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# Intrinsically Disordered Proteins in Biomineralization

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## 1. Introduction

Intrinsically disordered proteins (IDPs) have the potential to play a unique role in the study of proteins and the relationships between structure and function. Intrinsic disorder affects chemical and cellular events such as cell signaling, macromolecular self-assembly, protein removal and crystal nucleation and growth. This chapter explores the structural principles by which IDPs act and reveals the prevalence of IDPs in the field of biomineralization. It has been demonstrated that proteins involved in biomineralization are frequently very extended and disordered. Moreover, the disordered structure is integral to how these proteins fulfill their functions. We have focused on the analysis of polypeptide folding, the role of post-translational modifications, predictions of the structural disorder and the degree of disorder in secondary structures. Computational and biophysical strategies to analyze the secondary structures and evaluate the degree and nature of "disorder" in proteins are described. Biomineralization is the result of the orchestration of a series of protein-protein, protein-mineral and protein-cell interactions. Identifying unfolded functional domains in cell signaling may have a great impact in the study of tissue regeneration and biomineral formation. IDPs are typically organic components of biominerals. It is believed that they could act as a regulatory coordinator for specific interactions of many proteins, and thus many physiological processes such as formation of dentin and bone, the formation of sea urchin and crustacean exoskeletons. Here, we review what is currently understood about the molecular basis of biomineral formation. This includes protein interaction with metal ions, post-translational modifications, interactions with other proteins, or other factors that induce the formation of crystal shape and size along with the proper polymorph selection in relation to the role of IDPs.

## 2. Intrinsically disordered proteins

The history of IDPs goes back to the 1960s, with Linus Pauling's observation of the existence of regions in proteins with a disordered structure (Pauling & Delbruck, M., 1940). However, only a small group of researchers like Dunker, Uversky, Wright, Dyson, Tompa and others during the next forty years demonstrated that it was possible to depart from the paradigm that a protein's function is closely affiliated with its structure (Dyson & P.E. Wright, 2005; Tompa, 2011; Uversky & Dunker, 2010; P.E. Wright & Dyson, 1999). Currently, it is believed

that 20-50% of eukaryotic proteins contain at least one fragment belonging to the class of IDPs (Babu et al., 2011; Dunker et al., 2000). It is well known that globular proteins decrease their activity in a denatured state when a solution is subjected to high temperatures or chemical denaturants. The "structure-function" paradigm was not contested for many years until experimental data began to show that there was no stable three-dimensional structure for some protein fragments that had been attributed to particular functions. These proteins are in whole or in part, in contrast to globular proteins, heterogeneous ensembles of flexible molecules, unorganized and without a defined three-dimensional structure. According to these properties, the proteins are referred to as natively unfolded, intrinsically unfolded (IUP), intrinsically unstructured or intrinsically disordered (Dunker et al., 2005; Dyson & Wright, 2005; Tompa, 2005; Uversky, 2002). It has been previously shown that structural disorder is characteristic of proteins involved in important biological processes such as signal transmission, regulation of cell cycles, regulation of gene expression, activity of chaperone proteins, neoplastic processes, and biomineral formation (Dyson, 2011; Tompa, 2011; Uversky, 2010).

These processes require a series of dynamic macromolecular interactions and IDPs seem to be specially created for their functions. An IDP's meta-stable conformation allows it to bind to its protein partners as well as interact with high specificity and relatively low affinity. Furthermore, there is some experimental evidence showing that IDPs may interact with multiple partners, changing or adjusting the structures and functions of their partners (Tompa, 2005). Comparative analyses of the amino acid sequences of all currently known IDPs have shown common features. These proteins are characterized by amino acid compositions enriched with residues like A, R, G, Q, S, P, E and K that promote a disordered structure, with the small participation of other residues like W, C, F, I, Y, V, L and N, which simultaneously promote an ordered structure (Dunker et al., 2001). IDPs can be classified into five groups based on their relative functions: entropic chains, effectors, assemblers, scavengers, and display sites. Entropic chains act as flexible linkers between the globular domains of multidomain proteins. Effectors bind and modify the activity of a partner. Assemblers are able to simultaneously bind several ligands as multimolecular assemblies. Scavengers store or neutralize small ligands. Finally, display sites promote specific interactions within the active sites of enzymes that facilitate post-translational modifications (Tompa, 2002).

## 2.1 Methods for analyzing IDP structure

Based on the amino acid composition of IDPs, a number of algorithms have been proposed that predict regions containing a disordered structure. The most frequently used are PONDR (Romero et al., 2001), DISOPRED 2 (Ward et al., 2004a, 2004b), IUPred (Dosztanyi et al., 2005), GLOBPLOT 2 (Linding et al., 2004) and FoldIndex (Uversky et al., 2000). More algorithms can be found in the DisProt database (Sickmeier et al., 2007). These algorithms operate on the principle of a neuronal network "trained" using amino acid sequences belonging to experimentally confirmed IDPs. It has been observed that sequences that have low complexity or are abundantly charged and/or frequently post-translationally modified (e.g. phosphorylated) usually adopt a stretched, unordered conformation (Romero et al., 2001). IDPs are often characterized by charge-hydropathy

plots. Based on the normalized net charge and mean hydrophobicity, proteins can be categorized into either globular folded proteins or IDPs. IDPs are specifically localized within a unique region of charge-hydrophobicity proteins (Uversky et al., 2000). Figure 1A shows a charge-hydrophobicity plot for experimentally confirmed IDPs and globular proteins, while Figure 1B presents a charge-hydrophobicity plot for proteins involved in biomineralization.

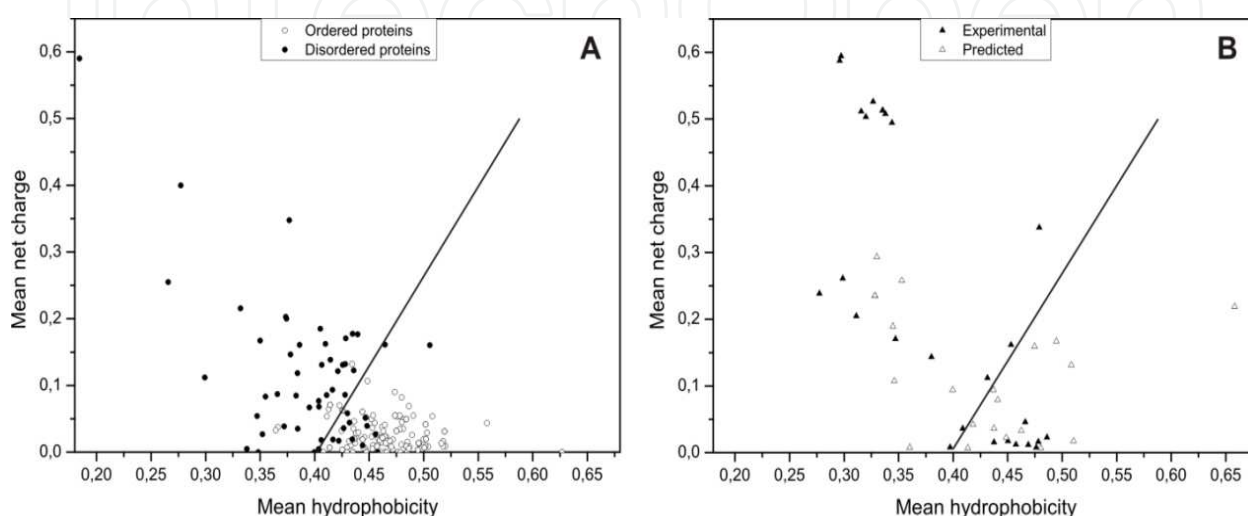


Fig. 1. Charge-hydrophobicity plots. The solid line represents the contractual boundary between disordered and ordered proteins. (A) Comparison of charge-hydrophobicity for IDPs (black dots) and globular proteins (white dots). (B) Proteins involved in biomineralization represented on the charge-hydrophobicity plot. Black triangles show experimentally confirmed IDPs, while white triangles represent proteins whose secondary structure has not been studied.

Even this simple analysis indicates that many proteins involved in biomineralization could be IDPs. However, computer predictions themselves can not be relied on as the sole evidence of the absence of structural order in a protein. Such evidence only invites further experimental study, which could include X-ray diffraction analysis (Dunker & Obradovic, 2001), multidimensional nuclear magnetic resonance spectroscopy (NMR) (Bai et al., 2001), circular dichroism spectroscopy (CD) (Tompa, 2002), differential scanning microcalorimetry (DSC) (Mendoza et al., 2003), X-ray scattering at small angle (SAXS) (Millett et al., 2002).

### 3. IDPs found in calcium phosphate related mineralization

In bone and dentin, collagen acts as a structural matrix whereas hydroxyapatite (HA) nucleation is regulated by the acidic phosphoproteins (Chen et al., 1992; Glimcher, 1989). These non-collagenous proteins (NCPs) play crucial roles in the organization of the collagen matrix and in the modulation of HA crystal formation (Ganss et al., 1999). NCPs

are often classified as IDPs. Some examples of IDPs engaged in HA formation are presented below.

### 3.1 SIBLINGs

Small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) with NCPs are involved in the mineralization of bone and dentin (George & Veis, 2008; Qin et al., 2004). Within the family of human SIBLINGs there is limited sequence similarity; however, they share common features, such as: (i) similar gene organization and chromosome localization, (ii) RGD (arginine-glycine-aspartate) motifs mediating cell attachment/signaling *via* their interactions with cell-surface integrins, (iii) extensive post-translational modifications like phosphorylation and glycosylation, (iv) abundance of acidic residues, (v) calcium ions and collagen binding ability (George & Veis, 2008; Qin et al., 2004), (vi) intrinsically disordered molecular character (Tompa, 2002). The SIBLINGs family includes osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE) and dentin sialophosphoprotein (DSPP). DSPP gives rise to two mature products, dentin phosphoprotein (also called phosphophoryn) (DPP) and dentin sialoprotein (DSP) (George & Veis, 2008; Qin et al., 2004).

#### 3.1.1 DMP1

The highly acidic protein (D and E constitute 29% of all residues) DMP1 acts as a nucleator for HA deposition *in vitro* (He et al., 2003a). The disordered character of DMP1 has been shown using several methods (Tab. 1). CD and FTIR measurements have shown that DMP1 has a random structure in solution, however upon calcium ions binding DMP1 undergoes a slight conformational change to a more ordered structure. SAXS and DLS confirmed a calcium-induced disorder-to-order transition in DMP1 leading to oligomerization (Gericke et al., 2010; He et al., 2003b). Moreover, it has been shown that the DMP1 molecule assumes an elongated shape (He et al., 2005a). Further studies have revealed that two specific acidic clusters (ESQES and QESQSEQDS) in DMP1 are responsible for the calcium-induced oligomerization and *in vitro* nucleation of apatite crystals (He et al., 2003b). This calcium-induced conformational change of DMP1 could be the structural basis for biocomposite self-assembly (He et al., 2003b).

The lack of a rigid structure enables DMP1 to serve multiple functions, not only in biomineralization, but also in osteoblast differentiation and maturation (Narayanan et al., 2003). Nonphosphorylated DMP1 is localized in the nucleus where it acts as a transcriptional component for the activation of matrix genes involved in mineralized tissue formation (Narayanan et al., 2003). It binds the DSPP gene promoter and activates DSPP gene expression. Calcium ions released from intracellular stores bind DMP1 and induce in DMP1 a conformational change and phosphorylation by casein kinase 2 (CK2). Finally, the phosphorylated protein is exported to the extracellular matrix, where it acts as a nucleator of hydroxyapatite (Narayanan et al., 2003). The DNA binding domain is localized within the C-terminal region of DMP1 (Narayanan et al., 2006). Extracellular DMP1 also has the ability to strongly bind the H factors, integrin  $\alpha\beta 3$  and CD44 (Jain et al., 2002), and it is specifically involved in signaling via extracellular matrix-cell surface interaction (Wu et al., 2011).

Organism	Protein	pI	Methods	Reference
Mammals	DMP1	4.0	CD, DLS, FTIR, SAXS	(Gericke et al., 2010; He et al., 2003a, 2003b)
	DPP	2.8	CD, NMR, SAXS	(Cross et al., 2005; Evans et al., 1994; Fujisawa & Kuboki, 1998; George & Hao, 2005; He et al., 2005b; Lee et al., 1977)
	BSP	4.1	CD, NMR, SAXS	(Fisher et al., 2001; Tye et al., 2003, 2005; Wuttke et al., 2001)
	OPN	4.4	CD, NMR, FTIR	(Fisher et al., 2001; Gorski et al., 1995)
	amelogenin	6.6	CD, NMR	(Buchko et al., 2010; Delak et al., 2009b; Ndao et al., 2011; Shaw et al., 2008)
	statherin	8.0	CD, NMR	(Long et al., 2001; Naganagowda et al., 1998; Raj et al., 1992)
	lithostathine	5.7	CD	(Gerbaud et al., 2000)
<i>Haliotis rufescens</i>	AP7	5.2	CD, NMR	(Kim et al., 2004, 2006a; Michenfelder et al., 2003; Wustman et al., 2004)
	AP24	5.3	CD, NMR	(Michenfelder et al., 2003; Wustman et al., 2004)
	Lustrin A	8.1	NMR	(Wustman et al., 2003; Zhang et al., 2002)
<i>Pinctada fucata</i>	n16	7.5	CD, NMR	(Amos et al., 2011; Collino & Evans, 2008; Kim et al., 2004, 2006b)
	ACCBP	4.7	CD	(Amos et al., 2009)
	PFMG1	7.9	CD	(Liu et al., 2007)
<i>Atrina rigita</i>	Asprich	2.7-3.5	CD, NMR	(Collino et al., 2006; Delak et al., 2009a, 2008; Kim et al., 2008; Ndao et al., 2010)
<i>Procambrus clarkii</i>	CAP-1	3.9	CD	(Inoue et al., 2007)
<i>Strongylocentrotus purpuratus</i>	SM50	10.8	CD, NMR	(Xu & Evans, 1999; Zhang et al., 2000)
	PM27	8.1	CD, NMR	(Wustman et al., 2002)
<i>Danio rerio</i>	Stm	4.1	CD, gel filtration	(Kaplun et al., 2008, 2009)

Table 1. IDPs involved in biomineralization of calcium carbonate and phosphate for which a disordered structure has been confirmed experimentally.

3.1.2 DSPP

DSPP undergoes proteolytic cleavage to DPP and DSP. DPP is most abundant in dentin, but also present in bone (George & Hao, 2005; Lee et al., 1977). At least 75% of the DPP sequence (isolated from dentin) is composed of S and D residues and 85-90% of the S residues are phosphorylated (George & Veis, 2008; He et al., 2005b; Huq et al., 2000). In solution DPP isolated from dentin behaves as a fairly extended, random-chain molecule due to electrostatic repulsion (Table 1), as has been shown by CD studies (Lee et al., 1977). The



presence of calcium ions reduces DPP solubility, which indicates aggregation of the protein (Lee et al., 1977). It has been demonstrated that nonphosphorylated DPP has lower calcium binding ability than the phosphorylated form and induces amorphous calcium phosphate formation, while the phosphorylated form promotes plate-like apatite crystals (He et al., 2005b). SAXS studies revealed a calcium-induced conformation change from an extended structure to a more compact one, but only in phosphorylated DPP. Nonphosphorylated DPP was disordered irrespective of the presence of calcium ions at various concentrations (George & Hao, 2005; He et al., 2005b). NMR spectroscopy also confirmed high mobility and flexibility of DPP in the absence of calcium ions, and decreased mobility in the presence of calcium ions (Cross et al., 2005; Evans et al., 1994). Solid-state NMR spectroscopy enabled investigation of the DPP structure when bonded to HA. The secondary structure of DPP bound to crystal was very extended and largely disordered. A majority of DPP residues interacted with crystal. (Fujisawa & Kuboki, 1998). This disordered molecular structure facilitates DPP’s extension across the surface of a crystal and allows it to cover the surface with only a small number of molecules, all of which results in a highly inhibitory effect on crystal growth (Fujisawa & Kuboki, 1998).

While DPP structure has been extensively explored, DSP structural studies are still unavailable. However, bioinformatic predictions strongly suggest that DSP is also an IDP (Table 2).

Organism	Protein	pI	Overall percent disordered		
			IUPred	DISOPRED2	PONDR
Mammals	DSP	4.5	100	100	73
	MEPE	8.6	95	60	64
<i>Picntada fucata</i>	Aspein	1.5	100	100	100
	Prismalin-14	3.9	0	0	9
<i>Patinopecten yessoensis</i>	MSP-1	3.2	97	98	96
<i>Pinna nobilis</i>	Calprismin	4.9	12	0	74
<i>Crassostrea nippona</i>	MPP1	2.2	100	98	97
<i>Nautilus macromphalus</i>	Nautilin-63	9.2	66	4	41
<i>Procambrus clarkii</i>	GAMP	4.2	63	73	63
	CAP-2	4.2	18	2	45
	Casp-2	4.3	33	8	68
<i>Orchestia cavimana</i>	Orchestin	4.5	58	34	69
<i>Cherax quadricarinatus</i>	GAP 65	5.0	2	0	11
	GAP 10	5.5	4	0	37
<i>Penaeus japonicus</i>	Crustocalcin	3.9	45	37	81
<i>Strongylocentrotus purpuratus</i>	SM30	6.2	25	0	28
	SM32	8.3	44	44	47
	SM37	10.4	60	43	56
	phosphodontin	3.9	96	94	94
<i>Oryzias latipes</i>	Stm-like	3.8	100	98	94
<i>Oncorhynchus mykiss</i>	OMM-64	3.5	97	96	92
<i>Gallus gallus</i>	Ovocleidin-116	6.6	91	57	78

Table 2. Bioinformatic predictions of a disordered structure in proteins involved in biomineralization.

All three bioinformatic tools (IUPred, DISOPRED2, and PONDR) predict that DSP is largely or completely disordered. DSP, similarly to DPP, also has a low pI value, which causes electrostatic repulsion that leads to an extended structure. However, this presumption requires experimental confirmation.

### 3.1.3 BSP

BSP is a multifunctional protein involved in cell attachment, signaling, HA binding, HA nucleation and collagen binding (Ganss et al., 1999). BSP is relatively specific to skeletal tissues and is the most abundant protein in bone (Bianco et al., 1991). NMR, CD and SAXS studies of BSP showed a high level of disordered structure (Table. 1) (Fisher et al., 2001; Tye et al., 2003, 2005; Wuttke et al., 2001). Post-translationally, unmodified BSP has lower rates of migration during SDS-PAGE, as do other IDPs (Stubbs et al., 1997). BSP visualized by electron microscopy after rotary shadowing was an extended monomer possessing a globular structure, probably on the C-terminus of the protein (Wuttke et al., 2001). BSP treated with 6 M Gdm-HCl lost its residual  $\alpha$ -helix and  $\beta$ -sheet structure. Structural studies indicated that BSP had IDP-like characteristics, but not a random structure (Wuttke et al., 2001). Calcium ions had a significant effect on BSP conformation, in contrast to DMP1 and DPP (Tye et al., 2003; Wuttke et al., 2001). The flexibility and plasticity of BSP may enable cell attachment to HA. Additionally, BSP has a strong affinity for HA (Fujisawa et al., 1997), while on the other hand, RGD sequences allow integrin binding (Fujisawa et al., 1997; Oldberg et al., 1988). Hence, the extension and flexibility of BSP may be advantageous to its function as a bridge for the cell attachment of HA (Tye et al., 2003). The intrinsic disorder of BSP seems to be important for interacting with type I collagen (Tye et al., 2005).

### 3.1.4 OPN

While DPP, BSP and DMP1 are relatively specific to bone and teeth, OPN is also present in the brain, kidney, smooth muscle, macrophages, inner ear and body fluids (milk, urine, bile) (George & Veis, 2008; Gericke et al., 2005; Kazanecki et al., 2007). Its ubiquitous expression pattern and variations in phosphorylation, glycosylation and sulphation suggest that OPN fulfills many different functions (George & Veis, 2008). Moreover, OPN controls the formation of calcium phosphate (Goldberg & Hunter, 1995), calcium carbonate (Chien et al., 2008), and calcium oxalate (Grohe et al., 2007) crystals. NMR, CD and FTIR studies demonstrated that OPN is an IDP in solution (Tab.1) (Fisher et al., 2001; Gorski et al., 1995). The addition of calcium ions had little effect on the CD spectrum of OPN, while increasing protein concentration led to a more organized secondary structure (Gorski et al., 1995). Hence, it was suggested that OPN conformation in solution may be different from the conformation of OPN adsorbed to HA, because the local concentration of OPN in the semi-solid matrix might be relatively high (Gorski et al., 1995). This theory is supported by the observation that antibodies do not recognize OPN bound to HA, while they are able to recognize OPN bound to plastic (Gorski et al., 1995). Moreover, the acidic peptide based on the OPN sequence, which was examined by RosettaSurface, was able to adsorb to a calcium oxalate crystal surface in multiple conformations (Chien et al., 2009).

OPN is a substrate of tissue transglutaminase (Prince et al., 1991). Investigations of polymeric OPN showed a 5-fold increase in OPN binding to collagen type I when it was a polymer than when it was a monomer (Kaartinen et al., 1999), which indicates that there is a conformational change in OPN upon cross-linking. CD measurements demonstrated that



cross-linked OPN has a more organized structure than monomeric OPN (Kaartinen et al., 1999). It seems that OPN needs molecular crowding to assume a more ordered structure.

OPN found in non-mineralized tissues may or may not be phosphorylated, while bone OPN is always phosphorylated (George & Veis, 2008; Veis et al., 1997). Low phosphorylated OPN from bone (38%) is an effective HA mineralization inhibitor in contrast to highly phosphorylated OPN from milk (96%), which promotes HA formation and growth. The dephosphorylated form had no significant effect (Gericke et al., 2005). The degree to which OPN affected HA formation depended on the level of phosphorylation (Gericke et al., 2005). It has been suggested that the binding of OPN to HA alters OPN conformation, facilitating recruitment and activation of macrophages that remove pathologic HA deposits (Steitz et al., 2002). It was also shown that dephosphorylated OPN loses its ability to inhibit smooth muscle cell calcification (Jono et al., 2000).

### 3.2 Enamel proteins

Amelogenins are a protein family derived from a single gene by alternative splicing and controlled, post-secretory processing. They are abundant in tooth enamel, but amelogenin has also been identified in dentin, bone, cartilage and nonmineralizing tissues such as those of the brain, salivary glands and macrophages (Gruenbaum-Cohen et al., 2009; Lyngstadaas et al., 2009). Amelogenin is involved in biomineralization as well as cell signaling events (Gruenbaum-Cohen et al., 2009; Lyngstadaas et al., 2009; Veis, 2003). The primary sequence of amelogenin is highly conserved, especially the N-terminal Tyr-rich and C-terminal charged regions (Delgado et al., 2005). Determination of amelogenin's secondary structure has been hampered by the self-association of the protein (Li et al., 2006). Far UV CD and NMR spectra demonstrated that amelogenin in a monomeric form is largely disordered (Table 1). There is no well-defined, continuous region with an ordered structure, but some residual secondary structure was observed. In addition, amelogenin exists in at least two conformations (Buchko et al., 2010; Delak et al., 2009b; Ndao et al., 2011). Amelogenin can interact with other important proteins engaged in enamel formation (Bartlett et al., 2006). Amelogenin binds to CD63 and LAMP1 receptors, which mediate signal transduction events and endo-, pinocytosis, and phagocytosis, respectively. Both partners interact with the same amelogenin motif, which is largely disordered and accessible to the external environment (Shapiro et al., 2007; Zou et al., 2007).

The large number of alternatively spliced variants, expression in different tissues and the intrinsically disordered character of amelogenin reflect the multifunctionality of the protein, which is probably a common feature among IDPs.

Ameloblastin is also involved in enamel biomineralization, interactions between the ameloblasts and enamel extracellular matrix, and regeneration of hard-tissue. No NMR or CD studies of ameloblastin are available; however, bioinformatic analysis and molecular modeling strongly suggest that the protein is an IDP (Vymetal et al., 2008).

### 3.3 Statherin

Statherin is an inhibitor of the nucleation and growth of HA in the supersaturated environment of saliva (Hay et al., 1984; Johnsson et al., 1991; Stayton et al., 2003). The N-terminal region of statherin contains highly acidic motifs (D<sub>Sp</sub>SpEE, where Sp indicates a

phosphoseryl residue), which are important for adsorption of statherin onto the surface of HA and the inhibition of HA growth (Raj et al., 1992). Statherin was reported as unstructured in solution using NMR techniques (Naganagowda et al., 1998). Far UV CD experiments showed that N-terminal regions of statherin had a strong tendency to adopt an  $\alpha$ -helical structure (upon the addition of TFE), while middle and C-terminal portions of this protein were flexible and preferred to adopt an unordered conformation (Naganagowda et al., 1998; Raj et al., 1992). Solid-state NMR applied to examine the structure of statherin bound to HA (Tab. 1) demonstrated, that under biologically relevant conditions, 12 residues of the N-terminus of statherin were in a helical conformation and that they were strongly adsorbed to the HA surface. In contrast, middle and C-terminal regions weakly interacted with HA and were highly mobile. The mobility of statherin on HA surfaces could allow it to effectively block more nucleation sites than a very rigidly bonded protein (Long et al., 2001).

#### 4. IDPs found in calcium carbonate related mineralization

Calcium carbonate biominerals are widespread in nature. They occur in vertebrates as well as invertebrates, where they fulfill various functions. Here, we present the characteristics of IDPs involved in calcium carbonate crystal formation.

##### 4.1 Lithostathine

Pancreatic fluid is supersaturated with calcium and carbonate ions; however, stones are observed only in cases of patients with chronic calcifying pancreatitis (De Caro et al., 1988; Moore & Verine, 1987). Lithostathine, a pancreatic glycoprotein, inhibits the growth and nucleation of calcium carbonate crystals. The C-terminal region of lithostathine is homologous with C-type lectins (Patthy, 1988). The crystal structure of lithostathine has been determined. However, the 13 residues of the N-terminus, including the glycosylation site, are flexible in the crystal (Bertrand et al., 1996). Far UV CD spectra of the N-terminal region are typical for random-coil structures (Table 1) (Gerbaud et al., 2000). Interestingly, the short N-terminal peptide of lithostathine is essential for the inhibition of nucleation and crystal growth. The glycosylated N-terminal peptide generated by limited trypsin hydrolysis inhibited crystal growth similarly to full-length lithostathine, while the C-terminal polypeptide was inactive. A synthetic N-terminal peptide, but not glycosylated was equally active (Bernard et al., 1992). Hence, it was postulated that backbone flexibility is essential for the inhibitory effect of the protein (Gerbaud et al., 2000).

##### 4.2 Mollusk shell proteins

The mollusk shell is a commonly used model system for studying calcium carbonate biomineralization. Some mollusks have developed a bilayer of composite materials. Interestingly, the two layers, prismatic and nacreous, are composed of different forms of calcium carbonate polymorph, calcite and aragonite, respectively (Evans, 2008).

Several acidic proteins from *Haliotis rufescens* have been identified and characterized. AP7, AP8 and AP24 (aragonitic protein of molecular weight 7kDa, 8kDa and 24kDa, respectively) have been identified from the demineralized nacre protein fraction (Fu et al., 2005; Michenfelder et al., 2003). *In vitro* mineralization studies showed that AP7 and AP24 interacted with the step edges of growing calcite, which inhibited growth (Michenfelder et

al., 2003). Analysis of the AP7 and AP24 sequence revealed that the N-terminus of both proteins (1-30 residues of the mature protein) is characterized by calcite binding domains. Both sequences are capable of affecting calcium carbonate crystal growth *in vitro* (Michenfelder et al., 2003). Far-UV CD studies of AP7 showed that the N-terminus of AP7 adopts a random-coil or extended conformation in solution (Tab 1.) (Michenfelder et al., 2003; Wustman et al., 2004), while the C-terminus is  $\alpha$ -helical (Kim et al., 2006a). Interestingly, AP7's C-terminus end did not influence crystal growth (Kim et al., 2006a), while AP7's N-terminus was responsible for interactions with calcium ions and led to an inhibition of calcite growth *in vitro* (Kim et al., 2004; Michenfelder et al., 2003). It should be noted that full-length AP7 protein had the highest effect on calcite crystal growth (Kim et al., 2006a). This observation suggests that the flexibility and plasticity of IDPs might be crucial factors which inhibit crystal growth, although the well-defined, ordered secondary structure may also be necessary. It should be noted that high concentrations of calcium ions led to the precipitation of AP7's N-terminus, which may indicate calcium-dependent oligomerization (Kim et al., 2004). Finally, full-length AP7 undergoes a conformational change to an  $\alpha$ -helical structure as a function of TFE concentration (Amos & Evans, 2009).

Another protein from *Haliotis rufescens*, Lustrin A, is a component of the intercrystalline organic matrix lying between layers of aragonite tablets. The postulated role of Lustrin A is to enhance nacre layers' resistance to fracture (Shen et al., 1997). NMR studies showed that the N-terminal end of Lustrin A contains loop regions, which behave as entropic springs that endow it with resilience and flexibility (Table 1). This sequence is believed to be one of several putative elastic motifs within Lustrin A (Zhang et al., 2002). Although the sequence adopts a relatively defined secondary structure, the plasticity of Lustrin A's loop regions seems to be crucial to its postulated role in resisting fracture. Further studies of model peptides based on Lustrin A sequences showed two domains (RKS<sub>Y</sub> and D4) existing largely in conformationally labile random-coil and extended states (Wustman et al., 2003). Possibly, these features permit side chain accessibility to exposed calcium carbonate crystal surfaces (Wustman et al., 2003). In addition, its D4 domain, which as the name implies contains 4 D residues, inhibits nucleation of calcite crystals *in vitro* (Wustman et al., 2003). The inhibitory effect strongly supports the theory that IDPs control biomineralization processes.

Another mollusk protein, n16, has been isolated from the nacre layer of *Pinctada fucata* (Samata et al., 1999). The analysis of n16's primary sequence revealed the requisite amino acid residues associated with a calcium carbonate modification domain within the C- and N-terminae of the mature protein (n16-C and n16-N, respectively) (Kim et al., 2004). Both sequences modify the morphology of calcium carbonate crystals and induce calcite growth *in vitro* (Kim et al., 2004). Far-UV CD and NMR studies demonstrated that n16 exists as a random-coil molecule; however, increasing the concentration of the peptide led to a conformational equilibrium between random-coil and  $\beta$ -sheet conformers (Tab. 1) (Kim et al., 2004). Interestingly, in the presence of TFE, n16-N adopted a  $\beta$ -sheet conformation, unlike AP7 and AP24, which adopted an  $\alpha$ -helical conformation. Thus, AP7, AP24 and n16N are conformationally labile, but exhibit different folding propensities (Collino & Evans, 2008). Moreover, random scrambling of n16-N abolishes its concentration-dependent conformational rearrangement capability, resulting in a decrease of its ability to affect crystal growth (Kim et al., 2006b). It was also shown that n16-N

simultaneously interacted with  $\beta$ -chitin and the nucleating mineral phase, finally leading to aragonite formation *in vitro* (Keene et al., 2010). Also noteworthy, n16-N alone induced calcite formation (Kim et al., 2004). It has been suggested that the binding of n16-N to  $\beta$ -chitin could trigger disorder-to-order transitions. In summary, the intrinsically disordered structure of n16-N facilitates interactions with its partners (like for example  $\beta$ -chitin) and disorder-to-ordered transition seems to be crucial for self-assembly (Amos et al., 2011; Collino & Evans, 2008; Keene et al., 2010).

ACCBP (amorphous calcium carbonate binding protein) was found in the extrapallial fluid of *Picnada fucata* (Ma et al., 2007), while PFMG1 (*Picnada fucata* mantle gene protein 1) controls calcium carbonate nucleation and is believed to assist in the formation of pearl nacre (Liu et al., 2007). Based on previously obtained results (Amos et al., 2009; Collino et al., 2006; Keene et al., 2010; Kim et al., 2004; Wustman et al., 2004) and bioinformatic predictions, the C- and N- terminal sequences of both proteins (PFMG1-C, PFMF1-N and ACCN) have been studied to find mineralization activity and conformational disorder (Amos et al., 2009; Liu et al., 2007). Far UV CD spectroscopy confirmed that PFMG1-C, PFMF1-N and ACCN possess a combination of a random-coil conformation and other secondary structures (Table 1). Moreover, the addition of TFE led to disorder-to-order transitions in all peptides (Amos et al., 2009; Liu et al., 2007).

Aspein is another extremely acidic (60.4 % of D) protein from *Picnada fucata* (Tsukamoto et al., 2004). The extraordinary acidic character of Aspein and the fact that SDS-PAGE overestimated its molecular mass (Takeuchi et al., 2008; Tsukamoto et al., 2004) suggested that Aspein is also an IDP. Bioinformatic tools predicted that Aspein is completely disordered (Table 2). Prismatic-14 from the prismatic layer of *Picnada fucata* also contains putative intrinsically disordered regions. Firstly, G/Y-rich regions might form glycine loop motifs, which may contribute to the elasticity of the molecule (Suzuki et al., 2004). This motif is responsible for interaction with chitin (Suzuki & Nagasawa, 2007). Secondly, D-rich regions within the N- and C- terminae of Prismatic-14, which are responsible for inhibiting calcium carbonate precipitation *in vitro* (Suzuki & Nagasawa, 2007), also might be intrinsically disordered. However, only PONDR predicted short intrinsically disordered regions in Prismatic-14. IUPred and DISOPRED2 didn't predict any disordered structure (Table 2).

Ten acidic Asprich proteins (51-61% of acidic residues) have been identified from *Atrina rigida* (Gotliv et al., 2005). The Asprich family might be the product of alternative RNA splicing of the same gene and are arbitrarily divided into six domains: (i) N-terminal signal peptide, (ii) basic domain, (iii) the acidic1 domain, identical in all the Asprich proteins, (iv) the variable acidic domain, which differs among Asprich proteins, (v) conserved DEAD repeat domain, (vi) and conserved acidic2 domain (Gotliv et al., 2005). All three conserved acidic domains (acidic1, DAED and acidic2) are intrinsically disordered and they control calcium carbonate crystal growth (Collino et al., 2006; Delak et al., 2009a; Kim et al., 2008). Far UV CD studies of acidic1, DAED and acidic2 domains demonstrated that these peptides exist in a random-coil conformation in equilibrium with other secondary structures. Interestingly, a significant conformational change was not observed at lower pH and in the presence of calcium ions, which suggests that structural lability is a result of more than just electrostatic repulsion (Collino et al., 2006; Delak et al., 2009a; Kim et al., 2008). NMR spectroscopy showed that calcium ions induced local perturbations in conformations of



acidic1, DAED and acidic2, but the structure was still very flexible (Table 1) (Delak et al., 2009a). Asprich proteins are able to adopt a more ordered structure in the presence of TFE, indicating that they might undergo a disorder-to-order transition (Ndao et al., 2010).

Several other acidic proteins have been identified from mollusk shells, for instance: MSP-1 (molluscan shell protein 1) from *Patinopecten yessoensis* (Sarashina & Endo, 2001), Caspartin and Calprismin from *Pinna nobilis* (Marin et al., 2005), moluscan phosphorylated protein 1 (MPP1) from *Crassostrea nippona* (Samata et al., 2008), P95 from *Unio pictorum* (Marie et al., 2008), and Nautilin-63 from *Nautilus macromphalus* (Marie et al., 2011). No secondary structure analyses of these proteins have been published, hence it is unknown if they are IDPs. However, MSP-1 and MPP1 are likely to be largely disordered, while Nautilin-63 probably consists of a combination of disordered and ordered regions (Table 2).

### 4.3 Crustacean associated IDPs

Most crustaceans possess a calcified exoskeleton that is periodically molted to permit growth. Hence, their calcium metabolism is tightly linked to seasonal molting cycles. Aquatic crustaceans take calcium from the water, whereas terrestrial species have developed several storage strategies (Luquet et al., 1996; Testeniere et al., 2002). During the premolting period, calcium is stored in the hemolymph, the hepatopancreas, sterna plates, gastroliths or posterior caeca, depending on the species (Luquet et al., 1996; Meyran et al., 1984). All calcified deposits correspond to biomineralized structures (Testeniere et al., 2002). After ecdysis, stored calcium is quickly reabsorbed and translocated to harden the new exoskeleton (Ishii et al., 1996). The exoskeleton of crustaceans consists of calcium carbonate and chitin-protein microfibrillar frameworks.

Calcification-associated peptide 1 (CAP-1) is an acidic protein isolated from the exoskeleton of *Procambrus clarkii* (Inoue et al., 2001). The C-terminal region is especially acidic and possesses a phosphoserine residue, which is essential for the inhibition of calcium carbonate precipitation and the calcium-binding activities of CAP-1 (Inoue et al., 2003, 2007). Far UV CD studies demonstrated that CAP-1 has a random-coil conformation in equilibrium with a residual  $\beta$ -sheet (Table 1). The N-terminal region contributes to maintaining the overall conformation of CAP-1 (Inoue et al., 2007). It has been suggested that the N-terminal fragment of CAP-1 inhibits crystal growth less efficiently, because it possesses a more ordered structure than the C-terminal fragment (Inoue et al., 2007). This hypothesis is supported by the observation that CAP-2, which has a sequence similar to CAP-1, but shorter by 17 residues at the C-terminus, is a less active inhibitor of calcium carbonate precipitation (Inoue et al., 2004, 2007).

There are more proteins identified from the calcium storage organs of exoskeleton in crustaceans like CAP-2 (Inoue et al., 2004), Casp-2 (calcification-associated soluble protein-2) (Inoue et al., 2008) and GAMP (gastrolith matrix protein) (Ishii et al., 1996) from *Procambrus clarkii*, orchertin from *Orchestia cavimana* (Hecker et al., 2004; Luquet et al., 1996), gastrolith protein 65 (GAP 65) (Shechter et al., 2008) and gastrolith protein 10 (GAP 10) (Glazer et al., 2010) from *Cherax quadricarinatus*, and crustocalcin (CCN) from *Penaeus japonicus* (Endo et al., 2004). Most of them are acidic; however, no secondary structure analysis has been performed. Bioinformatic predictions indicated that all of these proteins might possess intrinsically disordered regions (Table 2).



#### 4.4 Sea urchin larval spicule matrix protein IDPs

Sea urchins have an elaborate, calcified skeleton, which is a valuable model system for analyzing molecular regulation of biomineralization (Wilt & Etensohn, 2007; 2008). The skeleton is a network of calcified spicules, consisting of calcium and magnesium carbonate (in a 19:1 ratio), a surrounding extracellular matrix and small amounts of occluded matrix proteins (Wilt & Etensohn, 2007; 2008). It has been estimated that about 40-50 different proteins are associated with the spicule (Killian & Wilt, 1996). SM30, SM32, PM27, SM37, and SM50 have been identified in *Strongylocentrotus purpuratus* (Benson et al., 1987; George et al., 1991; Harkey et al., 1995; Katoh-Fukui et al., 1991; Lee et al., 1999). SM32, PM27, SM37, and SM50 are basic, non-glycosylated, closely related proteins. They contain SP, C-type lectin domains and a variable number of proline-rich repeats (Killian & Wilt, 2008; Wilt, 1999; Wilt & Etensohn, 2007; 2008).

SM50 is involved in the depositing initial calcite crystals as well as the elongation of the spicule elements, and it probably interacts with growing calcium carbonate crystals (Killian & Wilt, 2008). The C-terminal domain of SM50 contains two motifs exhibiting traits that are characteristic for IDPs. Firstly, GVGG and GMGG repeats are present, which are sequence homologs of elastin and spider silk protein repeats. NMR studies of the C-terminal fragment showed that it exists as a  $\beta$ -sheet with an extended structure (Tab. 1). This structure might play an important role in matrix assembly, protein stability, molecular elasticity and protein-crystal recognition within the spicule's mineralized matrix (Xu & Evans, 1999). Secondly, PNNP repeat motifs are believed to play an important role in mineralization. P-rich sequences often exhibit the extended, flexible structure responsible for protein-protein and/or protein-crystal recognition, while N residues represent putative sites for side chain hydrogen bonding (Zhang et al., 2000). PNNP repeats adopt an extended, twisted conformation consisting of a mixture of turn-like and coil-like regions, most likely interconverting with the coiled-coil state (Zhang et al., 2000). Several possible roles for PNNP have been suggested: (i) as a mineral recognition domain, (ii) as a self-assembly domain, (iii) as a molecular spacer (Zhang et al., 2000).

PM27 contains PGMG repeated motifs, which have been examined by far UV CD, and NMR spectroscopy (Table 1). It has also been shown that this motif exhibits a random-coil or loop-like structure characterized by the ongoing oscillations between different conformational states (Wustman et al., 2002).

Bioinformatic tools also predicted that SM30, SM32, and SM37 might be partially disordered (Tab. 2). SM30, the most abundant spicule matrix protein, an acidic glycoprotein, also contains signal peptide and C-type lectin domains (Killian & Wilt, 1996; Wilt, 1999). It is coded by six genes, which are variously expressed in different mineralized structures (Illies et al., 2002; Killian et al., 2010; Livingston et al., 2006). Another protein isolated from *Strongylocentrotus purpuratus* is phosphodontin. It is the major acidic phosphoprotein of the tooth matrix (Mann et al., 2010). Phosphodontin is probably an IDP. All three predictors indicated that phosphodontin is disordered (Table 2).

#### 4.5 Otolith related IDP

Otoliths are mineral deposits in the inner ear of fish, responsible for sensing gravity and detecting linear acceleration. They are mainly composed of calcium carbonate, but also contain

a small fraction of organic molecules (1-5 % w/w). The organic matrix acts as a template for depositing crystals, promoting crystal growth in certain directions, and inhibiting crystal growth in undesired directions (Allemand et al., 2007; 2008; Ross & Pote, 1984).

Starmaker (Stm) and otolith matrix macromolecule-64 (OMM-64) are acidic proteins from *Danio rerio* and *Oncorhynchus mykiss* otoliths, respectively (Sollner et al., 2003; Tohse et al., 2008). Both proteins have similar gene organization and 25% sequence identity, and because of this, it has been postulated that they are orthologs (Sollner et al., 2003; Tohse et al., 2008). It should also be noted that DSPP is the close human functional analog of Stm (Sollner et al., 2003). Stm is responsible for the size, shape and polymorph selection in otoliths (Sollner et al., 2003). It has been demonstrated by several methods that Stm is an IDP (Tab. 1) (Kaplon et al., 2008, 2009). Far UV CD spectrum of Stm yielded the signatures typical for a disordered protein. The extended conformation of Stm results in the large hydrodynamic radius of the molecule, as was shown by gel filtration and ultracentrifugation. Interestingly, calcium ions led to a lower hydrodynamic radius, probably by decreasing electrostatic repulsion. Also, solvent-induced denaturation of Stm indicated the lack of an ordered structure in the molecule (Kaplon et al., 2008, 2009). Recently, another homolog of *Starmaker* has been identified: *Starmaker-like* from *Oryzias latipes*. The deduced amino acid sequence of *Starmaker-like* is also rich in acidic residues (Bajoghli et al., 2009). No secondary structure analysis of Stm-like, or OMM-64, have been performed; however, bioinformatic predictions strongly suggest that these proteins might be completely disordered (Table 2).

#### 4.6 Egg shell related IDPs

An eggshell is a highly ordered natural, acellular composite bioceramic. It is one of the fastest and also one of the most structurally complicated biomineralizing systems. To produce an eggshell, 5 grams of calcium carbonate must be crystallized within 20 h (Arias et al., 2007; 2008). OPN, which is one of the eggshell proteins, was described above and is characterized as an IDP (3.1.4) (Chien et al., 2008; Panheleux et al., 1999). There are some proteins that are unique to eggshells. Ovocleidin-116 has been isolated and cloned from chickens (Hincke et al., 1999; Mann et al., 2002). Recent studies demonstrated that Ovocleidin-116 is an ortholog of mammalian MEPE, which belongs to the SIBLINGs family (Bardet et al., 2010). Although, structural studies of MEPE have not been available, it's possible that both proteins are IDPs, as was predicted by bioinformatic tools (Tab. 2). Several proteins identified from different avian species are C-type lectin-like proteins (Arias et al., 2007; 2008): ovocleidin-17 (chicken) (Hincke et al., 1995), ansocalcin (goose) (Lakshminarayanan et al., 2003), stuthiocalcin-1 and struthiocalcin-2 (ostrich) (Mann & Siedler, 2004), rheacalcin-1 and rheacalcin-2 (rhea) (Mann, 2004), dromaiocalcin-1 and dromaiocalcin-2 (emu) (Mann & Siedler, 2006). Structural studies of ovocleidin-17 and ansocalcin revealed that their three-dimensional structure is very similar to the structure of lithostathine (Bertrand et al., 1996; Lakshminarayanan et al., 2005; Reyes-Grajeda et al., 2004); however, the three proteins had different effects on calcium carbonate crystals. Lithostathine was an inhibitor, ansocalcin caused the formation of crystal aggregates, and ovocleidin-17 led to the crystals twinning (but not to an extensive aggregation of crystals like with ansocalcin) (Geider et al., 1996; Lakshminarayanan et al., 2005). It should be noted that N-terminal regions of ansocalcin and ovocleidin-17 are well folded, while lithostathine's N-terminus is disordered. Possibly lithostathine, ansocalcin and

ovocleidin-17 influence crystal formation in different ways because of the distinct structure of each of their N-termini. The disordered structure of lithostathine's N-terminus might be directly responsible for its inhibitory effect on crystal formation. Hence, based on the evidence presented in this review, it can be postulated that IDPs are crucial to the inhibition of crystal growth.

## 5. The innate specialization of IDPs in biomineralization

All of the above-mentioned examples strongly suggest that it is no accident that so many proteins involved in biomineralization exhibit an IDP-like character or are simply IDPs. Conformational instability is a common feature of proteins that bind to inorganic solids (Delak et al., 2009a; Kim et al., 2006a; Michenfelder et al., 2003). Intrinsic disorder provides benefits to these proteins and enables them to fulfill their functions.

Many IDPs involved in biomineralization have an unusual amino acid composition, for instance, they are rich in acidic residues. This unusual composition leads to strong electrostatic repulsion and results in the extended conformation of the IDP molecule.

The extended conformation of IDPs provides a much larger binding surface in comparison to the compact conformation of globular proteins. It is highly advantageous, especially when interacting with crystalline surfaces. Many proteins act as inhibitors of crystal growth by interacting with the crystal lattice and blocking nucleation sites. DPP's larger binding surface enables it to block more nucleation sites, hence one extended molecule can do the work of several molecules with a globular conformation (Fujisawa & Kuboki, 1998). Moreover, CAP-1, statherin and lithostathin structural studies clearly demonstrated that the highly flexible regions of these proteins were directly responsible for the inhibitory effects they had on crystal growth (Gerbaud et al., 2000; Inoue et al., 2007; Long et al., 2001).

Secondly, their extended conformation combined with their acidic character facilitates protein interactions with counter ions (Uversky, 2009). DPP and DMP1 bind relatively large amounts of calcium ions compared to calmodulin, which has a well-defined three-dimensional structure (He et al., 2003b, 2005b). Counter ion-binding seems to be crucial to weakening electrostatic repulsion and permitting the formation of  $\beta$ -sheets that lead to disorder-to-order transitions (He et al., 2005a). Asprich and AP7 proteins can bind calcium ions as well as other ions; however, this does not lead to significant global conformational rearrangement. It has been suggested that both the extended  $\beta$ -strand and coil segments enhance calcium binding and possibly determine the function of these domains (Collino et al., 2006; Delak et al., 2009a).

One of the most significant features of IDPs is their ability to interact with many different partners (Uversky & Dunker, 2010). Proteins involved in biomineralization have to bind many targets to properly fulfill their functions. They are responsible for macromolecular complex assembly, and they are engaged in cell attachment (Fujisawa et al., 1997; Stubbs et al., 1997), cell signaling (Gruenbaum-Cohen et al., 2009) and/or the regulation of gene expression (Narayanan et al., 2003, 2006). Macromolecular complex assemblies are crucial for proper biomineral formation. The extended conformation of IDPs provides a large binding surface for different partners, while the flexibility and plasticity of polypeptide chains permit IDPs to conformationally adapt to different targets (Collino et al., 2006; Delak et al., 2008). Moreover, proteins involved in biomineralization may undergo

disorder-to-order transitions as a result of binding with a partner, molecular crowding or other factors (Amos et al., 2010). Additionally, this disorder-to-order transition promotes macromolecular complex assembly.

Interactions with other macromolecules can also modulate the function of a protein. DPP in solution acts as an inhibitor of HA growth, while DPP bound to collagen acts as a nucleator of HA crystals. This phenomenon might be the result of conformational changes in DPP triggered by interactions with collagen. OPN also exhibits a different conformation when it is bound to HA from that of the solution state (Chien et al., 2009; Gorski et al., 1995). The function of n16 and OMM64 (which is also a putative IDP) is also modulated by interactions with  $\beta$ -chitin. OMM64, n16 and  $\beta$ -chitin in isolation can not induce aragonite formation *in vitro*. However, the combination of OMM46/ $\beta$ -chitin and n16/ $\beta$ -chitin molecules result in aragonite formation. Moreover, it has been suggested that the intrinsic disorder of n16 is a key factor allowing this protein to simultaneously interact with  $\beta$ -chitin and mineral face (Keene et al., 2010; Tohse et al., 2009).

Many proteins involved in biomineralization are able to self-assemble (Kaartinen et al., 1999; Lakshminarayanan et al., 2003). Monomer proteins are often IDPs, while the aggregation of a protein leads to the formation of a  $\beta$ -sheet structure. It is very possible that the extended structure of a protein in solution facilitates interactions with other macromolecules. These interactions may trigger disorder-to-order transitions, which enable macromolecular complexes to form (Buchko et al., 2008; Gorski et al., 1995; He et al., 2003b). Random scrambling of n16 led to a lower affinity for  $\beta$ -chitin and the disappearance of disorder-to-order transitions; this resulted in an inability to form aragonite *in vitro* (Keene et al., 2010). Interestingly, aggregation and conformational change is highly associated with ambient conditions; self-assembly of amelogenin depends on protein concentration, ionic strength, pH and temperature (Lyngstadaas et al., 2009), while DMP1 oligomerization is induced by calcium ions (He et al., 2003b).

A fascinating example of IDPs involved in biomineralization is DMP1. This multifunctional protein is able to interact with calcium ions (He et al., 2003a, 2003b), HA crystals (He et al., 2003a), collagen (Qin et al., 2004), DNA (Narayanan et al., 2006), H factors, integrin  $\alpha\beta 3$  and CD44 (Jain et al., 2002). Consequently, DMP1 is engaged in biomineralization, cell signaling and regulation of gene expression (He et al., 2003a; Jain et al., 2002; Narayanan et al., 2006). It exhibits different localization (nuclear, extracellular) according to post-translational modifications and its conformational state (Narayanan et al., 2003). This multifunctionality and interactions with numerous target are likely facilitated by the intrinsically disordered character of the protein.

It is known that proteins involved in biomineralization are extensively post-translationally modified, frequently being phosphorylated, glycosylated and proteolytically cleaved (George & Veis, 2008; He et al., 2005b; Hecker et al., 2003; Qin et al., 2004). A susceptibility to post-translational modifications seems to be associated with the extended and flexible structure that is characteristic of IDPs. The lack of a well-packed hydrophobic core and rapid fluctuations in the peptide chain lead to a greater amount of potential phosphorylation sites, more so than in the case of globular proteins. (Bertrand et al., 1996; Iakoucheva et al., 2004).

Using the IDP classifications (2.2), one can classify the proteins involved in biomineralization into several groups. Those most important for biomineralization are



molecular assemblers. This is supported by their ability to interact simultaneously with crystals, scaffold molecules (collagen,  $\beta$ -chitin) and their self-assembling properties. Furthermore, widespread counter ion-binding indicates that they may act as scavengers. We cannot exclude the function of these proteins as effectors, because they are also engaged in processes such as gene expression (Narayanan et al., 2003, 2006). Finally, numerous post-translational modifications suggest the presence of display sites.

It is apparent that intrinsic disorder is characteristic of proteins involved in biomineralization. The structure of many proteins has yet to be established, but there is a considerable likelihood that they are IDPs (Fig. 1B, Table 2).

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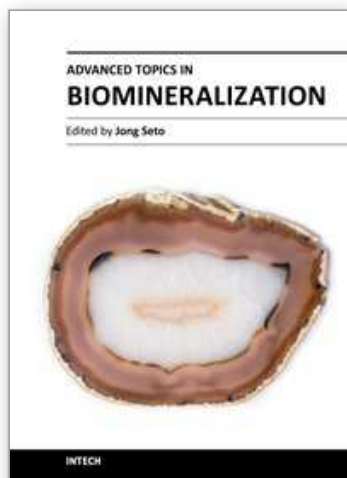
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