Glycosylation

Subjects: Biochemistry & Molecular Biology
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Post-translational modifications are one way that biomineral-associated cells control the function and fate of proteins. Of the ten different types of post-translational modifications, one of the most interesting and complex is glycosylation, or the covalent attachment of carbohydrates to amino acid sidechains Asn, Ser, and Thr of proteins. There are several biomineral-associated glycoproteins that have been characterized, and a subset of these have been the subject of intensive in vitro experimentation. These studies indicate that glycosylation does not alter the inherent function of the biomineralization protein; rather, it either accentuates or attenuates protein functionality. In essence, glycosylation gives the cell the “last word” as to what degree a biomineralization protein will participate in the biomineralization process.

Keywords: biomineralization ; glycosylation ; proteins

1. Introduction

Over the last forty years there has been a concerted effort to understand how organisms craft biomineralized skeletal structures for survival [1][2][3]. This effort has focused along two lines: first, how do mineral crystals or amorphous minerals form under biological conditions? Recent evidence points to a mineral precursor nucleation process involving nanoparticle synthesis followed by particle assembly into larger mineral mesoscale structures [4][5][6]. Second, what agents are biosynthetically created by these same organisms to manage the mineral formation process? With regard to the latter, it has been well documented that the genomes of biomineralizing organisms code for families of proteins that are mineral-specific and unique with regard to primary sequence construction and structure [7][8][9][10]. The appearance of these proteins in the extracellular matrix during mineral formation is a clear attempt by cells to regulate the nucleation and assembly stages that lead to the final mineral product of the skeletal elements that are necessary for organism survival. Thus, to understand how biominerals form into larger, useful structures, we must understand the role or function that these proteins play in nucleation and particle assembly.

In the majority of eukaryotic organisms, the overall complexity of the biomineral proteomes is augmented by a process known as post-translational modification [11][12][13]. In essence, once a nascent protein polypeptide chain is produced on the ribosomal complexes, in some cases the cells express enzymes that perform further covalent modifications of certain amino acid sidechains on the protein, thereby altering the functionality of these sidechains. These covalent modifications occur in compartments that are separate from the cell cytoplasm [e.g., Golgi apparatus, rough endoplasmic reticulum (rER)], intracellular vesicles)[11][12][13]. A summary of common post-translational modifications (Table 1)[12] indicates that certain amino acid sidechains are targeted by cells for covalent modification. These modifications are performed by

Table 1. Common post-translational modifications of proteins.

Post-Translational Modification	Type of Modification	Site(s) of Modification
Disulfide bond formation	Thiol oxidation to form -S-S- bond	Cys
Hydroxylation	Addition of -OH group	Pro, Glu
Ubiquitination	Addition of ubiquitin protein(s)	Lysine
Lipidation	Esterification to lipid group	Cys, Lys, N-terminal Gly
SUMOylation	Small Ubiquitin-like Modifier protein	Lys

Acetylation	Addition of acetyl group	N-terminus, Lys, Ser
Methylation	Addition of methyl group	Lys, Arg, termini
Phosphorylation	Addition of phosphate group	Ser, Thr, Tyr
Glycosylation	Addition of carbohydrate group(s)	Asn, Thr, Ser
Nitration	Addition of nitrogen	Tyr
Acylation	Addition of acyl chain	Cys, Gly, Ser, Thr, Lys

intracellular enzymes and in the majority of cases the finalized protein product is transported to its intended destination using vesicles [11][12][13]. In essence, genomic information is translated into proteomic information, and in some instances, this proteomic information can further be modified and thus diversified in terms of structure and ultimately function [11][12][13].

Perhaps the most complex post-translational modification process is *glycosylation*, or the addition of one or more carbohydrate monomers (known as monosaccharides) to specific amino acid sidechains on a protein, thus converting the polypeptide into a *glycoprotein* [11][12][13][14][15][16]. There are three classifications of glycoproteins, depending on which amino acids serve as attachment points for carbohydrates [11][12][13][14][15][16]: 1) O-linked, where the oligosaccharide attachment occurs on Ser and/or Thr residues and is performed in the Golgi apparatus; 2) N-linked, where the oligosaccharide attachment occurs on Asn and is performed within the endoplasmic reticulum (ER); and 3) hybrid, in which a glycoprotein has O-linked (Ser, Thr) glycans and N-linked (Asn) glycans.

Several features contribute to the overall complexity of glycosylation [11][12][13][14][15][16]: a) The number of carbohydrate groups added to a single amino acid sidechain site can vary; b) the number and type of amino acid sites for attachment on a given protein can vary; c) at a given attachment point on a protein, the carbohydrate groups can be constructed as linear or branching chains; d) the hydroxyl-rich carbohydrate groups themselves can be modified by the addition of chemical groups, such as carboxylate, sulfate, N-acetyl amino, and hydroxyl. Thus, unlike other post-translational modifications, glycosylation represents a unique opportunity for the cell to combine two very different macromolecular building blocks (amino acids, carbohydrates) into one macromolecule, which in turn may have a significant impact on the function and distribution of this protein class within a biomineralizing system.

2. Glycoproteins in biomineralization: An overview

Table 2 provides a summary of specific mineral matrix proteins that have been identified as glycoproteins and report the complete amino acid sequence [17][18][19][20][20][21][22][23][24][25][26][27][28][29][30]. Admittedly, this table is sparse, and at the time of this writing very few biomineral-associated glycoproteins have complete protein sequence data or oligosaccharide composition/sequence data available. Note that some studies have identified glycoproteins in the extracellular matrices of different organisms [31][32][33][34][35], but to date these proteins have not been sequenced nor rigorously characterized. The majority of the identified biomineralization glycoproteins are found in association with calcium-based biominerals [17][18][19][20][20][21][22][23][24][25][26][27][28][29][30][31][32][33][34][35]; however, it should be acknowledged that glycoproteins may eventually be identified in other non-calcium based biominerals, such as magnetite (Fe_3O_4)[36] or silicates (SiO_4)[37].

Table 2. Biomineral-associated glycoproteins.

Protein	Organism	Tissue	Associated Mineral Phase
SpSM30 A-F	*S. purpuratus* (sea urchin)	Embryonic spicule	Magnesium Calcite ($CaMgCO_3$)
AP24	*H. rufescens* (abalone)	Shell nacre	Aragonite ($CaCO_3$)
Enamelin	Vertebrates	Tooth enamel	Hydroxyapatite ($CaPO_4$)

SIBLING Family	Vertebrates	Bone, tooth dentine	Hydroxyapatite ($CaPO_4$)
EDIL3	Avian	Eggshell	Calcite ($CaCO_3$)
MFGE8	Avian	Eggshell	Calcite ($CaCO_3$)
Proteoglycans	Vertebrates	Bone, tooth dentine	Hydroxyapatite ($CaPO_4$)

3. The impact of glycosylation on protein function

Does the attachment of oligosaccharides affect the molecular behavior of a polypeptide chain? To answer this question, one could envision a comparative study wherein the function of an unglycosylated variant of a given protein is contrasted against that of a glycosylated variant, with each possessing the identical primary sequence. Here, the only variable would be the presence (or absence) of oligosaccharide chains.

Recently, this type of study was executed on two proteins, AP24 (aragonite nacre layer, Pacific red abalone *H. rufescens* [25] and SpSM30B/C (calcitic spicule matrix, *S. purpuratus*, purple sea urchin)[24][28][29][30]. Both proteins have been the subject of *in vitro* glycosylation studies in insect cells, where it was discovered that AP24 and SpSM30B/C belong to the hybrid classification, i.e., they consist of N- and O-linked linear and branching oligosaccharide chains [26][27]. Interestingly, the glycosylated variants of AP24 and SpSM30B/C both contain anionic monosialylated, bisialylated and monosulfated, bisulfated monosaccharides [26][27]. Given that both proteins inhabit a Ca(II)-rich environment *in vivo*, the anionic monosaccharides could serve as putative sites for Ca(II)-protein or mineral-protein interactions. To a certain extent, both proteins are similar in function: they are involved in the formation of the organic matrix, forming hydrogel particles that assemble mineral nanoparticles [26][27]. In addition, both protein hydrogels become occluded within calcium carbonates and modify the material and surface properties of the minerals they inhabit [26][27]. From these two studies we note a trend where glycosylation does not change the intrinsic function of the polypeptide chain; rather, the attachment of anionic oligosaccharide moieties either 1) attenuates specific functions or has no effect (AP24); or, 2) accentuates protein functionality [SpSM30B/C]. Other studies with multiple glycoproteins will hopefully confirm this trend or provide evidence of other effects that oligosaccharides impose upon polypeptides.

In addition to modulating the mineral formation process, a key role of biomineralization proteins is the assembly and organization of multiple proteins to form an organic matrix within which the nucleation and crystal growth processes take place. Studies were conducted on molluscan (AP7, AP24, *H. rufescens*)[28] and sea urchin (SpSM50, SpSM30B/C, *S. purpuratus*)[29][30] recombinant two-protein systems. Here, each pair of proteins is known to co-exist *in vivo* within the extracellular matrix [24]. Interesting contrasts were noted: a) AP7 and AP24 protein complexes form as a direct result of polypeptide – polypeptide chain recognition and not polypeptide – oligosaccharide recognition. However, the presence of anionic oligosaccharides on AP24 appears to modulate the intensity of AP7 – AP24 protein - protein interactions and potentially stabilizes the AP24 conformation upon binding to AP7. (b) The formation of a SpSM50 – SpSM30B/C complex requires glycosylation and, in contrast to the AP7 - AP24 study described above, these interactions were found to be *Ca(II) – independent* for both variants [29][30]. The glycosylation requirement clearly indicates that SpSM50 polypeptide sequence recognizes and binds to the glycan moieties on the surface of SpSM30B/C.Notably, the SpSM50 protein possesses a glycan-binding motif known as the C-type lectin-like domain, and it is believed that this region interacts with the glycan groups of SpSM30 (Figure 1)[29][30].

Figure 1. Predicted three-dimensional structures of *S. purpuratus* SpSM50 protein, in ribbon representation. Note that the SpSM50 protein possesses a surface-accessible C-type lectin carbohydrate binding domain, which presumably acts as a site for interaction with SpSM30B/C glycan groups.

4. Summary

From the foregoing, we can observe that glycosylation provides an additional degree of control over extracellular protein function by either accentuating or attenuating the intrinsic functionality of the polypeptide sequence. In a sense, the cell can have the “last word” as to the degree of participation within the biomineralization process. In some cases (e.g., AP24), the oligosaccharides stabilize the conformation of the glycoprotein, which is a known trait of N-linked oligosaccharides [12][13][14][15][16]. The author proposes that glycosylation can serve several purposes vis a vis the biomineralization process: 1) “tweak” or “tune” protein mineralization function to suit the situation or need; 2) act as a site for molecular recognition and binding with other matrix proteins; 3) conformationally stabilize a protein, thereby enhancing functionality; 4) create additional anionic sites for ionic [e.g., Ca(II)], mineral, or water interactions; 5) invoke cell activation or deactivation via binding to outer membrane receptor proteins. Clearly, there may be other benefits that arise from glycosylation, and thus this process represents a powerful method that cells can exploit to create skeletal elements under ambient or extreme conditions [1][2].

The biomineralization field is still in its infancy with regard to understanding the role that glycoproteins and their associated oligosaccharides play in the skeletal formation process. It is hoped that additional studies will provide more details about theser modified proteins.

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