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Biomineralisations in crustaceans: storage strategies

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Abstract

Crustaceans are a remarkable group of animals because of their ability to elaborate cyclically two kinds of calcified biomineralisations: an unstretchable exoskeleton (or cuticle) and also, for many species, depending of the way of life of the considered animal, transitory calcium deposits. They are in consequence subjected to a cyclic life and notably, to a periodical balance between two sources of calcium, exogenous and/or endogenous. The storage structures, essentially composed of calcium carbonate precipitated within a proteinaceous organic matrix, are very diversified. The calcium carbonate of the cuticle of most of the species is under a crystalline state and/or an amorphous form, whereas calcium deposits are always in amorphous form. The organic matrix is responsible for the mineral polymorph and the morphology of the calcified structures. The knowledge of the features of the components of organic matrices is a necessary prerequisite to understand how these calcified mineralisations are elaborated. Nevertheless, few organic matrix proteins involved in calcification are well characterized in crustaceans to date. Another interest is that the storage structures are elaborated by calcifying/decalcifying epithelia, which mimic vertebrate epithelia. Diverse enzymatic activities have been registered at this level, such as Mg^{2+}-ATPase, Na^{+}/K^{+}-ATPase, and carbonic anhydrase. Finally, crustaceans represent convenient models to study the hormonal control of mineralising systems because, besides the involvement of ecdysteroids, the vertebrate tripartite calcium hormonal system (calcitonin, parathyroid hormone, vitamin D) could also regulate the formation of the crustacean calcified structures. ***To cite this article: G. Luquet, F. Marin, C. R. Palevol 3 (2004)***.

Résumé

Biominéralisations chez les crustacés : stratégies de stockage. Les crustacés possèdent une remarquable aptitude à élaborer de manière cyclique deux types de calcification : un squelette externe (ou cuticule) inextensible, mais également, chez de nombreuses espèces, en fonction de leur mode de vie, des dépôts transitoires de calcium. Ils sont ainsi sujets à une balance calcique se produisant périodiquement entre deux sources de calcium, exogène et/ou endogène. Les structures de stockage, constituées en majorité de carbonate de calcium précipité au sein d'une matrice organique essentiellement protéique, sont très diversifiées. Alors que le carbonate de calcium se présente sous une forme essentiellement cristalline (pouvant coexister avec la forme amorphe) au niveau cuticulaire chez la plupart des espèces, il adopte une forme exclusivement amorphe au niveau des

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dépôts calciques. C'est la matrice organique qui est responsable du polymorphe et de la morphologie de la biominéralisation. C'est pourquoi la caractérisation des constituants des matrices organiques constitue un préalable à la compréhension de la formation des structures calcifiées : à ce jour, seules quelques protéines matricielles sont bien caractérisées chez les crustacés. Un autre intérêt des crustacés repose sur la présence d'épithéliums calcifiants (mais aussi décalcifiants), élaborant (et résorbant) les structures de stockage calcique, et dont l'étude permet de mieux comprendre le fonctionnement des tissus similaires rencontrés chez les vertébrés. De même, les crustacés constituent d'excellents modèles pour la compréhension de la régulation hormonale des systèmes calcifiants, d'autant qu'en plus d'un rôle probable de certains ecdystéroïdes, la trilogie hormonale contrôlant le métabolisme calcique des vertébrés (CT/PTH/Vit. D) pourrait être également fonctionnelle chez les crustacés. ***Pour citer cet article : G. Luquet, F. Marin, C. R. Palevol 3 (2004)***.

Keywords: Biomineralisation; Calcification; Calcium carbonate; Calcium Storage; Cuticle; Organic matrix

Mots clés : Biominéralisation ; Calcification ; Carbonate de calcium ; Cuticule ; Matrice organique ; Stockage de calcium

Version française abrégée

Le processus de biominéralisation, présent dans les trois règnes vivants (archées, eubactéries, eucaryotes), est apparu chez les eucaryotes aux environs de la limite Précambrien/Cambrien [76,81,114,147]. La calcification, processus de biominéralisation majoritairement rencontré chez les animaux, essentiellement au niveau de la formation d'un squelette (externe ou interne) [146], est un processus particulièrement développé chez les crustacés. En effet, si tous les arthropodes durcissent leur squelette externe (ou cuticule) par sclérotisation (liaisons protéines–saccharides), la majorité des crustacés rigidifient également leur exosquelette par calcification [47,48,56]. Pour permettre la croissance de l'animal, cette cuticule inextensible est donc renouvelée périodiquement au cours de cycles de mue, dont dépend toute la physiologie de l'animal, y compris sa reproduction.

À l'origine, toutes les espèces de crustacés étaient marines et se procuraient donc le calcium nécessaire à la calcification cuticulaire dans leur milieu environnant. Puis, d'autres espèces sont venues peupler les milieux dulçaquicoles, puis les milieux terrestres, où la disponibilité du calcium est très variable. C'est pourquoi certaines espèces de crustacés ont « développé » des stratégies de stockage leur permettant de disposer d'un minimum vital de calcium juste après l'exuviation (moment où l'animal se débarrasse de son ancienne cuticule ou exuvie). La nourriture, incluant l'exuvie, constitue également un apport calcique dont l'importance, très variable, reste cependant limitée.

La calcification de la cuticule, dont la synthèse est réalisée par le plus externe des trois feuillets épidermiques sous-jacents, a été particulièrement bien étudiée chez les crustacés décapodes [21,39,75,108,109,128]. Elle s'effectue par précipitation de carbonate de calcium et, en moindre proportion, de phosphate de calcium dans les trois couches les plus externes de la cuticule (épicuticule, exocuticule et endocuticule) constituées d'un réseau matriciel de chitino-protéines [19,33,40,109,128]. À l'exception de l'aragonite, les divers autres polymorphes du carbonate de calcium ont été décelés : forme amorphe, vatérite et surtout calcite. Une activité enzymatique très importante dans le domaine des biominéralisations calcifiées [64,114], à savoir l'activité anhydrase carbonique catalysant de manière réversible la réaction : $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$, serait impliquée dans la calcification de la cuticule [28,37,38,40,63,64].

La nouvelle cuticule n'est calcifiée qu'en période de post-mue, lorsque l'ancienne cuticule a été exuviée. Une partie du calcium de l'ancienne cuticule est résorbée en fin de pré-mue et relarguée dans l'environnement ou mise en réserve par l'animal. Le calcium stocké représente de 4 à 75% du calcium nécessaire à la calcification de la nouvelle cuticule.

Les lieux de stockage sont très diversifiés [47,48,56]. Il peut s'agir de l'hémolymphe, d'une glande digestive (l'hépatopancréas), de la paroi de l'estomac, des sternites (plaques cuticulaires ventrales), de l'intestin et/ou de diverticules de l'intestin, appelés cæcums postérieurs.

À l'exception des cirripèdes et des copépodes, chez lesquels aucune forme de stockage n'a été observée à ce jour, toutes les autres sous-classes de crustacés élaborent des réserves calciques [114].

Les crustacés de l'ordre des décapodes, par exemple, dont les représentants occupent des milieux aussi bien aquatiques que terrestres, sont capables de stocker du calcium dans des sites très diversifiés : dans l'hémolymphe sous forme de granules, comme chez certains crabes dulçaquicoles [57,84,99,117], ou au niveau d'une glande digestive appelée hépatopancréas (Fig. 1), sous forme de granules essentiellement constitués de phosphate de calcium [3, 48, 56] chez quelques crabes d'eau douce et crabes marins (comme *Cancer pagurus* et *Carcinus maenas*). Enfin, les écrevisses et les homards élaborent au niveau de la paroi stomacale une ou deux paires de disques calcifiés appelés gastrolithes (Fig. 2) [48,86,99,125,128,130,143]. Ils sont constitués de carbonate de calcium sous forme amorphe et ne constituent que 10% du calcium cuticulaire. Ce calcium stocké peut également servir à minéraliser le moulin gastrique et à réguler la calcémie en début de post-mue. Le transit de calcium de l'hémolymphe vers le lieu de stockage (et réciproquement) s'effectue à travers un épithélium spécialisé par la voie transcellulaire [125–127,130]. À ce niveau, les mitochondries constituent un lieu de stockage calcique transitoire, évitant la toxification cellulaire [96,131]. Des activités Ca^{2+}-ATPase et Mg^{2+}-ATPase ont d'ailleurs été décelées au niveau de la matrice mitochondriale [96,130–132].

Les crustacés isopodes possèdent un mode de mue biphasique particulier. Ils muent, en effet, en deux temps : tout d'abord la moitié postérieure de leur cuticule, puis la moitié antérieure [89,111,120,151]. Pendant la pré-mue, la plupart de ces isopodes élaborent des dépôts de carbonate de calcium (sous forme amorphe) au niveau des quatre sternites les plus antérieurs [14,148]. Ces plaques sternales, dont la formation est particulièrement bien étudiée chez le cloporte *Porcellio scaber* (Figs. 3 et 4), fourniront une partie du calcium permettant la minéralisation de la cuticule postérieure exuviée en premier [148–153].

Chez les crustacés amphipodes, deux sites de stockage ont été répertoriés [47, 48]. Par exemple, chez certaines espèces cavernicoles, comme *Niphargus*, on trouve, sous forme calcitique, des sphérolithes dans deux diverticules de l'intestin moyen, appelés cæcums postérieurs (CP), ainsi que des rhomboèdres dans l'intestin lui-même (Fig. 5). Chez d'autres espèces terrestres, le stockage s'effectue uniquement sous forme de concrétions calcaires dans les cæcums postérieurs. *Orchestia cavimana* en constitue un exemple particulièrement bien étudié par notre groupe à Dijon (Fig. 6) [42–55,90–95].

Chez *O. cavimana*, le calcium stocké, qui provient de la décalcification partielle de l'ancienne cuticule, fournira 60% du calcium de la nouvelle cuticule. Le stockage s'effectue pendant une quinzaine de jours, en pré-mue, sous forme de concrétions calcaires constituées de l'alternance de sphérolithes simples et de sphérolithes complexes (Figs. 6b et 7). Elles comprennent environ 95% de carbonate de calcium à l'état amorphe et 5% de phosphate de calcium, probablement aussi à l'état amorphe [102]. Le transit calcique s'effectue selon une voie paracellulaire au travers de l'épithélium cæcal unistratifié constituant les organes de stockage tubulaires. Venant corroborer cette observation, des activités Ca^{2+}-ATPase et Na^{+}/K^{+}-ATPase ont été enregistrées au niveau des membranes des cellules épithéliales cæcales. Après exuviation, le calcium stocké dans la lumière des CP est complètement résorbé en 48 h. Si, en pré-mue, le calcium traverse l'épithélium cæcal sous forme ionique, libre ou masqué, le calcium est réabsorbé en post-mue sous forme de sphérules calciques d'environ 1 μm de diamètre, élaborées à l'apex des cellules et résorbées à la base (Fig. 8). A ce niveau, les ions calcium libérés dans l'hémolymphe circuleront jusqu'à la cuticule pour y être précipités. Les sphérules de réabsorption sont également des structures biphasiques comprenant une matrice organique, où le calcium est précipité probablement là aussi sous forme amorphe, comme dans les concrétions.

Carapace et structures de stockage calciques sont des biominéralisations sensu stricto, c'est-à-dire qu'elles comportent un minéral précipité au sein d'une matrice organique, essentiellement constituée de protéines et de sucres chez les crustacés. L'étude de cette matrice est d'un intérêt considérable pour la compréhension, au niveau moléculaire, de l'élaboration d'une biominéralisation et de ses propriétés particulières comparativement au même minéral purement inorganique. Le point de départ en est l'identification et la caractérisation des constituants matriciels. À ce jour, si un certain nombre d'entre eux commencent à être caractérisés chez les mollusques et les oursins [16, 83, 145, 146], peu de protéines clairement impliquées dans la formation des structures calcifiées ont été identifiées chez les crustacés.

Si 33 protéines cuticulaires ont été séquencées à partir de cinq espèces de décapodes, très peu ont été bien caractérisées, et c'est essentiellement au regard de l'analyse de leur séquence que certaines d'entre elles sont suspectées d'être impliquées dans les processus de sclérotisation et/ou de calcification cuticulaire (Tableau 1) [10,30,68,98,121,137]. La seule identité de séquence observée entre certaines d'entre elles correspond à la présence d'un domaine dit de Rebers–Riddiford, synonyme de liaison à la chitine [105,106,144].

Deux d'entre elles cependant, CAP–1 et CAP–2 [69–71], extraites de la cuticule de l'écrevisse *Procambarus clarkii*, présentent des caractéristiques physicochimiques tout à fait intéressantes. En effet, il a été clairement démontré que ces polypeptides sont, non seulement capables d'interférer dans la précipitation du carbonate de calcium in vitro, mais également de lier le calcium ainsi que la chitine, suggérant leur implication dans les deux processus de sclérotisation et calcification. Ces protéines liées à la chitine pourraient fonctionner comme des nucléateurs du processus de calcification [33,71].

En ce qui concerne les structures de stockage, seules deux protéines sont caractérisées à ce jour (Tableau 1). L'une, GAMP [72,73,122,129], est un constituant de la matrice des gastrolithes élaborées par *Procambarus clarkii*. Cette protéine acide, ou une molécule immunologiquement apparentée, pourrait être également un constituant de la matrice cuticulaire. L'autre, Orchestine, est une calciprotéine phosphorylée extraite des concrétions calcaires élaborées par *Orchestia cavimana* [61,82,123]. Cette protéine, riche en Asp et Glu (environ 30% de la séquence), possède une aptitude à fixer le calcium qui dépend de la présence de phosphorylations sur des sérines. Elle est, par ailleurs, phosphorylée sur des résidus tyrosine. Si cette protéine n'est exprimée qu'au niveau de l'épithélium des organes de stockage, elle est un constituant de la matrice organique, non seulement des concrétions élaborées en pré-mue, mais également des sphérules de réabsorption formées en post-mue.

Tous les processus de biominéralisation sont biologiquement contrôlés, c'est-à-dire, entre autres, qu'ils sont hormonalement régulés. Chez les crustacés, de nombreux résultats suggèrent que le métabolisme calcique est contrôlé par l'hormone de mue, majoritairement la 20-hydroxyecdysone (ou 20E) [25,26,34]. Cependant, au niveau cuticulaire, seule la décalcification peut être régulée par la 20E. En effet, la calcification de la cuticule s'effectue en post-mue, alors que le taux de 20E est revenu au plus bas juste avant l'exuviation, processus régulé par une autre hormone, l'hormone d'éclosion, dont la synthèse est inhibée par la 20E. Si quelques changements dans le profil électrophorétique des protéines cuticulaires ont été observés après injection in vivo de 20E à des périodes stratégiques du cycle de mue, aucune cible directe ou indirecte de la 20E impliquée dans la décalcification ou calcification cuticulaire n'a été identifiée à ce jour. En revanche, les deux gènes codant les protéines GAMP et Orchestine sont régulés, mais de manière indirecte, par la 20E [122,129]. Cependant si le gène *orchestine*, est exprimé en post-mue, il ne peut l'être sous l'effet de la 20E. D'autres hormones sont alors suspectées de pouvoir réguler cette expression ainsi que, de manière plus générale, les processus de calcification de la post-mue. Il s'agit notamment de celles de la trilogie calcique des Vertébrés : calcitonine et/ou CGRP (*Calcitonin Gene-Related Peptide*), vitamine D, parathormone [22,35, 36,81, 88,94,95].

Les crustacés sont remarquables, du fait qu'ils sont capables d'élaborer, de manière cyclique, deux types de biominéralisations calciques : un squelette externe, mais aussi diverses formes transitoires de stockage. Ce dernier s'effectue majoritairement sous forme de carbonate de calcium, dont on rencontre tous les polymorphes à l'exception d'une forme cristalline, l'aragonite. Cependant, les structures de stockage semblent essentiellement constituées de $CaCO_3$ amorphe, état compatible avec la nécessité d'une remobilisation rapide du calcium stocké en vue de l'urgence de durcir certains organes vitaux (pièces masticatrices et carapaces protectrices). Ce carbonate de calcium amorphe étant plus facilement solubilisable qu'un polymorphe cristallin [20], la matrice des structures de stockage est beaucoup plus facilement accessible que celle de la carapace ou de l'exosquelette d'autres invertébrés (coquilles des mollusques ou squelette des coraux et des oursins). Il est à noter cependant que, compte tenu de l'instabilité du $CaCO_3$ purement chimique à l'état amorphe (par opposition à sa forme dite « biologique » rencontrée dans une biominéralisation), certains constituants moléculaires matriciels sont, non seulement responsables du polymorphe et de la forme adoptés [15,32], mais exercent très certainement aussi un effet stabilisant [2,6,7]. La découverte des molécules et des

interactions moléculaires en jeu dans le développement d'un type particulier de biominéralisation, mais aussi dans l'effet stabilisateur de l'état amorphe, représente un challenge d'autant plus intéressant que l'on découvre que l'état amorphe est beaucoup plus répandu dans le monde vivant que ce que l'on supposait jusqu'alors [2,138]. Par ailleurs, il s'avère que, dans de nombreuses structures calcifiées, cohabitent une forme cristalline et la forme amorphe [8], ou que certaines structures définitives sont élaborées à l'état amorphe, se transformant avec le temps en un polymorphe cristallin [8,17,103,104]. Il semblerait que ces deux processus se retrouvent également au niveau de la calcification de la cuticule de nombreux crustacés.

Un autre intérêt des modèles crustacés repose sur la présence d'épithéliums calcifiants mimant ceux rencontrés chez les vertébrés [101]. Les épithéliums élaborant les structures calcifiées transitoires semblent les plus intéressants, du fait qu'ils sont unistratifiés et relativement isolés comparativement à celui de la cuticule, inclus dans un feuillet multicouche. À ce niveau, on y trouve les deux modes de transit calcique, paracellulaire ou transcellulaire, ainsi que des pompes à calcium, l'échangeur Na^+/ Ca^{2+} et autres activités enzymatiques, telles des Ca^{2+}-ATPase [4, 5, 23, 108, 140], Mg^{2+}-ATPase, Na^+/K^+-ATPase et anhydrase carbonique.

Enfin, les crustacés représentent des modèles utiles à la compréhension du contrôle hormonal des systèmes minéralisants ainsi que de la balance calcique se produisant entre deux sources de calcium, d'autant plus que ces processus se produisent de manière cyclique.

1. Introduction

Biomineralisation is a phenomenon, widespread in the five realms, which appeared firstly in Bacteria [81,114]. Among the animals, numerous invertebrates are able to secrete biominerals, the first major function of which is the hardening of a skeleton, a structure that provides support and/or protection against environment [81,114,147]. The fossil species, discovered so far, revealed that the first calcified metazoan exoskeletons were probably elaborated at the end of the Precambrian (Proterozoic) [77,147]. The reason for the appearance of such a process remains speculative, but several hypotheses have been evoked, among which protection against predators or against the ionic composition (notably in calcium) of the primitive ocean evolving with time. In the same time, the ocean became rich in phosphates and carbonates, promoting the precipitation of calcium as calcium phosphates and/or carbonates. Thus some biomineralisations may have been elaborated as a consequence of a neutralization process against external toxic ions and/or molecules. The calcifications externally elaborated could have been favourable to the survival of species, which were then protected against chemical and physical environmental pressures.

The biomineralisation process is especially well developed in arthropods, currently the largest group of animals on Earth. As a consequence of the presence of a rigid exoskeleton, also called cuticle, the growth and the whole physiology of these animals are tightly linked to moulting cycles, each of which is characterized by the complete replacement of the exoskeleton. Moreover, arthropods harden their new cuticle by a process called sclerotization (proteins-polysaccharides cross-linking), whereas most of the crustaceans proceed also by calcification.

The major source of calcium is exogenous: the water in which most crustaceans live. In seawater, calcium concentration is very high, but some groups also live in freshwater or on land, where the availability of calcium at ecdysis (moment where the animal leaves its old cuticle) ranges from great to complete absence. These animals have developed different strategies to solve the problem of calcification, notably by storing calcium during the pre-moult period [48,56], a phenomenon particularly well developed in the terrestrial species. Thus, the source of calcium used to mineralise the cuticle depends on the way of life of the considered crustacean. The food, a possible source of calcium for each crustacean, represents a minor contribution to the calcification.

Crustaceans are notably interesting because of their particularly active calcium metabolism and their ability to form cyclically not only an external calcified structure, but also, for several species, calcium storage forms.

2. Mineralisation of the crustacean carapace

In crustaceans, a cellular hypodermis of at least three layers underlies the carapace. The outer layer is

responsible for the elaboration of the exoskeleton. This cuticle comprises four layers, from the external to the most internal: the epicuticle, the exocuticle, the endocuticle, and the membranous layer [108,128]. Except for the innermost layer, the three other layers are mineralised, essentially by precipitation of calcium carbonate and, to a lesser extent, of amorphous calcium phosphate into the twisted lamellar structure of the chitin–protein cholesteric matrix [19,40,108,128]. Calcification of the carapace has been particularly well studied in Decapoda [21,39,75,108,109,128]. It occurs at different sites within the cuticle: in the chitin–protein fibres, at the level of interprismatic septa and pore-canals. The latter are vertically extensions of the outer epithelial cells, which pervade the whole cuticle in very numerous sites. They are responsible for the transport of components necessary for the sclerotization as well as for the calcification of the cuticle in post-moult (quinone, phenoloxidase, calcium and carbonic anhydrase, for example). This route can also be taken by molecules responsible for the partial resorption of the old cuticle in pre-moult (protease, chitinase, and also carbonic anhydrase).

The carbonic anhydrase (CA) is a particularly important enzyme involved in the calcification processes in crustaceans, as in all the invertebrates elaborating calcified biomineralisations [62,114] by catalysing the reversible reaction: $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$. This enzymatic activity has been first evidenced in crustacean in the barnacle mantle by Costlow in 1959 [28] and in the carapace by Giraud in 1977 [37], where it has been localized at the calcification sites described above [38,39]. Implication of CA in cuticle calcification has not been extensively studied so far [63,64]. Except for aragonite, the various polymorphic forms of calcium carbonate (amorphous, calcite, vaterite) have been found in the crustacean cuticle. Nevertheless, the crystalline calcite seems to be the major one. The part of the new cuticle synthesized by the hypodermis during the pre-moult period is not mineralised before the old cuticle has been discarded. A portion of the minerals that made up the old cuticle are resorbed during the pre-moult period and lost into the environment or stored as calcium reserves in different organs. It is worth noting that to avoid toxification of the epidermal cells, calcium ions resorption does not occur via the cytoplasm: transport mechanisms used are pinocytosis vesicles or paracellular channels (extracellular dilated network delineated by cell membranes). Moreover, calcium is in general in bound or precipitated form. The same anti-toxification strategy is used by the most part of epithelial cells comprising the storage organs. Nevertheless, it is worth noting that at this level, calcium transport may also occur via a transcellular pathway using Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger mechanisms [4,5,108].

The amounts of excreted or stored calcium vary considerably from one species to another. The stored calcium represents 4% to 75% of the calcium needed for the complete calcification of the exoskeleton [48,56,114]. This calcium is accumulated during pre-moult and thus available at ecdysis. Whatever its contribution for the cuticle mineralisation, this stored calcium is used firstly before any other exogenous sources, sometimes restricted to the cuticle of some primordial organs such as the masticatory parts. In addition to this endogenous calcium, water, and food, which includes the rejected old cuticle (also called the exuviae), are exogenous sources of calcium used in post-moult.

For aquatic species, the loss of calcium at ecdysis is related to their ability to remove quickly a great amount of calcium from their environment. However, losses are limited in supralittoral or terrestrial crustaceans. They do not excrete, but store the calcium originating from their old cuticle. Storage sites are diverse and may occur in common or peculiar organs, such as haemolymph, hepatopancreas, cardiac stomach wall, sternites, or posterior ceca [48,114].

3. Calcium turnover and storage in crustaceans

Calcium, from an endogenous or exogenous source, reaches the cuticle via the haemolymph. Similarly to the outer layer of the hypodermis, responsible for the synthesis of the cuticle, the organs storing calcium are generally formed by an unstratified epithelium across which calcium is translocated. These calcium-transporting epithelia are the subject of extensive studies [4,5,97,139,141,142] because they represent interesting models as compared to similar vertebrate ones such as the intestine, kidney, placenta, and chick chorioallantoic membranes [101]. Moreover, at this level, calcium transport involves, as in vertebrates, activation of calcium pumps, such as Ca^{2+}-ATPases

pumps [23,108,140] and other common ion pumps such as H^+-ATPase and Na^+/K^+-ATPase [114].

Except for the cirripeds (among which barnacles) [114] and copepods, where calcium storage has not been reported so far, all the other crustaceans have developed mineral storage strategies related to their moulting cycle.

3.1. In Decapoda

The order of decapods represents the largest group of crustaceans living as well on land as in water, and storage strategies are very diversified in decapod species.

Calcium translocation between an endogenous or exogenous source and the cuticle occurs via the haemolymph. Also the haemolymph calcium rate is increasing periodically, when calcium is resorbed from the cuticle in pre-moult and during the cuticular calcification in post-moult [48].

But there are some cases in which this inner medium may be considered as a storage site. In the freshwater crab, *Holthuisana transversa*, the haemolymph calcium content increases 150-fold in pre-moult because of the presence of a great amount of small-size calcified granules [117]. Nevertheless, it is to notice that calcium is also stored by this crab as medium (calcium carbonate) and large (calcium, potassium and phosphate) granules not released in the haemolymph, but trapped by thin extension of connective tissue cells or by epidermal cells in intercellular spaces or at the level of the basal lamina [57]. This stored calcium corresponds to about 65% of the exoskeleton calcium content. It seems that other crabs such as *Sesarma dahaani* [99] and *Paratelphusa guerini* [84] also transitorily store cuticular calcium as granules in their haemolymph, because of the similar milky appearance of this inner medium.

The hepatopancreas is a midgut gland whose main function is related to digestion. But in some marine decapods [48,56], like *Carcinus maenas* or *Cancer pagurus* (Fig. 1), and in the freshwater crab *Paratelphusa hydrodomous* [3], calcium and phosphate are accumulated into this organ during the pre-moult period as calcified granules, essentially composed of calcium phosphate. The stored calcium probably is used in the calcification of the skeleton, whereas phosphate seems necessary to chitin synthesis [107]. A detoxification role has also been proposed for these hepatopancreatic granules [58,65].

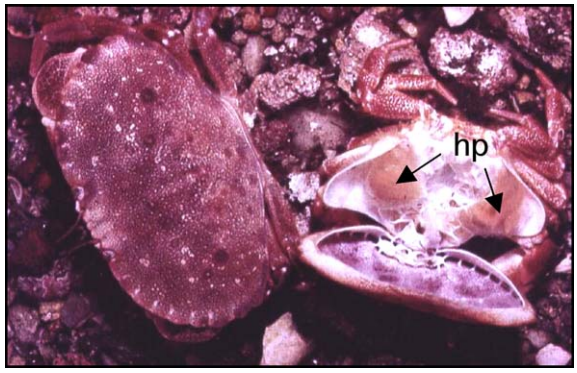

Fig. 1. *Cancer pagurus*, a marine decapod that stores calcium as granules in the hepatopancreas. At right, a specimen where hepatopancreas (hp) are well visible after the removal of the dorsal carapace.
Fig. 1. *Cancer pagurus* (crustacé décapode marin) stocke du calcium sous forme de granules dans l'hépatopancréas. À droite, spécimen où l'hépatopancréas (hp) est bien visible.

Gastroliths are calcified structures formed in the cardiac stomach wall [48,86,99,125,127,130,143]. They appear as paired discs in some decapods such as crayfishes, lobsters and as four more irregular concretions in gecarcinid land crabs (Fig. 2) [18,48,85]. Calcium is stored mainly as calcium carbonate probably in the amorphous form [122]. Gastrolith calcium is in general less than 10% of the body content [48] and is also used to mineralise the gastric mill [110] and to maintain the calcemic rate, especially in the beginning of the post-moult period [24]. Calcium transport between the haemolymph and the storage site is performed through a specialized epithelium, which possesses the reversible function of secretion and reabsorption [125–127,130]. Four types of epithelial cells have been described; the change from one type to the other is correlated to the different periods of a moulting cycle, notably the elaboration and resorption of gastroliths. Moreover, it has been demonstrated that mitochondria play a role of transient calcium storage sites during the transcellular calcium transport [96,131], in relation with Ca^{2+}-ATPase and Mg^{2+}-ATPase activities detected in the mitochondria matrix [96,130–132].

3.2. In Isopoda: Porcellio scaber as a model

Isopods possess a particular biphasic mode of moulting: they shed first the posterior half of their

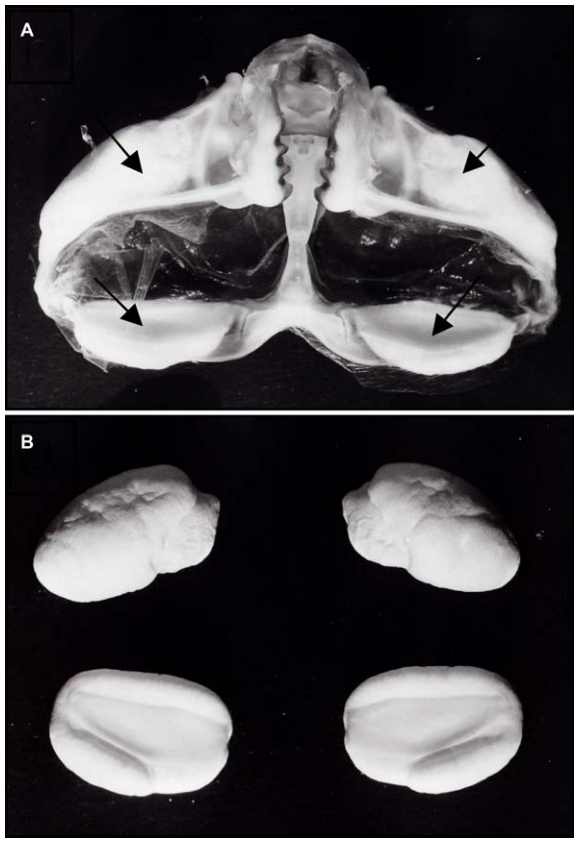

Fig. 2. Formation of gastroliths in a land crab of the genus *Gecarcinus*. **(A)** Gastric mill where four gastroliths (arrows) are being elaborated. **(B)** View of the four irregular gastroliths (10-mm long) composed of amorphous calcium carbonate.
Fig. 2. Formation des gastrolithes chez un crabe terrestre du genre *Gecarcinus*. **(A)** Moulin gastrique où quatre gastrolithes (flèches) sont en cours d'élaboration. **(B)** Vue des quatre gastrolithes d'aspect irrégulier (10 mm de long), composés de carbonate de calcium amorphe.

Fig. 3. *Porcellio scaber* (terrestrial isopod), which first moults the posterior part of its cuticle, then the anterior one. This specimen is eating its anterior old cuticle (the exuviae) that it has just shed.
Fig. 3. *Porcellio scaber*, crustacé isopode terrestre qui mue en deux temps : tout d'abord, la partie postérieure de sa cuticule, puis la partie antérieure. Ce spécimen est en train d'ingérer la partie antérieure de sa cuticule qu'il vient d'exuvier.

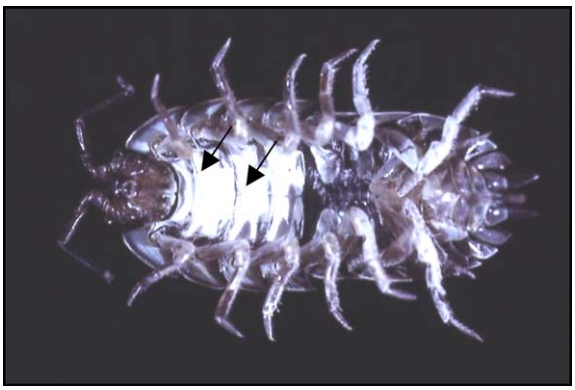

Fig. 4. Ventral view of *Porcellio scaber* showing sternal plates (arrows), storage calcium structures elaborated in pre-moult at the level of the four most anterior sternites.
Fig. 4. Vue ventrale d'un cloporte, *Porcellio scaber*, montrant les plaques sternales (flèches), structures de stockage calcique, élaborées en pré-mue au niveau des quatre sternites les plus antérieurs.

cuticle and then the anterior part (Fig. 3) [89,111,120,151]. During the pre-moult period, almost all the terrestrial isopods elaborate calcified deposits in the four anterior sternites between the cuticle and the hypodermis (Fig. 4). The formation of such sternal plates has been particularly well studied in a woodlouse, *Porcellio scaber*, by the team of Andreas Ziegler. These calcified storage structures are composed of amorphous hydrated calcium carbonate precipitated within an organic matrix [14,148]. Ultrastructural study of these $CaCO_3$ deposits revealed the existence of three types of sternal deposits [150]. These types, a combination of a homogeneous and/or a spherular layer of calcium carbonate, are correlated to the degree of adaptation to the terrestrial environment. The formation and resorption of these deposits are closely related to the biphasic moulting cycle of these crustaceans. The calcium deposits, fully developed before the ecdysis of the posterior part, are completely resorbed before the ecdysis of the anterior part. Transported by the haemolymph, the stored calcium is used to calcify the posterior cuticle [31,120,150,151]. *P. scaber* represents also a good model to study Ca^{2+} epithelial transport [41,60,149]. For the deposition and resorption of calcium deposits, a great amount of calcium

ions has to cross the anterior sternal epithelium. Recent findings, such as the expression of a Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger at the level of the plasma membrane and of the K^{+}-ATPase in the basolateral membranes [152,153], suggest a transcellular pathway [113]. Furthermore, to avoid toxification of the cells during this transepithelial transport, the smooth endoplasmic reticulum plays a role of transient storage site [59,60]. Finally, the SERCA (Smooth Endoplasmic Reticulum Ca^{2+}-ATPase) molecule of *P. scaber* has been characterized and its gene expression, evidenced in the sternal epithelium, seems transcriptionally regulated in relation with the calcium translocation process [60].

3.3. In Amphipoda: Orchestia cavimana as a model

Finally, Amphipoda represent a peculiar group in which a specific storage is known [48,56]. These animals store calcium in paired diverticula of the midgut called posterior ceca (PC), and also, for some of them, in the gut itself. As in other taxa, storage is greater in terrestrial species than in aquatic ones. Nevertheless, cave-dwelling amphipods like *Niphargus*, which live in hard water, store calcium (44% of the new cuticle content) not only as calcite spherulites into the PC, but also as rhomboidal calcite structures in the midgut (Fig. 5). As regards the aquatic way of life of these animals, this storage appears almost abnormal, but it is also found in other freshwater cave-dwelling isopods or decapods and may have two explanations. First, in relation to their way of life, the metabolism of such animals is very slow. That is in conflict with the urgency to quickly calcify the new cuticle after ecdysis. Storage of a great amount of calcium immediately available is a way to solve this problem [48]. Second, this storage may be considered as a resistance system related to the possibility of a dramatic environmental change such as draining of the underground water.

Terrestrial crustaceans of the genus *Orchestia* store calcium originating from their old cuticle in two posterior ceca (PC). *Orchestia cavimana* (Fig. 6), the only European amphipod living in moist biotopes, cyclically elaborates and resorbs calcareous concretions in

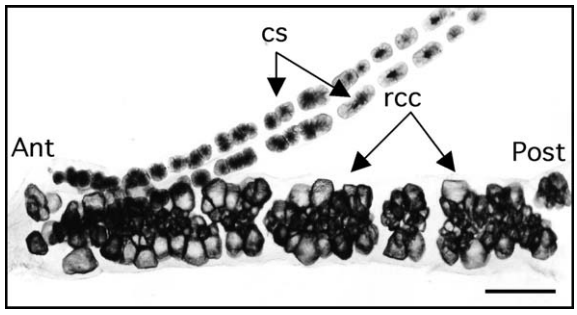

Fig. 5. Storage of calcium as calcite spherulites **(cs)** into the posterior ceca and as rhomboidal calcite structures **(rcc)** in the midgut of a cave-dwelling amphipod, *Niphargus virei*. Scale bar: 500 µm.
Fig. 5. Stockage de calcium sous forme de sphérolithes calcitiques **(cs)** dans les cæcums postérieurs et sous forme de rhomboèdres calcitiques **(rcc)** dans l'intestin moyen d'un crustacé amphipode cavernicole, *Niphargus virei*. Échelle: 500 µm.

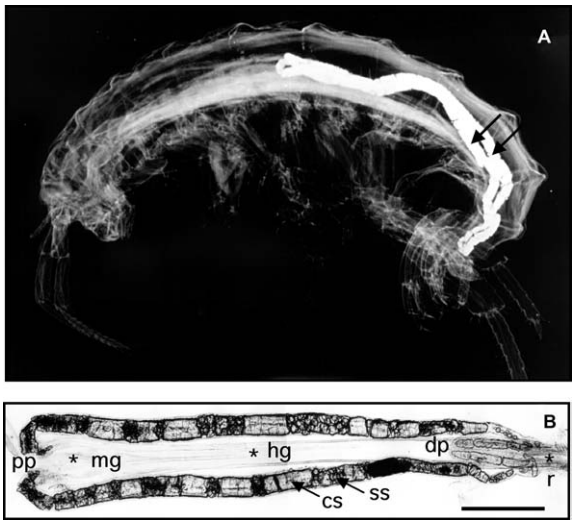

Fig. 6. *Orchestia cavimana* (terrestrial amphipod). **(A)** X-ray photograph of a specimen just after ecdysis showing in situ the paired storage organs, the posterior ceca, filled with calcareous concretions (arrows) elaborated during the pre-moult [42]. **(B)** Dorsal view of posterior ceca, 7 h after ecdysis, showing the alternation of compound **(cs)** and single **(ss)** spherulites. The posterior ceca are proximally connected to the midgut and distally blind. **cs**: Compound spherulites, **dp**: distal part, **hg**: hingut, **mg**: midgut, **pp**: proximal part, **r**: rectum, **ss**: single spherulites. Scale bar: 1 mm.
Fig. 6. *Orchestia cavimana* (crustacé amphipode terrestre). **(A)** Radiographie d'un spécimen qui vient d'exuvier, montrant in situ la paire d'organes de stockage, les cæcums postérieurs, remplis de concrétions calcaires (flèches) élaborées pendant la pré-mue [42]. **(B)** Vue dorsale des cæcums postérieurs, 7 h après l'exuviation, montrant l'alternance de sphérolithes complexes **(cs)** et de sphérolithes simples **(ss)**. Les cæcums postérieurs sont connectés proximalement à l'intestin moyen **(pp)** et sont distalement aveugles, recourbés sur eux-mêmes **(dp)**. **cs** : sphérolithes complexes, **dp** : partie distale, **hg** : intestin postérieur, **mg** : intestin moyen, **pp** : partie proximale, **r** : rectum, **ss** : sphérolithes simples. Échelle : 1 mm.

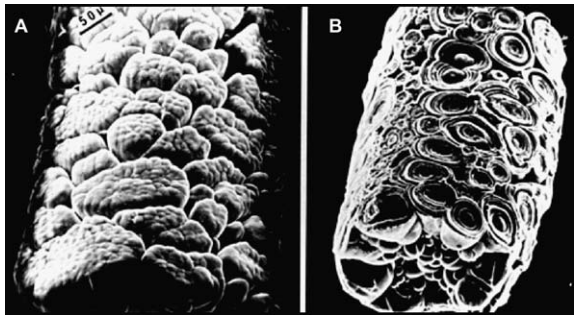

Fig. 7. Calcium storage forms in *Orchestia cavimana*: a compound spherulite just before **(A)** and 10 h after ecdysis **(B)**. Scale bar: 50 µm.

Fig. 7. Structures de stockage calcique chez *Orchestia cavimana* : aspect d'un sphérolithe complexe, juste avant **(A)** et 10 h après l'exuviation **(B)**. Échelle : 50 µm.

its PC (Fig. 7) [43,45]. Although they first were considered as excretion forms, the real function of these structures as transitory calcium storage deposits related to the moulting cycle of this crustacean has been demonstrated [42].

The storage organs, the PC, are distally blind cylindrical tubules delineated by an unstratified epithelium and connected proximally to the midgut [50].

During the pre-moult period, calcium is transported in ionic form and is precipitated in the PC lumen within an organic matrix synthesized by the storage organ cells [45,51,91]. Calcareous concretions are composed of single spherulites, the association of several of them forming complex spherulites (about 1 mm long by 400 µm in diameter; Fig. 8). Concretions are formed by addition of successive concentric layers of organic matrix, some of which being mineralised, alternating with non-mineralised ones. They are composed of calcium carbonate essentially in amorphous form [102]. After ecdysis, calcium reabsorption occurs through successive generations of calcified spherules that form at the apical part of the PC epithelium and dissolve at the basal part of the extracellular PC network (Fig. 8) [43–45,49,52,92]. Although the same amount of calcium transits through the epithelium during the pre-moult and post-moult periods, the storage occurs regularly during a 16-day mean period concomitant to the partial demineralisation of the cuticle, whereas dissolution of concretions is performed in less than 48 h, in relation with the urgency of the calcification of the exoskeleton. Thus, the intraepithelial transit as calcified spherules during the post-moult period may be explained by the constitution of a potent and rapid calcifying system, while avoiding the risks of cellular saturation and toxicity [52,92]. All the calcium translocation is entirely performed by a paracellular pathway in a dilated extracellular network of lateral membranes [45]. This has been corroborated by the localization of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase at the apical and baso-lateral level of the epithelial cells [90,113]. Moreover, carbonic anhydrase was ultrahistochemically localized throughout the membranes of the epithelial cells, as well as within the pre-moult concretions and the post-moult calcified spherules. This suggests the involvement of this enzyme in the calcification/decalcification processes occurring in *Orchestia cavimana* [93].

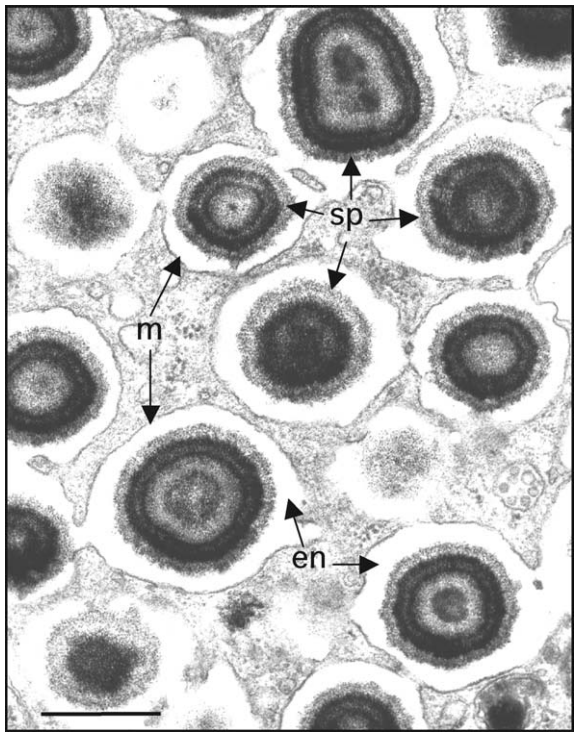

Fig. 8. Calcium resorption spherules **(sp)** elaborated by *Orchestia cavimana* during the post-moult in a dilated extracellular network **(en)** delineated by the lateral membranes (m) of the storage organ epithelial cells. Scale bar: 1 µm.

Fig. 8. Sphérules de réabsorption calcique **(sp)** élaborées par *Orchestia cavimana* pendant la pré-mue dans un réseau extracellulaire dilaté **(en)**, délimité par les membranes latérales (m) des cellules de l'organe de stockage. Échelle : 1 µm.

4. The organic matrix of the crustacean calcified structures

Carapace and storage structures are biomineralisations, which means that they contain an organic matrix. The knowledge of the features of the matrix components, the study of the interactions occurring between these molecules to construct the molecular framework within which calcium is precipitated, and the discovery of the chemical interactions occurring at the interface between the matrix and the mineral are of first importance. That could lead us to understand the remarkable properties (elasticity, mechanical resistance...) of these minerals when comparing their 'biological' form to their inorganic form, as well as to better understand in a general way why and how biomineralisations are elaborated.

Molluscs and echinoderms represent two invertebrate groups where the characterization of proteins involved in biomineralisations is considerably increasing since a decade [16,83,145,146]. In crustaceans, few molecules have been characterized to date (Table 1) [10,30,61,68–74,77,82,98,121–123,129,137].

Previous investigations have shown that the crustacean cuticle contains numerous proteinaceous components [9,116,136,144]. At this time, 33 proteins from calcified parts of the cuticle (also called hard or solid cuticle contrarily to the membranous arthrodial cuticle called the soft cuticle) have been completely sequenced. Twenty-six cuticular matrix proteins have been sequenced by the team of Andersen from Copenhagen: one from the cuticle of the shrimp *Pandalus borealis* [74] (another one from the same origin has been characterized by Suzuki et al. [121]), 13 from the carapace of the American lobster *Homarus americanus* [77,98] and 12 from the exoskeleton of the crab *Cancer pagurus* [10]. Nevertheless, nothing is clearly known about the involvement of these cuticular proteins in the sclerotization or the calcification process. The chemical features of some of them only suggest that they could participate to the calcium precipitation and/or bind chitin.

The group of Nagasawa, from Tokyo, recently characterized and sequenced, from the exoskeleton of the crayfish *Procambarus clarkii*, two interesting polypeptides, named CAP-1 and CAP-2, both with anti-calcification (CAP-1 inhibits in vitro calcium precipitation), calcium-binding and chitin-binding properties (presence of Rebers–Riddiford motifs in the sequence and positive in vitro chitin-binding assay) [69–71]. These proteins could both be linked to chitin and act as nucleator of the calcification process, as suggested [33,71].

Finally, four cDNAs were sequenced, which encode crustacean proteins of the exoskeleton of the prawn *Penaeus japonicus* (DD4, DD9A, DD9B and DD5) [30,68,137]. Among these, only DD4 [30], encoding an acidic protein is clearly calcium binding and presents similarities with calphotin (a calcium-binding protein from *Drosophila* photoreceptor cells). DD9A and DD9B correspond to acidic proteins (20% of the amino-acids) with a Rebers–Riddiford motif and DD5 comprises a tandem repeat domain (of 93 to 98 a.a.) also with chitin-binding property.

As regards the storage structures, only two proteins have been evidenced, sequenced and extensively studied. First, an insoluble matrix protein, named GAMP, extracted from the gastroliths of the crayfish *Procambarus clarkii*, was characterized by Nagasawa's team. GAMP is rich in acidic amino acids and its molecular weight was estimated to 94 kDa on SDS-PAGE and to 50.5 kDa by mass spectrometry. GAMP is suspected to play a role in the calcification process of the storage structures [72,73,129]. An immunolocalization study revealed that GAMP is present into the gastroliths, as expected, and in the stomach epithelial cells responsible for the elaboration of gastroliths. Moreover, this protein (or a immunologically-related molecule) seems also present as a component of the cuticle organic matrix; except for the epicuticle, all the other layers expressed a GAMP-immunoreactivity [122]. This study also demonstrated that calcium carbonate is present in its amorphous form in gastroliths, whereas only calcite is detected in the cuticle of *P. clarkii*. The authors suggest that GAMP could have a function in the precipitation of calcium carbonate in the amorphous form in gastroliths.

Second, a soluble organic matrix protein, Orchestin, from the terrestrial crustacean *Orchestia cavimana* has been well characterized [61,82,123]. Analysis of the proteinaceous components of the organic matrix of the storage forms elaborated by this crustacean revealed that this matrix obtained after dissolution in an EDTA buffer is composed of a soluble fraction (SM) and an insoluble fraction (IM) [82]. The electrophoretic pattern of the SM fraction, which contains most of the

Table 1
Sequenced matrix proteins from crustacean mineralisations
Protéines de matrices de structures calcifiées séquencées à ce jour chez les crustacés

Protein	Species	Origin	Known characteristics	References
H1a	Shrimp: *Pandalus borealis*	Cuticle	16 kDa (SDS-PAGE)/12.7 kDa (mass spectrometry)	[74]
Pb Cp-12.7	″	″	12.7 kDa, lipophilic,adsorption to chitin	[121]
HaCP4.6[a], HaCP11.6[a]	Lobster: *Homarus americanus*	Cuticle	chitin-binding[b]	[76]
HaCP4.4, HaCP4.5	″	″	chitin-binding[b]	[98]
HaCP5.6, HaCP5.9[a]	″	″	chitin-binding[b]	[98]
HaCP6.3	″	″	chitin-binding[b]	[98]
HaCP9.3, HaCP11.8	″	″		[98]
HaCP12.7, HaCP14.2	″	″		[98]
HaCP18.8, HaCP20.2[a]	″	″		[98]
CpCP4.34, CpCP4.59	Crab: *Cancer pagurus*	Cuticle	chitin-binding[b]	[10]
CpCP4.63, CpCP4.66	″	″	chitin-binding[b]	[10]
CpCP4.98, CpCP11.58	″	″	chitin-binding[b]	[10]
CpCP12.43, CpCP12.46	″	″	chitin-binding[b]	[10]
CpCP/AMP11.14	″	″	present in both the calcified and the membranous cuticles	[10]
CpCP5.75	″	″		[10]
CpCP14.99, CpCP18.76	″	″		[10]
CAP-1	Crayfish: *Procambarus clarkii*	Cuticle	8.7 kDa, anti-calcification activity, chitin-binding[c]	[69,70]
CAP-2	″	″	7.4 kDa, anti-calcification activity, chitin-binding[b], calcium-binding, 44% homology with CAP-1	[71]
DD4	Prawn: *Penaeus japonicus*	Cuticle	57 kDa, pI 3.53, calcium-binding	[30]
DD5	″	″	13.5 kDa, pI 5.33, chitin-binding[b]	[68]
DD9A	″	″	12.5 kDa, pI 3.93, chitin-binding[b]	[137]
DD9B	″	″	12.5 kDa, pI 3.99, chitin-binding[b]	[137]
GAMP	Crayfish: *Procambarus clarkii*	Gastroliths	94 kDa (SDS-PAGE)/50.5 kDa (mass spectrometry)	[72,73, 122,129]
Orchestin	Terrestrial amphipod *Orchestia cavimana*	Concretions	23 kDa (SDS-PAGE)/12.4 kDa (from the sequence), pI 4.4, calcium-binding, phosphorylated on Ser and Tyr	[61,82,123]

[a] presents two isoforms.
[b] possesses the consensus motif of Rebers-Riddiford (or a variant) in their sequence [105,106].
[c] presents positive reaction in an *in vitro* chitin-binding assay. N.B.: The number associated with the names of the HaCP- and CpCP- proteins corresponds to the molecular mass of these proteins.

constituents, as generally observed in transitory biomineralised structures, showed about 12 polypeptides with acidic isoelectric points. Comparison of this pattern with those corresponding to proteins extracted at different stages of the moulting cycle led to evidence of a SM-specific 23 kDa polypeptide [82]. Biochemical characterization of this polypeptide, called Orchestin, revealed that it is an unglycosylated but phosphorylated calcium-binding acidic protein [123]. Furthermore, its ability to bind calcium is linked to phosphorylations on serines [61]. The corresponding cDNA and gene were sequenced [123]. The molecular mass calculated from the deduced sequence of the protein is 12.4 kDa. The discrepancy of this calculated mass with the SDS-PAGE deduced one is attributed to the richness of the protein in acidic amino acids (30%) and the

presence of post-translational modifications [123]. The same kind of explanations can be applied to the other proteins listed in Table 1, with which such a divergence was observed.

The spatio-temporal expression of the gene encoding Orchestin was studied by in situ hybridisation and Northern blotting [61]. The gene is expressed only in the posterior ceca at a rate that increases from negligible during the inter-moult through the pre-moult period to culminate at the end of the pre-moult concomitant to the calcium storage process. After ecdysis, the expression remains not negligible and decreases until the basic rate is attained at the beginning of the next inter-moult. Contrarily to GAMP, no Orchestin-immunoreactivity was observed at the level of the cuticle. Recent ultrahistochemical investigations by electronic microscopy have led to the conclusion that Orchestin is also a component of the organic matrix of the transepithelial post-moult spherules of calcium resorption.

The only common feature of all these sequenced crustacean matrix proteins are that they are acidic, as generally observed for matrix proteins of calcified structures. Comparison of the sequences of these molecules does not permit to find homologies, identities, nor particular domains, except for the Rebers–Riddiford (RR) motif [105,106,144]. This RR consensus region is suspected to adopt a two-stranded β-sheet conformation, which dictates the helicoidal structure of the cuticle [66,67,106]. The possible presence of such a domain could lead to define two groups of cuticular proteins: those linked to the chitin filament system and the others, involved in the calcification process or simply in the structural elaboration of the matrix framework. Nevertheless, the characteristics of CAP-1 and CAP-2 suggest that a protein could be both calcium-precipitation interfering and chitin binding [70,71].

5. Hormonal regulation of calcium turnover and storage in crustaceans

Whatever the origin of the calcium used for the hardening of the carapace, calcium metabolism is of great intensity in crustaceans and results from a very precise and hormonally controlled balance between the cuticle and the environment or the storage sites, coordinated with the moulting cycles. Little is known about the hormonal regulation of such calcium translocations.

In arthropods, most events of a moulting cycle are under ecdysteroid control [25,26,34,87,88,115,118]. If other active ecdysteroids have been found, 20-hydroxyecdysone (20E) can be considered as the active moulting hormone in most of the crustaceans, as in many insects. The moulting hormone is generally synthesized, from cholesterol, in cephalic organs called Y-organs as an inactive precursor, the ecdysone for the most crustaceans. During inter-moult, ecdysone secretion in Y-organs is inhibited by another hormone, MIH (Moult-Inhibiting Hormone), synthesized in the eyestalks by the X-organs, a group of neurosecretory neurones, and released from an adjacent neurhemal organ, the sinus gland [25,26,34,47,78,115].

One of the target tissues of the moulting hormone is the epidermis. Moreover, it has been shown that this hormone has implications in the general crustacean calcification and/or decalcification processes, but the mechanism of control, probably indirect, is still unknown [46,47,56,81,88,139]. It is worth noting that, in general, ecdysteroid titre becomes very low just before ecdysis, which permits the synthesis of the eclosion hormone responsible for this event. This ecdysteroid level remains very low during the post-moult period. Thus, calcification of the cuticle occurring during this period is surely independent of the moulting hormone. At the molecular level, only some changes of protein expression in the cuticle epidermis have been observed after in vivo injection of the moulting hormone, 20E [80,100,119,124,133]. Nevertheless, if some proteins stimulated by the moulting hormone have been characterized, none of these seems to be involved in the cuticle calcification or decalcification process [27,29].

The role of the moulting hormone in the elaboration of gastroliths has been suspected for many years [85–87,143], notably by eyestalk removal experiments. Ueno and colleagues [134] have clearly shown the presence of ecdysteroid-binding sites in the stomach and gastrolith epithelium (in the cytoplasm, then the nuclei) during the elaboration and resorption periods of the gastroliths. More recently, Nagasawa et al. [129] have shown, by culture of gastrolith discs with their surrounding tissues, that expression of the gene encoding GAMP, the insoluble protein from the matrix gastrolith described above [72,73,122,129], is induced by 20E.

Previous experiments have shown a possible involvement of ecdysteroids in the calcium turnover in *O. cavimana* [46,47,114]. The ecdysteroid titre was measured at different stages of a moulting cycle in both haemolymph and whole body extracts by radioimmunoassay [53]. This titre increases during the pre-moult period concomitant to both calcium storage and expression of the gene coding for Orchestin [53,123]. Thus, we tested the involvement of 20-hydroxyecdysone (20E), the moulting hormone of *Orchestia*, in the regulation of the elaboration of concretions. First, we demonstrated that injection of 20E in animals at the beginning of the pre-moult not only induced an early ecdysis, but also, 6 h later, an electrophoretic pattern of the whole PC proteins similar to a late pre-moult one [82]. Second, study of *orchestin* expression showed a significant stimulation of the gene by 20E, but this effect is probably indirect as demonstrated by the inhibitory action of cycloheximide (a protein synthesis inhibitor) on this stimulation [123]. In summary, it seems that if ecdysteroids are involved in the cuticle calcification and calcium storage control, this control is certainly indirect.

The involvement of other specific crustacean hormones in calcium metabolism is not clear. Nevertheless, some vertebrate-type hormones [35,54] have been suspected to play a role in crustacean calcium metabolism.

For example, hormones that resemble the calcitonin (CT) and the calcitonin gene-related peptide (CGRP) of vertebrates have been detected in some crustaceans [11,12,22,36,54,79,135]. Some results showing a possible involvement of these hormones in calcium metabolism were obtained.

In *Orchestia*, a CT-like molecule was detected and its titre calculated at different stages of a moulting cycle [54]. Effect of in vivo injection of salmon CT on calcium haemolymph rate suggests that this hormone could induce hypocalcemia [112]. A strong CT-immunoreactivity detected in the central nervous system of *Orchestia*, and a weaker one at the level of the posterior ceca led the authors to hypothesize that a CT-like hormone could be synthesized in the SNC as a neurohormone, and then participate to the regulation of calcium translocation in the storage organs [55].

Arlot-Bonnemains and associates have purified from a lobster a cysteine protease, which is immunologically related to salmon CT and human CGRP, which interacts with the CT and CGRP specific receptors, stimulates adenylate cyclase, and induces hypocalcemia and hypophosphatemia when injected in young rats [13].

Finally, other molecules that resemble hormones regulating vertebrate calcemia, such as parathyroid hormone (PTH) or vitamin D, could be active in crustaceans [81,88,94,95]. Nevertheless, the function of such molecules, which are not chemically characterized so far in crustaceans, remains unclear.

6. Conclusion

Crustaceans are a remarkable group of animals as regards the biomineralisation world. Firstly, these animals are able to elaborate cyclically a mineralised exoskeleton. Furthermore, they can also synthesize cyclically other calcified structures, not only different in their morphology but also in their mineralogical composition and in the adopted polymorph (crystalline and/or amorphous, for example). The biomineralisations elaborated as calcium storage deposits are transient structures, reservoirs of calcium ions quickly available after ecdysis, and, for this reason, the storage structures are essentially in amorphous form. To understand why these amorphous mineralisations are stabilized in time in comparison with the same very unstable purely chemical mineral is also of great interest [6,20]. In fact, it seems that amorphous calcium carbonate (ACC) is more widely distributed than previously suspected [2,138]. On the one hand, many invertebrates seem to elaborate calcium carbonate structures firstly under the ACC precursor form, which is transformed into one of the crystalline calcium carbonate polymorphs [17,103,104]. On the other hand, it has been shown that ACC can coexist with one of the calcium carbonate crystalline forms: ACC and calcite coexist in well-defined domains in the exoskeleton of ascidians and calcareous sponges, for example [8].

It seems that both of these processes, transformation with time of one polymorph into another and coexistence of two polymorphs, could be involved in the calcification of the crustacean cuticle.

The organic matrices of crustacean biomineralisations are relatively accessible, notably those from the storage structures (because of their mainly amorphous form easier to decalcify than their crystalline counter-

parts). Unfortunately, few molecules really involved in calcification have been well characterized so far in crustaceans. Moreover, if numerous proteins begin to be sequenced in other invertebrates, as in vertebrates, their actual function is not fully understood. The knowledge of the physical and chemical features of each matrix component is a prerequisite to clarify, at the molecular level, how a biomineralisation is elaborated, how the matrix molecules influence the nature of the polymorph obtained, as suggested [1,6–8,15,32, 104], and also to determine, by means of comparative studies, why and how calcification could have emerged on Earth. The sequencing of the genes encoding these constituents could lead us to the understanding of the appearance of calcification processes and of the strategy used by evolution to construct different mineralising systems: convergence of different biological systems, adaptation to similar mineralising roles, or exaptation of an ancestral biomineral system in many separate lineages?

Other interesting characteristic of calcium metabolism in crustaceans is that calcium transport is performed by epithelia very similar to some vertebrate ones, and in this way they represent good models to understand how they function. Similarly, calcium pumps and enzymatic systems associated with the vertebrate calcium-transporting epithelia seem operational in the same kind of crustacean tissues.

Finally, crustaceans represent useful models for the understanding of hormonal regulation of the balance occurring cyclically between two sources of calcium, and more generally of hormonal control of mineralising systems. Notably, the tripartite vertebrate calcium-controlling system (CT/PTH/Vitamin D), which probably has emerged from invertebrates, could regulate crustacean calcium metabolism.

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