant bivalve molluscs, abundant brachiopods, gastropods (snails), asteroids (starfish), echinoids (sea urchins) and crinoids (sea lilies). Blockley Station Quarry (Simms *et al.* 2004), in particular, shows a diverse fish fauna that includes both holostean and teleostean bony fishes, and sharks and rays. Belemnites were also abundant (both sites) and along with fishes were probably the main food of *Ichthyosaurus* (Lingham-Soliar 2003b).

3. MATERIAL AND METHODS

Ichthyosaurus GLAHM V1180a (Glasgow Hunterian Museum; figure 1a) is represented by a partially preserved head that lay within a mudstone nodule (Lingham-Soliar 1999). The preserved soft tissue overlies the dentary and surangular and includes fibre bundles oppositely oriented in at least two layers of the integument (see Lingham-Soliar (1999) for details). However, the small fragment of approximately 1 cm², which had chipped off the edge of the fossilized soft tissue, appears to have contained the least well-preserved fibre bundles in the specimen¹ (figure 1c cf. b).

Previous investigations of the dermal fibre architecture of ichthyosaurs (Lingham-Soliar 1999, 2001; Lingham-Soliar & Plodowski 2007), as with the extant thunniform groups (Hebrank & Hebrank 1986; Pabst 1996; Lingham-Soliar 2005a,b), involved optical examinations of fibre bundles (ranging in diameter in those studies from approx. 50–1000 µm) rather than fibres. It is clear that references to

'fibre(s)' in such cases were simply ones of convenience (Lingham-Soliar 2003a, 2008). Ultrastructural details of true fibres (usually approx. 4–20 µm in diameter), were usually not evident in, nor relevant to, those studies. Thus, in the present study, the distinction is necessary.

The chip was examined optically by means of a Zeiss Axiostar binocular microscope. An area approximately 8×2.5 mm showed whitened areas and traces of a buff-coloured material (figure 1c), which suggested replacement of organic material by calcium phosphate (Allison 1988). For the SEM study, the latest Carl Zeiss field-emission scanning electron microscopy (FE-SEM) Ultra 55 was used. Pretreatment, for example, sputter-coating with metal or carbon was avoided so as not to alter the size of structures in the sample, vital if measurements were to be made. This was weighed against a potential loss of some quality of image although efforts were made to minimize this. Charge build-up, for example, was minimized by viewing the sample at an accelerating voltage of 1 kV and by applying two-sided carbon tape immediately around the area being examined. A number of technical adjustments were necessitated with respect to using the FE-SEM Ultra 55 under these constraints, which most experienced SEM microscopists will be familiar with and consequently will not be discussed further here.

SEM–energy dispersive X-ray spectroscopy (EDX) was used for analysis of the elemental composition of specimen GLAHM V1180a. It was performed in 12 locations, each approximately 1.5 mm², across two broad bands (six locations per band; see figure in the electronic supplementary material), which covered the major part of the sample (the same sample used for the SEM fibre investigation; electronic supplementary material, table 1).

4. RESULTS

SEM investigation along narrow buff-coloured tracts (remains of the fibre bundles, see arrows in figure 1c) in our specimen revealed fibres. Well-preserved fibre bundles were shown in earlier optical studies (Lingham-Soliar 1999; figure 1b, inset). We explain poorer preserved fibre bundles here by the very small mineralized area under study, location nearer the edge of the fossilized soft tissue and to degradation. Despite this, two relatively well-preserved fibres (figure 2a,b) of approximately 20–30 µm

Figure 2. *Ichthyosaurus* (GLAHM V1180a). (a) Part of a 30 µm long fibre packed with fibrils in remarkable preservation. Inset shows *D*-bands or 67 nm repeat axial bands. (b) The sheared tip of another fibre; numerous well-preserved fibrils are evident. Inset (i): detail from circle showing well-preserved *D*-bands. Inset (ii): cultured heart muscle showing *D*-bands (courtesy of Dr Martin Stolz 2007). Scale bar, (a,b) 2 µm, insets, 1 µm.

Figure 3. *Ichthyosaurus* (GLAHM V1180a). (a) Part of the fibre in figure 2b further along towards its mid-length; the fibrils are disorganized and cross over in places, presumably as a consequence of severe breakdown of the glue that binds them (see text). Inset shows *D*-bands preserved in fine detail; white semicircle shows possible group of ?sub-fibrils (see text). (b) Fibre sheared across shows the rounded cross section. Fibrils can be seen on the fibre surface and densely packed in cross section. Scale bars, (a) 2 µm, inset, 0.5 µm, (b) 2 µm.

length, with rounded cross sections (figure 3b) of approximately 4 µm in diameter were identified (4.69 K $\times$ magnification). At high resolution (30 K $\times$), numerous component fibrils were observed (figure 2a, inset and b, inset i) and an axial band periodicity was evident in many. The bands ($n=329$) were measured along each fibril ($n=38$; mean = 66.12 nm; s.d. = 4.35 ($n=329$)) using UTHSCSA IMAGE TOOL v. 3 software. The mean period of 66.12 nm is consistent with *D*-band measurements in recent collagen (Smith 1968; Kadler *et al.* 1996). Figure 3a, inset, shows striking detail of the *D*-bands. A crossed fibril pattern (figure 3a, inset) is presumably a consequence of twisting of the fibres during degradation and probably unconnected with a recent model of twisted rope-like substructure of the fibrils (Bozec *et al.* 2007). It is difficult to determine conclusively the width of the fibrils, since it is unclear whether the individual strands (figure 3a, inset) are complete fibrils or whether they are sub-fibrils comprising a fibril (figure 3a, inset, semicircled). It has, however, been shown that

fibrils may consist of molecules up to 10 nm in diameter, which may assemble to form into fibrils of greater diameter from 50 to several hundred nanometres (Bozec *et al.* 2007). In the light of this, we suggest that the smallest units observed in our material are fibrils (approx. 60 nm in diameter) and that they are very similar to those seen, for example, in fresh collagen (Reichlin *et al.* 2005; Stolz *et al.* 2007; figure 2b, inset ii). On the other hand, the *D*-band ultrastructure that characterizes collagen (types mentioned above), which is independent of fibril diameter (Bozec *et al.* 2007), is positively identified in GLAHM V1180a.

Although fibre preservation is restricted to the lower jaw in GLAHM V1180a, identical fibres were found in the dermis of the closely related thunniform ichthyosaur, SMF 457, where they were more widely preserved in several parts of the body as well as in the dorsal and caudal fins (Lingham-Soliar 2001; Lingham-Soliar & Plodowski 2007). It, therefore, seems a reasonable speculation that they comprised much of the dermis in *Ichthyosaurus*.

SEM–EDX shows that calcite (CaCO_3), pyrite (FeS_2), a clay (containing a number of elements (Al, Mg, K, Fe) $2\text{Si}_2\text{O}_5(\text{OH})_4$) predominate in the organically transformed material. There was partial replacement of the organic content with iron pyrite. Calcite formed the nodule/concretion. Relatively small amounts of phosphate were also present across the two broad bands. The matrix was apparently a clay (probably mostly kaolinite) with a calcite cement (electronic supplementary material, table 1). The two broad bands, representative of most of the sample, were considered more informative than an elemental map, which was dark and for most elements, except for a few dominant ones, did not establish any reliable impression of the areas they were present in nor their gross quantities.

5. DISCUSSION

Although we were unable to investigate the nature of the fibres in *Ichthyosaurus* by chemistry (see §1), our findings represent the first evidence that collagen ultrastructure is preserved in the ichthyosaur skin, i.e. the first demonstration of the 67 nm axial repeat *D*-banding of the fibrils, which characterizes collagen, in fossilized vertebrate integument (soft tissue) *per se*. Some of the problems associated with the preservation of such fine structures over long periods of time are considered below.

(a) General problems associated with the preservation of soft tissue in fossils

Biomineralized tissues (shells, bones and teeth) have the highest preservation potential. During the process of fossilization, inorganic mineral components are replaced and organic components are commonly considered to be destroyed or rendered uninformative by the changes accumulated during geological time (Curry 1990). In a recent study, however, it was proposed that not all organic components are destroyed (Schweitzer *et al.* 2007) and that the apparent survival of original protein in *Tyrannosaurus rex* bone allowed authentic sequencing that demonstrated a phylogenetic closeness with modern birds (Asara *et al.* 2007; Schweitzer *et al.* 2007). By contrast, Buckley *et al.* (2008) found that not all collagen had been authentically preserved and that the collagen sequences in *T. rex* only allowed a comparison with amphibians rather than birds. They (Buckley *et al.* 2008) concluded that the unusual fragmented nature of the reported *T. rex* sequence (Asara *et al.* 2007) does not make it amenable to standard model-based phylogenetic analysis.

In this study, however, the much rarer fossilized integumental dermal fibres were investigated, which obviously lacked any relationship with the mineral phases of bone and the concomitant implications with respect to the preservation of original protein discussed above. Rather, in all probability, all the dermal fibres in GLAHM V1180a had been diagenetically mineralized (see below).

‘Soft-bodied’ fossils in contrast to biomineralized tissue are a relatively rare occurrence, highlighted in the concept of Konservat–Lagerstätten (Seilacher 1970). In particular, the skin as the outer covering of most animals is most exposed to degradation and may at first glance be considered, by virtue of its exposure and relatively low thickness compared with other structures in the body, the least likely to leave traces in the fossil

record. Yet, besides bone, it is the integument alone that has been fossilized in some of the best preserved Jurassic ichthyosaurs from the Posidonia Shale of southern Germany. The problem is amplified with respect to preservation of collagen in the integument as opposed to bone. In fossil bone, survival of original protein may depend on a synergistic relationship between the apatite and organic phases of bone (Schweitzer *et al.* 2007). The rate of degradation of collagen in bone is slow because the mineral ‘locks’ the components of the matrix together, preventing helical expansion, which is a prerequisite of fibril collapse (Buckley *et al.* 2008). Added to which, in the absence of such a matrix, as in the integument, the breakdown of a ‘glue’ that binds the collagen fibrils in a parallel fashion (Lewis & Johnson 2001; Lingham-Soliar 2003a) may be expected to hasten their degradation and disorganization (figure 3a). Hence, fossilization of the two well-preserved integumental fibres in GLAHM V1180 would have required rather extraordinary circumstances and raises some key questions with respect to their taphonomy: how was the ichthyosaur integument, in particular the fibres, preserved? What was the depositional environment of their preservation? How rapidly did their fossilization take place? An attempt is made to answer these questions below.

(b) Taphonomic and diagenetic conditions leading to the extraordinary preservation of collagen ultrastructure in GLAHM V1180a

The depositional environment of a carcass may affect organic preservation in many ways (Allison 1988). One proposal for the excellent preservation of Posidonia Shale ichthyosaurs is rapid mineral transformation of organic matter, perhaps within hours of the animal’s death (Martill 1993). The high fidelity of the *D*-banding structure in *Ichthyosaurus* GLAHM V1180a suggests that biodegradation and diagenetic mineralization in the Lower Lias of Gloucestershire were also rapid. However, there is little published data on the preservation of GLAHM V1180a. Thus, the SEM–EDX results are particularly interesting (electronic supplementary material, table 1). They show that the most commonly occurring diagenetic minerals in our sample are those associated with exceptional preservation of soft tissue, i.e. pyrite, carbonates (such as calcite), silica and phosphate (Allison 1988). X-ray diffraction analysis of the clays and mudrocks of the Lower Lias of Gloucestershire also showed pyrite, despite significant loss due to weathering, together with high calcite concentrations (Floyd *et al.* 2003), although no record of phosphate was given. The proportion of pyrite is a function of the depositional and diagenetic conditions—a reducing environment and organic matter (see above) are required to form pyrite. This reducing environment would also have favoured the formation of early diagenetic mineral precipitates such as phosphate. The presence of phosphate in our sample is considered important for two reasons: (i) that mineralization may have been exceedingly rapid, predating pyrite and carbonates and (ii) because it is usually associated with exceptional preservation of volatile structures such as collagen fibres (Allison 1988; Briggs & Kear 1993). Admittedly, our detection of phosphate is fairly low but

it should be considered in the context of the small amount of fibres present in our sample. In summation, SEM–EDX (electronic supplementary material, table 1) indicates that preservation of the dermal collagen ultrastructure of *Ichthyosaurus* GLAHM V1180a is probably a consequence of replication by an early calcite that was probably associated with phosphate, enabling high-fidelity three-dimensional reproduction. Following mineralization, the calcareous nodule that GLAHM V1180a was encased in would have helped preserve the three-dimensional fibre bundles during compaction.

There were two probable sources for the phosphate in GLAHM V1180a: (i) from within the animal very early in the diagenetic sequence, for example, during microbial degradation of the underlying tissue, as a consequence of which phosphate would have been precipitated (Allison 1988; Briggs & Kear 1993; Dornbos *et al.* 2005) and (ii) from the external environment of the Lower Lias of Gloucestershire that was rich in animal life (Simms *et al.* 2004).

The high level of preservation of the ultrastructure of collagen from the most exposed soft tissue of a 200 Myr ichthyosaur (Lingham-Soliar 1999) must be considered extraordinary. The collagen fibril ultrastructure (figure 3a, inset) ranks in definition with that from modern investigations of fresh collagen (Reichlin *et al.* 2005; Stolz *et al.* 2007). Results on the exceptional preservation of the ichthyosaur integument have interesting implications for the preservation of soft tissue in a variety of fossil animals including the dinosaurs. Although it has been reasonably proposed that SEM studies could be damaging to valuable soft tissue fossils (Allison 1988; Lingham-Soliar *et al.* 2007), the present study shows that the quantity of material required for SEM tests may be much smaller than previously thought. Beyond this obvious advantage with respect to prized museum specimens, the identification of collagen ultrastructure, i.e. of a unique and ubiquitous molecule in the animal kingdom, tremendous opportunities for future ultrastructural investigations of fossilized integumental structures are envisaged. For instance, SEM provides a tool for resolving the highly contentious evolutionary problem of whether integumental structures in Chinese dinosaurs relate to a key evolutionary intermediate structure, a protofeather (presumed to be keratin; Chen *et al.* 1998; Currie & Chen 2001), or relate to dermal fibres (presumed to be collagen; Feduccia *et al.* 2005; Lingham-Soliar *et al.* 2007).

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ENDNOTE

¹The sparse nature of the fibres and degradation warranted many hours of SEM viewing (approx. 4–5 hours) before fibres of the quality described herein were found. Several more fibres were observed than those described here but they lacked the quality needed for the ultrastructural measurements.

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