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Review

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Inorganic Polyphosphates As Storage for and Generator of Metabolic Energy in the Extracellular Matrix

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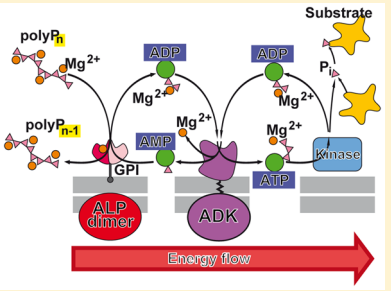
ABSTRACT: Inorganic polyphosphates (polyP) consist of linear chains of orthophosphate residues, linked by high-energy phosphoanhydride bonds. They are evolutionarily old biopolymers that are present from bacteria to man. No other molecule concentrates as much (bio)chemically usable energy as polyP. However, the function and metabolism of this long-neglected polymer are scarcely known, especially in higher eukaryotes. In recent years, interest in polyP experienced a renaissance, beginning with the discovery of polyP as phosphate source in bone mineralization. Later, two discoveries placed polyP into the focus of regenerative medicine applications. First, polyP shows morphogenetic activity, i.e., induces cell differentiation via gene induction, and, second, acts as an energy storage and donor in the extracellular space. Studies on acidocalcisomes and mitochondria provided first insights into the enzymatic basis of eukaryotic polyP formation. In addition, a concerted action of alkaline phosphatase and adenylate kinase proved crucial for ADP/ATP generation from polyP. PolyP added extracellularly to mammalian cells resulted in a 3-fold increase of ATP. The importance and mechanism of this phosphotransfer reaction for energy-consuming processes in the extracellular matrix are discussed. This review aims to give a critical overview about the formation and function of this unique polymer that is capable of storing (bio)chemically useful energy.



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1. INTRODUCTION

Any kind of chemical or biochemical reaction follows the thermodynamic laws. While chemical systems tend to reach an equilibrium between reactants and products, the reactants in living biochemical systems are usually in a nonequilibrium state.[1] Living systems remain in the latter state due to thermodynamic processes that dissipate energy. This fact implies that in biological systems energy-generating/providing circuits are coupled to endergonic reactions. Intracellularly it is ATP that is capturing and transferring free energy.[2,3] Under standard conditions, enzymatic hydrolysis of the terminal high-energy $\beta-\gamma$ and $\alpha-\beta$ phosphoanhydride bonds in ATP to ADP and ADP to AMP, respectively, results in the release of -30.5 kJ mol^{-1} of Gibbs free energy change (ΔG^0). This released free energy is used as fuel for driving energetically unfavorable processes, like channeling of molecules against a concentration gradient via ATP-powered pumps, movement of cilia, contraction of muscle or synthesis of biopolymers. In animal cells, and most nonphotosynthetic cells, fatty acids and sugars are metabolized during aerobic oxidation to CO_2 and H_2O, and the energy liberated is, at least partially, stored as biochemically usable energy in phosphoanhydride bonds of ATP. In eukaryotes, the final stages of aerobic oxidation occur in mitochondria, whereas in prokaryotes those reactions proceed on the plasma membrane. In addition, substrate-level phosphorylation steps take place during which ATP or GTP is formed directly from ADP or GDP. In plants, light energy is converted to chemical energy and stored in the chemical bonds of carbohydrates.

The intracellular cytosol has a sol consistency which thwarts especially the low-molecular weight molecules to diffuse freely. This highly dynamic and yet exquisitely organized cytosol undergoes sol−gel transition (gelation) in response to changing pH conditions and changing ions/molecules supplementation.[4] Already this property allows a fairly stable regional organization of the cytosolic metabolic pathways. In the next higher hierarchical level of complexity, the cytosol in eukaryotes harbors insoluble suspended particles, like ribosomes and organelles. Semipermeable membranes around the organelles build a barrier and implement a further possibility for control of the different metabolic cycles, the intracellular transport or metabolic energy production/utilization.[5] In addition, this compartmentalization builds a protection mechanism against the activity of lytic enzymes, like those of the lysosomes.

One prerequisite for the emergence of life was the separation of the cellular environment from the surrounding, external environment, which was accomplished by the cytoplasmic membrane with a width of ∼6−10 nm built of a phospholipid bilayer into which (glyco)proteins, glyoclipids, and cholesterol molecules are embedded too.[6] These hydrophobic structures undergo self-assembly and exclude water interactions between the cell interior and the extracellular space.[7] ATP-dependent as well as ATP-independent transporters establish and maintain a membrane asymmetry. The exchange of molecules between the cytoplasm and the extracellular milieu is enabled by both passive (without energy consumption) and active transport (energy-dependent transport against a concentration gradient or an electrochemical gradient) using a variety of channels and transporters.[8] Mitochondria are the major intracellular ATP generator producing ∼32 mol of ATP from 1 mol of glucose which is metabolized via glycolysis and the citric acid cycle. ATP synthesized in mitochondria or during glycolysis is found to play parallel and/or separate, distinct roles during differentiation and migration.[9,10]

ATP cannot diffuse across the plasma membrane into the extracellular space unless it is channeled through, for example, the conductive ATP-releasing pathways.[11,12] In addition, a series of ATP-permeable channels have been identified that are grouped to the connexin channels, hemichannels, pannexin 1, Ca^{2+} homeostasis modulator 1, volume-sensitive outwardly rectifying anion channels, and the maxi-anion channels. In spite of these varieties of ATP export channels, the intracellular ATP concentration is much higher and can reach up to 100 mM in the neuronal synaptic vesicles, compared to the extracellular environment with 10 nM (see refs 11 and 13). In human blood, the ATP levels are in the range of 20 and 100 nM.[13] In this context, it is interesting to mention that mammalian erythrocytes do not contain any mitochondria with the consequence that the level of ATP in those cells is low with 360 nM, compared to 8500 nM (average level for 10^6 cells) in T lymphocytes.[14] It should be noted that precise measurements of the low, nanomolar ATP levels in the tissue interstitium are technically difficult, in particular, close to the cell surface where ATP seems to increase.[13]

While the energetics within cells is quite well understood the extracellular energetic cycle(s) are poorly addressed. In general, the extracellular space does not contain mitochondria, with the exception of the blood platelet environment during inflammatory responses. During this state extracellular mitochondria have been identified together with neutrophils in vivo, triggering neutrophil adhesion toward the endothelial wall.[15] In most human tissues the volume occupied by cells is substantially smaller compared to the volume of the surrounding extracellular matrix (ECM), like in cartilage which contains about 1−2% of cells[16] or calcified bone which comprises about 25% organic matrix (of which 2−5% contributes to cells), 5% water and 70% inorganic mineral.[17] Now, the pressing question emerges, which addresses the fueling of the dynamic and complex ECM with metabolic energy, a space which does not contain ATP-generating mitochondria. Even more, the ECM is, as outlined above, poor in ATP, and if present in this compartment, this nucleotide is exposed to the extracellular or cell membrane-bound alkaline phosphatase (ALP [EC 3.1.3.1]) and the cell membrane glycoprotein-1 phosphodiesterase [EC 3.1.4.1]/nucleotide

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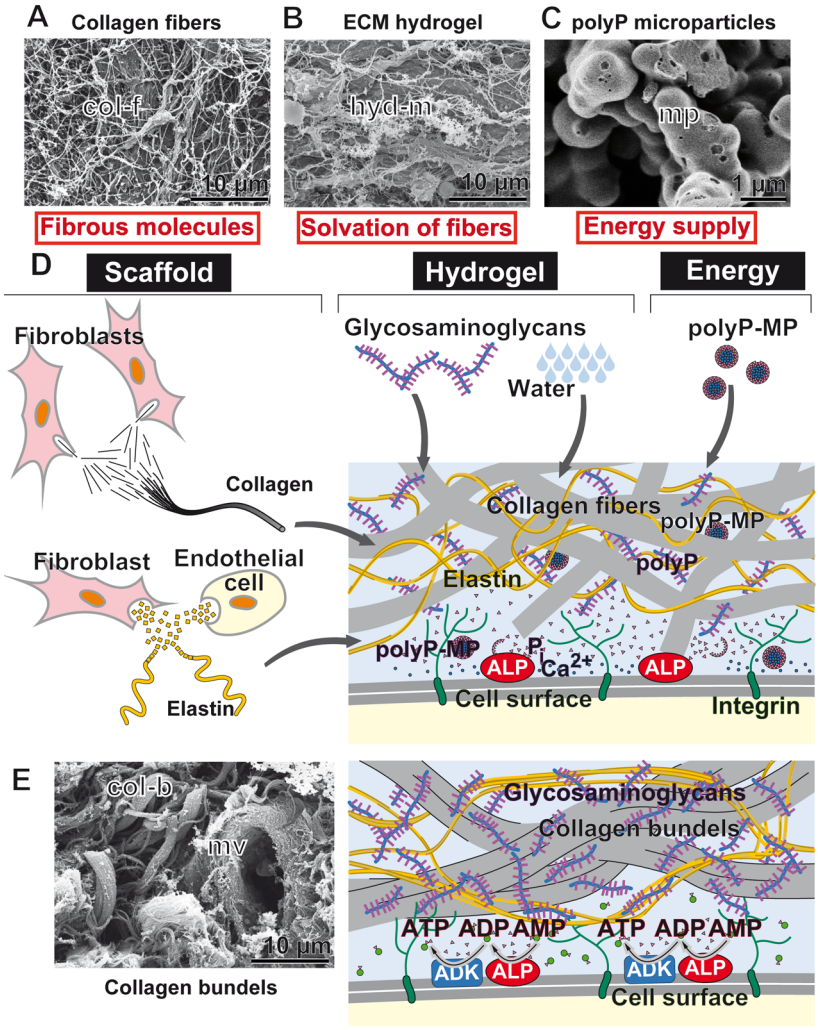

Figure 1. Extracellular matrix (ECM) as a hydrogel-scaffold into which structural fibrous molecules are embedded in a functionally organized manner. (A) Initially, the collagen fibrils/fibers (col-f) are quite randomly arranged, and (B) become incorporated into a hydrogel matrix (hyd-m), depicted as contracted globular deposits. (C) Microparticles (mp), either released from the blood platelets or added as artificial particles, like Ca-polyphosphate microparticles (polyP-MP), integrate into the scaffold and function as storage for and generators of metabolic energy. (D) The three phases of the hierarchical organization of the ECM scaffold molecules; first, synthesis of the structural macromolecules (scaffold) such as collagen from the fibroblasts and elastin from fibroblasts and endothelial cells, second, formation of the hydrogel molecules such as proteoglycans and glycosaminoglycans, and third, the compartmentation of these scaffold molecules into functional tissue units, which requires organization processes driven by supramolecular assembly/interaction and metabolic energy, like ATP, as stressed in this review. It is proposed that enzymatically controlled hydrolysis of Ca-polyP-MP via ALP generates inorganic phosphate (P_i), Ca^{2+}, and metabolic energy that, in the presence of adenylate kinase (ADK), is stored in ADP and ATP. (E) Organized arrangement of collagen bundles (col-b) that are often associated with microvessels (mv). [(A–C,E) SEM; scanning electron microscopy].

pyrophosphatase [EC 3.6.1.9].[18] The diffusion level of ATP in the protein-rich ECM as well as in the cytosol is lower than the one in water;[19] for example, the diffusion in the cytosol amounts to approximately 5×10^{-10} m^2 s^{-1}.[20] An extracellular ADP/ATP carrier has not been identified. It is widely acknowledged that the maintenance of the organization of the ECM in general and especially in cell-poor organs is a high-energy-demanding process. It is very obvious that tissues which contain only a small number of cells, like cartilage or intervertebral discs, receive some of their metabolic energy from their cells by metabolic respiration during glycolysis and mitochondrial oxidative phosphorylation.[21] Hence it must be assumed that nutrients/glucose can only be supplied by diffusion from blood vessels to the margins of the cartilage/discs through the dense ECM of that tissue. Quantitative determinations revealed that the values of the diffusion coefficients in cartilage vary within the range from 0.3×10^{-11} to 30×10^{-11} m^2 s^{-1};[22] the diffusion is higher in damaged/degenerated tissue compared to normal cartilage.

Until now, no quantitative determinations are available that support the view that ATP released from cells is sufficient to maintain the coordinated complex ECM structure. Perhaps one reason for this inappropriate situation is the lack of data on energy requiring and energy consuming reactions that proceed in the extracellular space. These, or at least some of those, will be outlined in this review. Furthermore, besides ATP, a new candidate physiological metabolic energy-storing compound in eukaryotes will be introduced, inorganic polyphosphate (polyP). This polymeric polyanion consists of unbranched chains of three up to 1000 phosphate (P_i) residues, linked by high-energy phosphoanhydride bonds. PolyP has been conserved in all biotic organisms from bacteria to animals

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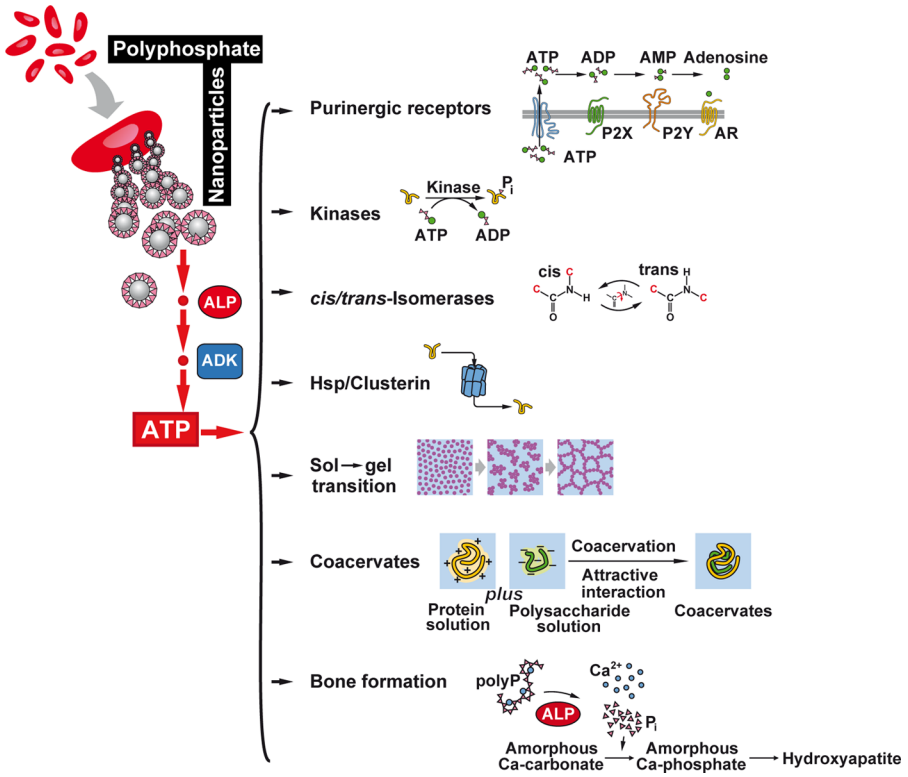

Figure 2. ATP-requiring processes in the ECM. It is proposed that polyP in the ECM occurs as nano/microparticles that undergo hydrolytic cleavage via the ALP. In concert with the ADK, ATP (ADP, AMP) is synthesized that acts as a metabolic energy reservoir. In comparison, it is shown that ATP, exported from the cells, can act in the extracellular space as signal/ligand for purinergic receptors. During this reaction ATP is not used as metabolic fuel but activates purinergic receptor(s), P2X, P2Y, or adenosine receptors (AR). The ATP metabolizing systems in the ECM include the ECM kinases that control metabolic processes by phosphorylation of key molecules. ATP might also be required during peptidylprolyl *cis–trans* isomerase reactions. Surely, heat shock proteins (HSP), like clusterin, are also present in the ECM that are involved in the physiological folding of functional polymeric molecules. In addition, processes like sol to gel transitions during supramolecular polymer organization are critical organization principles in the ECM involving exergonic reactions. The transition processes during coacervation likewise follow an energy-favorable reaction pathway. Bone and cartilage formation in the extracellular space are prominent energy-requiring reactions. During bone mineralization P_i and ADP/ATP are generated by enzymatic hydrolysis of polyP via ALP. The released P_i is driving the transition of amorphous Ca-carbonate "bio-seeds", initially formed during bone mineralization, to amorphous Ca-phosphate and the final deposition of hydroxyapatite.

and can reach in yeast vacuoles levels as high as 20% with respect to dry weight.[23] It has been proposed that already in the prebiotic environment polyP was abundantly formed during volcanic eruptions. Emerging evidence is now available, which qualifies polyP, being an abundantly present polymer in metazoan tissue, due to the presence of high-energy phosphate bonds/acid anhydride linkages (reviewed in refs 24–27), as a polymer that can transfer Gibbs free energy to AMP or ADP under formation of ATP after enzymatic cleavage of the polymer by ALP (reviewed in refs 28–30).

2. EXTRACELLULAR MATRIX ORGANIZATION

It is conceivable that the formation of the bulky ECM of metazoan tissues, most prominent with bone [70% contributes to the bone mineral and the remaining organic matrix, consisting of proteoglycans, glycosaminoglycans, and collagen, and only 25% is water],[31] cartilage [<20% are cells],[32] eye lens [<10% cells],[33] and cornea [<10% cells],[34] is a strongly energy-dependent process. The reason is that the (macro)molecular components of the ECM exist in a highly ordered, functional arrangement (Figure 1). These extracellular structural molecules are hierarchically composed and have a modular composition. The organization can be operationally grouped into three phases (Figure 1D). First, the synthesis of the macromolecules building the scaffold,[35,36] such as collagen extruded from fibroblasts, elastin released from fibroblasts and endothelial cells, and fibronectin secreted from hepatocytes. These fiber-forming or rubber-like structural molecules of the ECM share the property of being dynamic and adapting to the physicochemical conditions of their environment. They are spiral-like and are loosely held together by noncovalent hydrophobic forces. Second, the elasticity of the ECM molecules is a prerequisite for the next step of complexity, the structural and functional organization of these components in the ECM. At this stage the macromolecules become embedded in a hydrogel consisting of proteoglycans and glycosaminoglycans (hyaluronan or heparin) and, to some extent, the fibrous proteins collagen, elastin, laminin, and fibronectin.[37] Third, at least some of these organization processes are certainly driven by exergonic reactions, which are kinetically controlled by the flow of energy along metastable morphologies, assembled from the same or adjacent molecular building blocks.[38] In addition, the self-assembly of these blocks into regional, tissue-specific, and distinct ECM tissue units requires metabolic energy, especially in the form of ATP. Exemplary highlighted: the transparent cornea with the stromal ECM as the major refractive element of the eye is composed of homogeneous small diameter collagen fibrils which are regularly packed in a highly ordered hierarchical way.[39] Initially, in the developing cornea, collagen fibrillo-

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genesis involves multiple molecules interacting in a sequential way, a process which is driven by a cell-directed deposition of aligned collagen fibrils. In the next step tuned interactions between keratocytes and stroma matrix components follow. In parallel, collagen fibrils nucleate and form bundles within cell surface channels or fuse to form fascicles of uniaxial collagen.[40] While the mechanism of interaction, e.g., between the small leucine-rich proteoglycans and the collagen fibrils, has been well addressed also with respect to the temporal and spatial organization, the underlying energetics have only been poorly studied. It has been proven that ATP in the extracellular space promotes not only ECM biosynthesis, mediated by cell-based molecular pathways, but also increases the intracellular energy supply.[41] In turn, the maintenance of the functional organization of the ECM requires metabolic energy, surely in the form of nucleoside triphosphates, mainly of ATP, as well. Some of those ATP-dependent processes will be mentioned (Figure 2).

It should be stressed here that ATP, as well as polyP, is expected to be associated, especially in the extracellular space, with binding proteins. However, only very fragmentary and rudimentary first data have been gathered in this field.[42,43] ATP- and polyP-binding proteins could have the role of protecting these metabolites toward degrading enzymes, interfering with potential functional receptors, or allowing their transportation.

2.1. Purinergic Receptors

The plasma membrane comprises integrated purinergic receptors, purinoceptors, that respond to extracellular nucleosides (like adenosine) and nucleotides (ADP, ATP, UDP, or UTP), which act as signaling molecules.[44,45] They are involved in learning and memory, locomotion/movement, feeding behavior, as well as in sleep.[46] As typical signaling molecules these nucleosides and nucleotides react locally with the purinergic receptors.[47] As an example, ATP released from aggregating blood platelets acts as a signaling molecule and elicits endothelium-dependent vasodilatation. During this process nucleosides and nucleotides are released from intracellular organelles and stores and act locally around the extracellularly exposed receptor(s).[48] ATP acts as a signaling molecule on the purinergic receptors and, as such, needs to exist only at defined, usually low concentrations, triggering intracellular metabolic reactions.[49]

Important to note that polyP, present in the mammalian brain, acts in micromolar concentrations as a gliotransmitter between astrocytes $P2Y_1$ purinergic receptors.[50] The cells respond with an activation of phospholipase C, followed by a release of Ca^{2+} from the intracellular stores. Furthermore, besides neural cells, the P2Y purinergic receptors are also found at the surface of cardiomyocytes[51] as well as on platelets and other hematopoietic and nonhematopoietic cells with, e.g., the subtype $P2Y_{12}$.[52]

In addition to the function of ATP/ADP as a signaling molecule and a link in an autocrine signaling loop, the nucleotides are fed as substrates for metabolic energy-requiring processes in the ECM space. Some examples are given below.

2.2. Kinase Reactions

The intracellular signaling is dependent on amplifiers that potentiate extracellular signals (like hormones) via enzyme reactions (kinase reactions). Over 500 kinases have been described in humans[53] and about 30% of the intracellularly existing proteins are phosphorylated.[54] Initiated by analyses of the mammalian phosphoproteome, a series of secreted, extracellular proteins with phosphotyrosine units has been disclosed,[55] like the vertebrate lonesome kinase (VLK). It is secreted in the ECM as a Tyr kinase which phosphorylates proteins both in the secretory pathway and outside the cell.[56] Evidence has been presented that this kinase is physiologically regulated during platelet degranulation and enzymatically active.

2.3. Peptidylprolyl *cis*–*trans* Isomerases

The triple-helical protein collagen represents the most abundant ECM component. A series of enzymes, molecular chaperones, and post-translational modifiers facilitates the maturation of collagen. Among them are the peptidyl-prolyl cis–trans isomerases (PPIases) which catalyze an essential step during trans isomerization of the peptidylprolyl bonds, a rate limiting step in protein folding.[57] The PPIases cover three families of structurally unrelated proteins, the cyclophilins, FK506-binding proteins, and parvulins. Spontaneous isomerization of peptidylprolyl bonds is a rather slow process at physiological pH conditions; the PPIases substantially accelerate this reaction.[58] The first evidence that PPIases are present in the extracellular space came from studies with hensin, an ECM protein that forms 50- to 100 nm-long fibers.[59] On the basis of theoretical consideration, it has been proposed that the cis–trans interconversion does not require ATP but instead is driven by energy derived from conformational changes in the protein.[58,60] However, more experimental data are required to rule out that ATP is not indirectly involved in those PPIases processes, since for the FK506-binding protein binding of ATP to the domain II has been identified, which has been shown to be functionally active, while GTP is not.[61] It remains also to be studied if a functional interplay between prolyl isomerases and ATP consuming HSPs, like with HSP60, during mitochondrial protein import exists, allowing a reduction of the number of ATP binding and release cycles and, in turn, making the folding more efficient and less energy-consuming.[62]

Interestingly, the formation/assembly of collagen type I monomers into higher organization complexes, into SLS (segment long spacing) crystallites[63] with a length of ∼300 nm, is accelerated in the presence of ATP under in vitro conditions.[64]

2.4. Heat Shock Proteins/Clusterin

Heat shock proteins (HSPs) support and restore not only intracellularly but also extracellularly correct protein folding.[65] The chaperones can be grouped into different families, according to their structure, complexity, and regulation.[66] First, energy-independent chaperones, which function as monomers, dimers, or oligomers, and second, complex multichaperone machines, which bind to ATP, hydrolyze the nucleotide, and regulate cochaperone as well as substrate protein binding and a subsequent release (reviewed in ref 67) are key regulators of proteostasis and include TRiC, HSP70, and HSP90. Surprisingly, it has been disclosed that polyP shares protein-like chaperone properties.[68] The authors demonstrated that at micromolar concentrations, polyP efficiently prevent protein aggregation during stress conditions, like oxidative stress and higher temperature. Even more, it has been reported that in the presence of polyP, enzymes are stabilized as soluble micro-β-aggregates with amyloid-like properties.[69] In continuation of this line, the authors showed

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that polyP prevents misfolding of the proteins by rendering them in a β-sheet-rich, amyloid-like, soluble conformation.[69]

Solid evidence exists that HSP70 is present in the extracellular space and released via the MyD88/NF-κB signal transduction pathway.[70] Since HSP70 shows functional activity in the presence of ATP, it is more than hypothetical to suppose that HSP70 also binds ATP in the extracellular space, allowing energy-dependent protein folding.[71]

Perhaps more experimental data on the potential function of ATP toward the extracellularly present HSP clusterin are needed.[72] For this protein it has been suggested that it functions independently of ATP hydrolysis,[73] even though it comprises the ATP binding domain.[74] Another example of an extracellular chaperone with ambiguous interaction with ATP is α-crystallin.[72] This molecule protects vertebrate eye lens proteins from adverse protein aggregation. In addition, this protein exists also in extra-lenticular organs, including heart, kidney, and brain.[75] In an experimental approach, it could be demonstrated that αB-crystallin, as a molecular chaperone, increases its chaperone functions in the presence of ATP.[76] Further experiments are needed to clarify if those mentioned ATP-independent folding processes are not indirectly coupled with ATP consuming reactions.

2.5. Sol to Gel Transition: Supramolecular Polymers

Basically the ECM can be considered as a dense matrix which appears, at first glance, as an impermeable barrier for cells to infiltrate and to migrate into. In accordance with the gelation theory, the sol−gel transition theory,[77] it is assumed that gelation starts when soluble polymer chains randomly bind together under formation of growing soluble clusters which assemble together and form a gel. This transition state is critical and fast and only depends upon a slight variation in the bond quantity.[78] The directed navigation of cells through the ECM requires a series of intracellular biochemical signaling pathways which are probing the microenvironment through integrin-mediated adhesion circles. These cellular physiological processes must be coordinated with phase transition changes in the ECM during which the thermodynamical equilibrium shifts to a partition. This process requires free energy.[79,80] It is known that within cells, ATP-driven processes cause fluctuations which usually result in random movements that can become directional if driven by motor proteins.[81]

Sol−gel transition processes often accelerate the coassembly of two components in supramolecular systems, like in hydrogels, the ECM, or in the cytoplasm. Supramolecular polymerization is caused and mediated by noncovalent interactions, which are usually weaker than covalent ones,[38] but in contrast to covalent ones more wide-ranging and more specific. Even more, because of their property to be highly flexible in their organization, the molecules associated with functional units in the ECM are rarely used for a static stabilization of the cells. Specifically, the supramolecular polymers are prime candidates allowing a transitionally membranous scaffold formation in the ECM in order to set up extracellular functional compartments.[82] In addition to those, some transport systems lined with epithelia, transcellular, pericellular, and paracellular transport pathways are characterized by a high flexibility in the ECM.[83] Examples are the claudin−claudin cis interfacial structures that organize the dynamic and flexible nature of tight junction strands.[84] Likewise, transcellular functional organization of aligned scaffolds is relevant for the formation of transcellular units and, by that, for an efficient transmission of information between neurons[85] and basically between any other cell systems. Those flexible structures, based on supramolecular polymers, are energy requiring and energy consuming. If those structures are not fueled by metabolic energy, the transport systems should stall. In consequence, the mentioned self-adaptive supramolecular organizations and cycles are energy dissipating systems. As shown for micelles, the growth and breakdown of supramolecular associations are ATP-dependent.[86] More generally, it can be proposed that a failure of ATP-driven transient supramolecular interactions in the ECM, like in the intracellular environment, results in a collapse of the metastable state of all living systems.

2.6. Coacervation

Related to the sol to gel transition process is the coacervate phase transition during which organic-rich droplets are formed via liquid−liquid phase separation, resulting in an association of oppositely charged polymeric molecules.[87] This process requires the availability of free energy and entropy in the system.[88]

2.7. Bone and Cartilage

It is well-established that mechanical energy is stored as elastic energy in both nonmineralized and mineralized components in the ECM.[89] Besides those energy-storing and dissipating systems in collagens and tendons,[90] the less flexible human skeleton, contributing with about 15% to the total body weight, requires metabolic energy, like for released elastic strain energy, for the formation as well as continual maintenance and repair of this dynamic tissue. It is indicative that during the onset of osteoblast mineralization, ATP levels in those cells peak, a process which is paralleled with the accumulation of mitochondria with high-transmembrane potentials in those regions;[91] subsequently, fully differentiated osteoblasts revert to glycolysis to maintain ATP production.[92] These findings indicate that the development and the bioenergetic programs of the osteoblasts are coupled with the mineralization state. Furthermore, osteocalcin, secreted solely by osteoblasts, increases insulin sensitivity and modulates an array of genes involved in energy expenditure, like for *Ucp2*. Its protein product causes mitochondrial uncoupling of oxidative phosphorylation under dissipation of energy as heat.[93]

In addition, polyP turned out to act as a source of inorganic phosphate needed during bone mineralization for the transformation of the initially formed amorphous calcium carbonate deposits ("bio-seeds") into amorphous calcium phosphate (for a review, see ref 30). While the calcium carbonate bioseed formation is enzyme-catalyzed by a membrane-associated carbonic anhydrase (CA-IX), the exchange of carbonate by phosphate in the amorphous calcium carbonate proceeds nonenzymatically but requires prior hydrolysis of polyP mediated by ALP. The amorphous calcium phosphate subsequently undergoes a phase transition to hydroxyapatite (Figure 2).

During bone and cartilage formation, like during wound healing, vascularization is one important process to happen and is essential for tissue construction and repair. During the initiation of this process, endothelial cells have to form initial small vessels. During this initial phase, the orientation, guiding, and ring formation of endothelial cells is mediated by ATP which is released into the extracellular space and acts as a chemotactic gradient along which the cells migrate.[10,94]

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In conclusion, among the above-mentioned processes in the ECM which are evoked by ATP, some of them are most likely dependent on metabolic energy, generated during the cleavage of the energy-rich bonds of the nucleotide. Examples are the ATP-requiring processes during the following reactions: phosphorylation reactions driven by ECM kinases, the peptidylprolyl cis−trans isomerase process during collagen packaging, some heat shock protein-mediated actions, coacervation, sol to gel transition processes during supramolecular polymer organization, and finally formation of bulky extracellular tissue, like in bone and cartilage.

In section 13 experimental evidence is given, underscoring that extracellular ATP is enzymatically generated from polyP.

3. STRUCTURE OF POLYP

Every pro- and eukaryotic cell contains inorganic polyphosphate (polyP) with a chain length that can reach up to several thousands of orthophosphate (P_i) residues (Figure 3; see refs 24, 25, 95−97). The tetrahedral P_i units forming this polymer are linked together by high-energy phosphoanhydride bonds similar to those found in ATP. While microorganisms usually contain very long-chain polyP, ranging in size from hundreds to thousands of P_i residues, eukaryotic cells synthesize polyP chains which are much less heterodisperse and comprise polymer lengths of ∼60−100 P_i units.

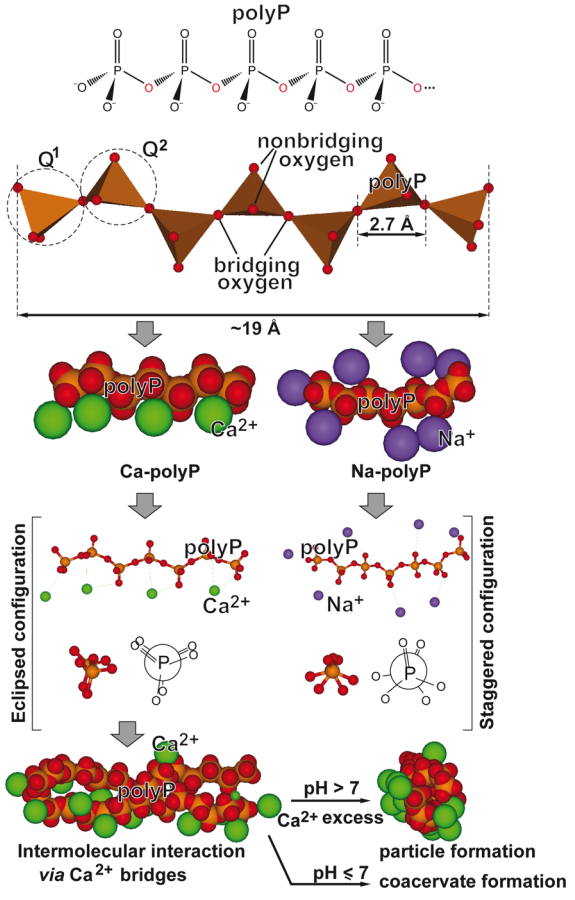

Figure 3. Structure of the polyP molecule composed of interconnected PO_4 tetrahedra, linked via their bridging oxygen atoms. The linear polyP consists of both Q^1 (one bridging oxygen) and Q^2 (two bridging oxygens) tetrahedra. Because of the rotational flexibility of the P−O−P (phosphoanhydride) bonds, the polymer can occupy various steric conformations (staggered and eclipsed), in dependence on the counterion. The space-filling models and ball-and-stick models of Ca-polyP and Na-polyP are shown. The divalent Ca^{2+} salt of polyP assumes an eclipsed configuration, while for the monovalent Na^+ salt of polyP a staggered configuration is found. Higher-ordered structures formed by an intermolecular Ca^{2+} bridge formation comprise both solid particles (at alkaline pH and Ca^{2+} excess) and coacervates (at lower pH values).

3.1. PolyP Chain

In accordance with the Q^i nomenclature (i = number of bridging oxygens), the linear polyP is composed of both Q^1 and Q^2 tetrahedra with one (terminal P_i units) and two bridging oxygens (internal P_i units), respectively[98] (Figure 3). Branched polyP chains ("ultraphosphates") with Q^3 tetrahedra (three bridging oxygens) are not found in living organisms. The P−O bond lengths within the P_i units are between 1.62 and 1.66 Å for the single bonds[99,100] and 1.52 Å for the P═O double bond.[101,102] The length of the P═O bond is much shorter than the sum of the two atomic single-bond radii (P: 1.07 Å and O: 0.66 Å)[100] due to the difference in electronegativity (P = 2.1; O = 3.5). On the basis of these values and the bond angles of P−O−P and O−P−O bonds (P−O−P: 130° and O−P−O: 102°),[103] the maximum length (longitudinal size) of the linear polyP molecules can be estimated from the size of each phosphate unit (2.7 Å); e.g., the maximum size of $polyP_{40}$ (usually used for the formation of biomimetic polyP nanoparticles; see below) amounts to 108 Å, while the maximum size of the long chain polyP produced by bacteria (around 1000 P_i units), which accomplish structural properties, is in the range of $\sim 3 \times 10^3$ Å (∼300 nm). Since the bond angles within the P_i units can vary between ∼120° to 180°,[104] and the polymer is physiologically complexed to divalent cations, a smaller value for the space-filling area is, however, more realistic.

3.2. PolyP Conformations

In addition and due to a high degree of rotational flexibility, the polyP chain can occupy various conformations, staggered and eclipsed,[104] depending on the arrangement of the tetrahedral PO_4 units (Figure 3). In this way, the polymer can adjust to the charge and the coordination requirements of the respective counterions which can be monovalent (e.g., Na^+ or K^+), divalent (e.g., Mg^{2+} or Ca^{2+}), or trivalent (e.g., Gd^{3+}) cations. The eclipsed configuration seems to be optimal for divalent Ca^{2+} ions, as confirmed by modeling studies with pyrophosphate,[105] while the monovalent Na^+ salt of polyP prefers a staggered configuration. The higher binding energy of the fully charged polyanionic polyP to divalent cations is the major factor that determines the preferential binding of Mg^{2+} and Ca^{2+} at physiological pH; at a lower pH/charge density of the polymer, monovalent cations increasingly bind to the polyP chain.[106] Based again on modeling studies, Ca^{2+} ions are able to form with polyP molecules organized aggregates/particles whereby—in the presence of a small number of polyP chains—the divalent cations occupy a more peripheral position (Figure 3).

4. DIFFICULTIES AND CHALLENGES IN ANALYSIS OF CELL-ASSOCIATED POLYP AND IN IDENTIFICATION OF POLYP METABOLIZING ENZYMES

The polyP polymer has been identified comparably late after the discovery of ATP, first described as adenyl pyrophosphoric acid, by Lohmann.[107] Initially named RNA,[108] the basophilic

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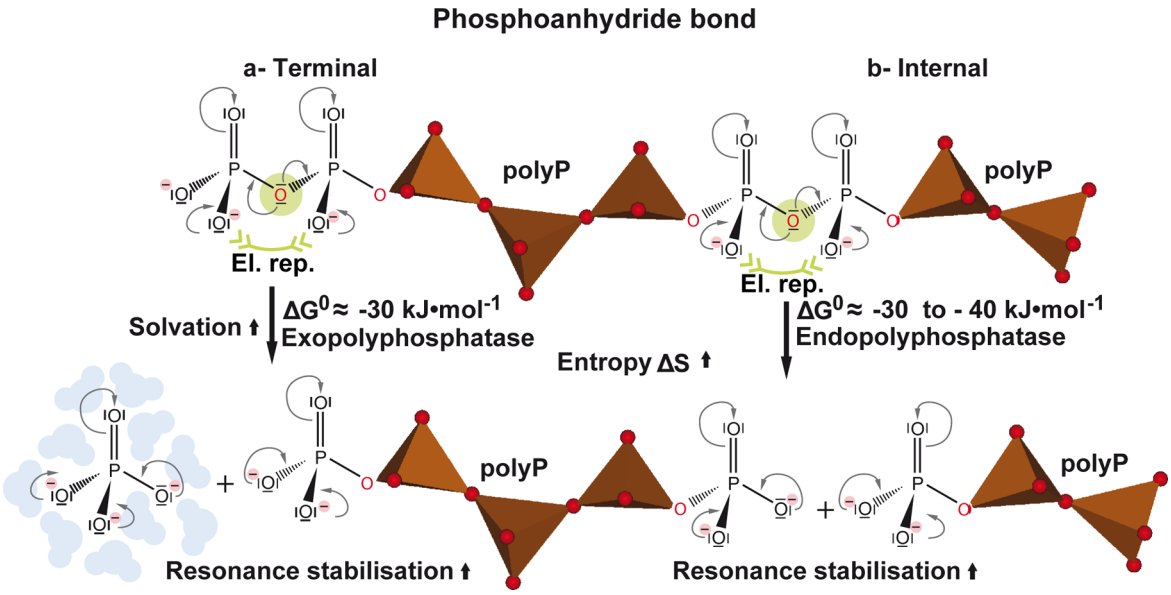

Figure 4. Various factors contributing to the large amount of Gibbs' free energy released during polyP hydrolysis by exopolyphosphatases and endopolyphosphatases. El. rep., electrostatic repulsion.

polymer was later identified as metaphosphate by Schmidt et al.[109] and later as polyphosphate.[110] The first suggestion that polyP can serve as an energy-rich phosphate came from Ebel in 1952 (see ref 111). The first attempts to understand the polyP metabolism were performed by Liss and Langen.[112]

4.1. Chemical Analysis of polyP

Despite the increasing importance of polyP in medicine and biotechnology as well as in environmental research, the chemical and biochemical analytics of the polymer are not well developed.[113,114] Starting with biological and environmental samples, a significant disadvantage is seen in the isolation procedure because during the extraction of the polymer other phosphate-containing compounds are coisolated. Initially, polyP was adsorbed to charcoal, followed by LiCl desorption.[115] After delipidation and alkali treatment, the material was neutralized with perchloric acid. The polyP in the fractions was determined after hydrolysis in acid and corrected for the orthophosphate content.[116] In addition, especially if an accurate chain length is attempted to be determined, an interference with the ALP is a serious pitfall since this enzyme readily hydrolyzes the polymer during and after the extraction process from living tissue, especially from eukaryotic cells.[117]

An improvement was reached by application of a sequential extraction procedure, by isolation first of acid-soluble polyP and second of long-chain polyP at neutral pH.[118] After removal of proteins using phenol-chloroform and nucleic acids by precipitation of polyP at neutral pH, the polyP factions (short chain and long chain) were collected by ion exchange chromatography. The polymer was quantitatively assessed after charcoal adsorption, by a total phosphate colorimetric assay, including also the toluidine blue colorimetric reaction.[118,119] This basic extraction concept is still applied today[97,120] and includes extraction after breaking up the cells with a chaotropic agent, like guanidinium thiocyanate, followed by enzymatic digestion with proteinases, phenol/chloroform mixture extraction, and anion exchanger-based chromatography. The chain length of polyP was determined by thin layer chromatography (short- to medium-chain polyP), ion-exchange chromatography, gel-filtration, electrophoresis on urea-polyacrylamide gels or ^{31}P NMR spectroscopy.[119−123] The calibration of the systems was achieved with either fluorescently or radiolabeled polyP markers.[124−126] Introduced is also the overall determination of polyP, using the Fourier transform spectroscopy (FTIR),[30] a technique which can be used also quantitatively.[113] A less practical but comparably sensitive method for the quantitation of polyP was introduced with the fluorescence Ca^{2+} indicator fura-2.[127] The fluorescence intensity in this system was found to be abolished with increasing polyP concentrations. This method turned out also to be applicable for the determination of polyP catabolic enzymes.

4.2. Determination of polyP in Intact Cells

It is of serious disadvantage that for polyP a specific dye does not exist. Very often the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) is used for in situ detection of polyP within cells or tissues.[128] However, this reaction is not specific since DAPI stains also nucleic acids with only slightly different characteristics. The dye toluidine blue is less frequently used.[129]

4.3. Assays for polyP Metabolizing Enzymes

Due to the lack of precise polyP quantification techniques, faithful methods for the quantification of enzymatic reactions of polyP metabolizing enzymes are also not available. As an example, the photometric determination of the polyP kinase both acting as a polyP synthesizing enzyme and as a polyP hydrolyzing enzyme might be mentioned.[130,131] In the first system, an enzyme-coupled assay with pyruvate kinase and lactate dehydrogenase has been elaborated, while for the reverse reaction, the determination of NADH consumption is used as a changing parameter. Furthermore, a similar assay has been introduced for the polyP-AMP-phosphotransferase reaction[132] during which ATP, ADP, and AMP in combination with luciferase and luciferase/adenylate kinase is quantified. Finally, the purine-nucleoside phosphorylase has been used as an enzyme indicator for the quantification of polyP in the reaction.[119]

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In all these assay systems, inaccuracies can creep in especially during the preceding isolation of the polyP product/substrate and the indirect approaches used for quantification of the product. Therefore, more reliable and specific methods have to be developed in the future suitable for a more precise quantification of polyP in cells and also during the enzyme reactions. These remarks should also indicate that some of the existing enzymatic and functional data should be taken with some caution.

5. PHYSIOLOGICAL ROLES OF POLYP

Because of the presence of multiple energy-rich phosphodiester bonds, long-chain polyP is able to store large amounts of metabolically utilizable energy, much more than an ATP molecule. The energy (ΔG^0; standard conditions) released during hydrolysis of a single phosphoanhydride bond within this polymer is similar to that of hydrolysis of the $\beta-\gamma$ or $\alpha-\beta$ phosphoanhydride bond in ATP (-30.5 kJ mol^{-1}) or even higher ($\Delta G^0 \approx -40$ kJ mol^{-1}).[133]

As sketched in Figure 4, several factors, similar to ATP hydrolysis, may contribute to the highly exergonic character of the hydrolytic cleavage of the phosphoanhydride bonds of polyP, which is catalyzed by polyP exo- and endopolyphosphatases, such as the extensive electrostatic repulsion of the negatively charged oxygen atoms on the phosphate groups,[134] changes in entropy[135] or resonance stabilization[136,137] and a higher degree of solvation of the reaction products,[135] compared to polyP (Figure 4).

It should be noted, however, that under physiological conditions, the actual ΔG values, as with ATP, may be influenced by a number of factors, such as pH, temperature, divalent cations, and ionic strength.[138−143] For example, physiologically, polyP (like ATP)[144] is coordinated with divalent cations, like Ca^{2+} and Mg^{2+}.[25] Therefore, it is expected that the actual ΔG values for polyP hydrolysis will be significantly different from ΔG^0, similar to ATP hydrolysis. For example, in the case of ATP hydrolysis, the actual ΔG values, for example, in the liver, are between -59 and -53.5 kJ mol^{-1}, and in the heart, between -61.7 and -59.5 kJ mol^{-1};[141,145] for in vivo data (human brain and muscle), see ref 146.

In contrast to microorganisms, the biological and biochemical pathways of polyP in higher eukaryotes have been largely unknown for a long time, although this polymer is detectable throughout the entire animal kingdom. This situation changed more recently when it became obvious that polyP has a functional role during bone mineralization[147] as well as affects the energy balance within the cells and also in the surrounding ECM.[148−150] First indications came from an observation that the amount and turnover of polyP is considerably higher in tissues with high metabolic rates and requirements like the brain or heart.[150−152] However, caution is advisible about the reliability of those data since the isolation, purification, and analysis methods used are sometime immature, as pointed out.[153]

Microorganisms such as bacteria and yeast can accumulate large amounts of polyP. Multiple functions of polyP in these organisms have been demonstrated, e.g., being a source of energy, a donor for sugar and adenylate kinases, and also acting as a chelator for divalent cations and working as a functional buffer against alkaline stress or as a regulator of development. Likewise, the enzymes catalyzing these reactions have been identified (see refs 25 and 26). However, also vertebrates contain significant amounts of polyP. This polymer has been identified in every animal tissue investigated so far, from mouse heart (~114 μM), brain (95 μM), and lungs (91 μM) to kidney (64 μM). Lower amounts of polyP are found in the liver (38 μM), while high levels are present in mast cells and especially in blood platelets (up to 1.1 mM).[121,154] It might be stressed that blood platelets comprise ~5%, after erythrocytes (~85%) but before bone marrow cells (2.5%) and lymphocytes (1.5%), the second most abundant cells in the human body.[155] In human blood, a concentration of 1−3 μM polyP has been calculated.[96] Also in other body fluids, like in synovial fluids, polyP exists.[156] Within mammalian cells, this polymer has been identified in lysosomes,[157] dense granules of the acidocalcisomes,[121] mitochondria, and nuclei.[158]

Focusing on the polyP effect within the eukaryotic, mainly mammalian cells, it could be elucidated that polyP is likely to be involved in the following pathways.

5.1. Energy Production and Permeability Transition Pore Induction

In the mitochondria, polyP was found to increase in response to the activation of the metabolic respiration and be reduced by their inhibitors.[159] Oligomycin, a known inhibitor of complex V/F_1F_O-ATP synthase, abolishes the proton gradient at the inner mitochondrial membrane and strongly interferes with mitochondrial polyP metabolism.[159] The crucial role of polyP in mitochondrial energy metabolism has been demonstrated by enzymatic depletion of the mitochondrial polyP content using polyP-hydrolyzing yeast exopolyphosphatase PPX1; a significant decrease in mitochondrial membrane potential required for F_1F_O-ATP synthase-mediated ATP production was observed.[160] The major role of mitochondrial polyP at certain pathological conditions associated with ischemic cell death like stroke and myocardial infarction[161,162] has been intensively studied. These pathological conditions are associated with a Ca^{2+}-induced activation of the mitochondrial permeability transition pore (mPTP), a large, voltage-dependent multiprotein channel complex in the inner mitochondrial membrane, causing opening of the channel and leading to increased membrane permeability, decreased mitochondrial membrane potential, abolition of ATP production, and ultimately cell death (for a review, see refs 154 and 163). Especially reperfusion/reoxygenation therapy of ischemic tissue can result in excessive Ca^{2+} accumulation inside the mitochondria. Already in the first report on the effect of polyP, evidence has been presented that this polymer is closely associated or a component of the mPTP complex.[164] In this study, the isolation of a polyP/Ca^{2+}/poly-3-hydroxybutyrate (PHB) complex from mammalian (rat liver) mitochondria was reported,[164] a complex which was first identified in bacteria.[165] On the basis of its properties, it was concluded that this complex might act as the ion-conducting module of mPTP.[164] The proposed involvement of the polyP/Ca^{2+}/PHB complex in mPTP opening was supported by polyP depletion experiments that revealed a strong inhibition of mPTP function,[159,166] resulting in prevention of mitochondrial Ca^{2+} accumulation and cell death (see refs 154 and 167). The effects of polyP are correlated with the chain length of the polymer. While long chain polyP ($polyP_{120}$) can induce/activate PTP and cell death as well as mitochondrial depolarization in astrocytes, medium- ($polyP_{60}$) and short-chain polyP ($polyP_{15}$) failed to cause this effect.[168] This result has been supported by findings revealing that short and medium polyP does not cause

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apoptosis while long chain polyP activates the apoptotic cascade in neurons and astrocytes.[168] In a more recent study, a highly purified preparation has been obtained from mammalian mitochondria that contained besides the polyP/Ca^{2+}/PHB also the C-subunit of the ATP synthase.[169] The properties of this isolated channel-forming complex were quite similar to the native mPTP. The data also showed that polyP, PHB, and the C-subunit are intimately associated as an integral component of the Ca^{2+}-induced mPTP.[169] This conclusion was also supported by the finding that the formation of this channel-forming complex is inhibited by cyclosporine A, an inhibitor of mPTP.[169] Interestingly, polyP has also been found to be an modulator (activator) of mTORC1 (target of rapamycin complex 1),[170] a complex that acts as an intracellular homeostatic ATP sensor by activating a series of anabolic metabolic pathways.[171] Recently, it has been reported that a decrease of mitochondrial membrane potential by opening mPTP pores is associated with a rise in calcification of polyP-treated MC3T3-E1 osteoblastic cells.[172]

5.2. Cell Sensation

The receptor-activated nonselective cation channel TRPM8 (transient receptor potential melastatin subtype 8), a cold-activated channel, is involved in sensing of extracellular temperature, such as coolness, but is also activated by certain chemicals such as menthol. Whole-cell patch-clamp and fluorescent calcium measurements with human embryonic kidney and F-11 neuronal cells revealed that the TRPM8 protein complex forms a stable association with polyP and PHB resulting in a modulation of the sensitivity threshold.[173] A related but mechanistically distinct function of polyP, acting on the nociceptive sensation, has been described.[174] The TRPA1 receptor (transient receptor potential A1) is expressed in sensory afferent neurons and requires polyP as a soluble factor to become functionally active. In contrast to TRPM8 which copurifies with polyP, TRPA1 only weakly interacts with polyP.

5.3. Cell Metabolism

The overall cell metabolism of human fibroblast cells is activated by polyP.[175] This effect has been deduced from the findings that the mitogenic activities of both the acidic fibroblast growth factor (FGF-1) and the basic fibroblast growth factor (FGF-2) are enhanced by this polymer. In addition, polyP increases the adhesion capacity of the cells via their cell surface receptors. Support came also from more recent studies with Caco2/BBE cells.[176] These authors described that polyP induces *HSP27* gene expression in vitro and causes in vivo a protection of mice against inflammation, elicited by Na-dextran sulfate. Finally, it should be mentioned that higher levels of polyP are present in myeloma plasma cells, which are assumed to modulate the transcriptional activity, catalyzed by RNA polymerase I.[177]

5.4. DNA Synthesis and Repair

Using yeast cells as a model, polyP was found to increase the intracellular pool of deoxynucleotide triphosphates (dNTPs). These "extra" dNTPs were identified to protect the cells against DNA damage and extend the longevity of those compromised cells.[178] Subsequent studies with mammalian HEK293 cells and in human dermal primary fibroblasts confirmed this effect by demonstrating that after deprivation of those cells for polyP the cells become more prone to DNA damage.[178] In turn, the authors claim that polyP increases the intracellular metabolic energy in the form of ATP needed during the DNA repair process. In the bacterial system *Pseudomonas aeruginosa*, a close coupling of polyP synthesis with the completion of cell cycle exit during starvation has been demonstrated.[179] This aspect of the interaction of polyP with the DNA replication machinery is, at the present state, not completely understood.

5.5. Blood Clotting

It has been suggested that after release from activated blood platelets, synthetic polyP initiates clotting of plasma via the contact pathway on the level of factor XII.[120] However, subsequent studies disclosed that when platelets become activated only physiologically occurring short-chain polyP is released, which has only a very low capacity of direct activation of factor XII (see ref 180).

5.6. Bone Mineralization and Cartilage Formation

Among the most energy-consuming processes proceeding in the extracellular space in humans are the bone and cartilage anabolic processes. An extensive review on this subject has been very recently published,[30] and it is also discussed here in the context of energy homeostasis during bone and cartilage formation in the body.

Increasing evidence indicates that polyP can also act as an important mediator of pro-inflammation and pro-coagulation.[181] Both activities are beneficial in innate immune response and during at least the initial stages of wound healing, e.g., accelerating the sealing process for severe injuries.[182] These authors identified in rat basophilic leukemia mast cells polyP with a chain length of 60 P_i units which they attribute to the serotonin-containing acidocalcisomes. It has been described that those polymeric molecules are secreted from platelets/acidocalcisomes upon activation.[120,121]

The supporting role of ATP and adenosine being an important metabolite that controls the activity of chondrocytes in rat tibia explants was demonstrated in mice lacking the adenosine A_{2A} receptor.[183] Animals with this defect develop spontaneous osteoarthritis, a degenerative joint disorder. Supplementation with adenosine via intra-articular injection prevented the development of the disease.

6. POLYP METABOLISM IN PROKARYOTIC AND EUKARYOTIC CELLS: ENZYMES

Although polyP is an abundantly present inorganic polymer found in all cells from prokaryotes to eukaryotes, the way of synthesis and degradation of polyP in the different kingdoms of life appears to be distinctly different. This partially depends on the different functions of polyP in these organisms. In (eu)bacteria, polyP takes an active/functional role in survival at the stationary phase, stress responses, activation of ATP-dependent serine peptidases, Lon proteases, as well as during virulence[184] (reviewed in ref 185) but also acts extracellularly as an intercellular communication/signaling molecule during quorum sensing processes, and biofilm formation.[186] Also in the budding yeast *Saccharomyces cerevisiae* and the social amoeba *Dictyostelium discoideum*, polyP acts as a signaling molecule during pH homeostasis[187] and growth control.[188] In animals, polyP is also released into the extracellular space, especially from the platelets, and functions there as a regulator of a series of regeneration processes, e.g., in wound healing,[189,190] inflammation,[189] and coagulation.[180,191]

The chain length of the polyP has a decisive effect on the biological effects of the polymer. This has been reported both

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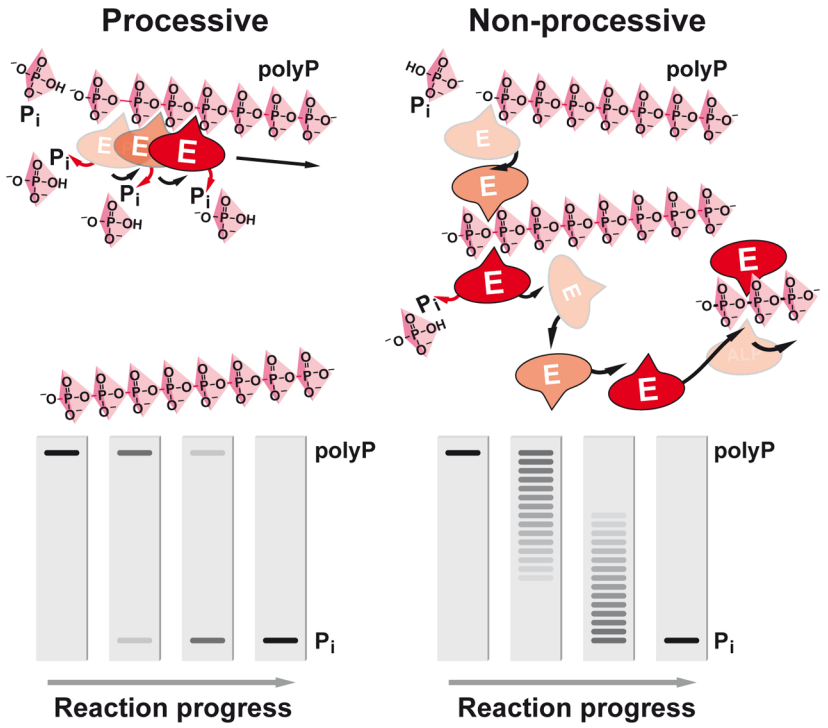

Figure 5. Processive versus nonprocessive degradation of polyP. In the processive mechanism (left), the enzyme (E) remains bound to the polyP substrate until the degradation is completed, while in the nonprocessive reaction (right), the enzyme dissociates from the substrate after each catalytic cycle and then reassociates either with the same or another polyP molecule.

for intracellular (e.g., mitochondrial) and extracellular polyP. Therefore, the control of the size of the polymer seems to be of utmost importance. In principle, the polyP chain length can be controlled both on the level of polyP synthesis, catalyzed, e.g., by bacterial polyP kinases (PPK) [EC 2.7.4.1], and on the level of polyP degradation. In yeast and higher eukaryotes, different mechanisms for polyP synthesis have been developed, as discussed later.

polyP degradation can occur either by hydrolysis of the polyP chain, catalyzed by polyphosphatases, both by exopolyphosphatases [EC 3.6.1.11] which hydrolyze the polyP chain from the ends of the polymer under liberation of orthophosphate (P_i) and by polyP endopolyphosphatases [EC 3.6.1.10] which cleave polyP within the polymer chain, or by transfer of the terminal P_i groups to an acceptor molecule, mediated by a phosphotransferase. Examples of the latter group of enzymes are a polyP-AMP phosphotransferase [EC 2.7.4.B2] which catalyzes the phosphotransfer from polyP to AMP,[192] or a polyP glucokinase [EC 2.7.1.63] which phosphorylates glucose by polyP.[193] While in the first case, the energy of the energy-rich phosphoanhydride bond is liberated in the form of heat, this energy is, at least partially, conserved in the latter case in the newly formed phosphoanhydride or phosphoester bond at the acceptor molecule.

In addition, two principal mechanisms can be discerned through which degradation (but also synthesis) of polyP can proceed: processive or nonprocessive (Figure 5).[24,25,117] In the processive reaction, the enzyme (here an exopolyphosphatase) remains bound to the polymer until the degradation of the polyP chain is completed. Analysis of the products on high-percentage polyacrylamide gels will reveal only two bands, the undegraded polyP substrate, which decreases during the reaction, and the product (e.g., P_i), which increases in the course of the reaction. No intermediate polyP chain lengths can be seen. In the nonprocessive mechanism, a successive association and dissociation of the enzyme from the polyP during each catalytic step takes place. In turn, the enzyme continually "jumps" from one polyP molecule to another until the substrate is completely turned over. Consequently, polyacrylamide gel analysis will show a ladder-like pattern reflecting the occurrence of shorter polyP chains between the polyP substrate and P_i product in the course of the reaction. It should be noted that some polyphosphatases break down the polyP to a certain chain length or change from a processive to a nonprocessive mechanism beyond a certain chain length; in the latter case, a laddering pattern is observed below that size of the polyP molecule.

6.1. Eubacterial Enzymes

The intracellular, enzyme-driven polyP anabolism and catabolism, especially with respect to the energy metabolism, is well understood in bacteria. In the center of the bacterial energy transfer reactions are the polyphosphate kinases (PPKs),[26,194] which synthesize polyP by using ATP as a substrate[194] (Figure 6). Besides in bacteria, the PPKs have been identified in archaea, fungi, yeast, and algae (reviewed in ref 185) but not yet unequivocally in animals.[159] The PPKs are reversible acting enzymes transferring energy-rich bonds in both directions.[185]

Due to the comprehensive studies of the Kornberg's group, the enzymatic metabolism of polyP in prokaryotes has been resolved. The initial ATP-dependent step of polyP synthesis is mediated by the polyP kinase 1 (PPK1 [EC 2.7.4.1]), most likely the dominant enzyme in bacteria (reviewed in refs 195 and 196). Experimental data indicate that the metabolic energy, stored in polyP, can be reutilized in bacteria to synthesize ATP. A second bacterial polyP kinase, the PPK2

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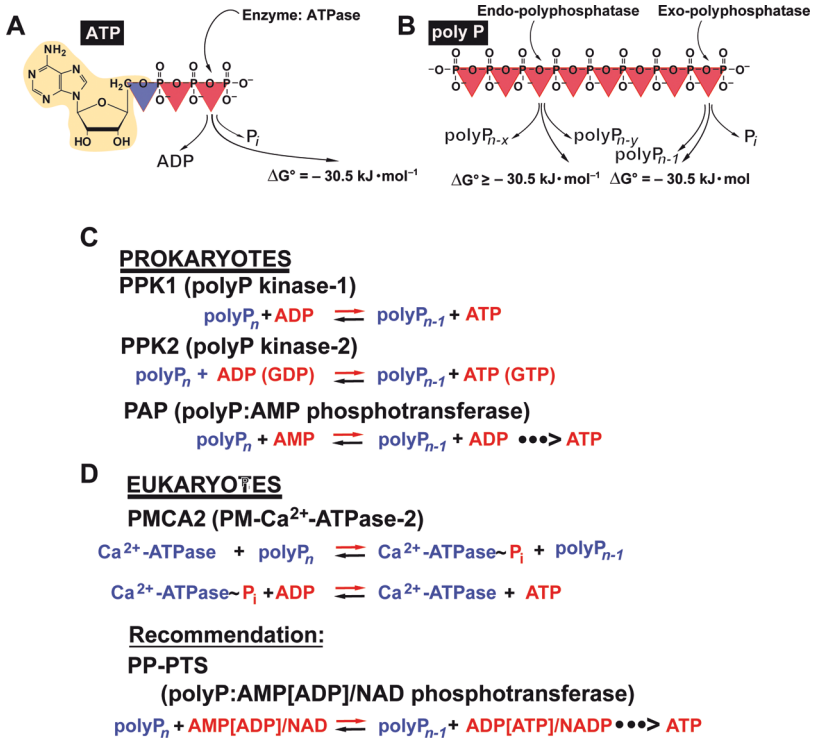

Figure 6. Enzymes involved in energy storage and energy supply by polyP. (A) ATP as the intracellular energy generator, and (B) polyP as an intra- and extracellular energy source and energy generator during the enzymatic hydrolysis by exo- or endopolyphosphatase(s). In both compounds, energy-rich bonds are present that comprise a ΔG^0 of about −30.5 kJ mol^{-1} per bond. The polyP metabolizing enzymes that catalyze reversible reactions are present in both (C) prokaryotes and (D) eukaryotes. In prokaryotes, the initial ATP-dependent step of polyP synthesis is catalyzed by polyP kinases; the reactions catalyzed by polyP kinase 1 (PPK1) and polyP kinase 2 (PPK2) are shown. These reversible reactions can also serve for the formation of ATP (GTP) from polyP. Additionally, it has been suggested that polyP-AMP-phosphotransferases are using polyP as a substrate for the synthesis of ATP via AMP and ADP. For eukaryotes, it has been described that the plasma membrane associated Ca^{2+}-ATPase (PMCA) acts as an ATP-polyphosphate transferase and polyphosphate-ADP transferase. Finally, an ALP is present, which is involved in both polyP hydrolysis and ADP formation from AMP, as well as a NAD-kinase which catalyzes the transfer of the terminally located energy-rich phosphate units of the polyP chain to NAD^+ resulting in $NADP^+$ production. We recommend to group these phosphotransferases which also includes the PPK (reversible reaction) as polyP-phosphotransferase system [PP-PTS].

[EC 2.7.4.1][197] has been described that shows similarity to the mammalian thymidylate kinase.[198] In addition, several bacterial PPKs have been identified that use preferentially pyrimidine nucleotides as cosubstrates; they were termed PPK3 [EC 2.7.4.1].[199]

polyP degradation in bacteria is catalyzed both by the polyP kinases (reversible reaction of PPKs) and exopolyphosphatases which only hydrolyze the polymer chain.[200,201] A well investigated polyP degrading exopolyphosphatase is the bacterial exopolyphosphatase (PPX [EC 3.6.1.11]) from *Escherichia coli* a highly processive enzyme that preferentially hydrolyzes long polyP chains (≥1000 P_i residues).[125,201,202] The enzyme is a dimer[201] which produces four discrete intermediates: $polyP_2$, $polyP_3$, $polyP_{14}$, and $polyP_{50}$. In a model proposed,[125] each monomer of the PPX dimer contains two catalytically active *N*-terminal sites and three polyP binding sites located at a distance of 3, 14, and 50 P_i residues from the active site.

Two further examples of an enzyme that produces intermediates with a specific chain length during degradation are the *E. coli* polyP guanosine pentaphosphate phosphohydrolase (GppA) [EC 3.6.1.40][203] and the polyP glucokinase [EC 2.7.1.63] of *Propionibacterium shermani* which phosphorylates glucose by polyP.[193,204] Both enzymes degrade long chain polyP by processive hydrolysis of the terminal phosphates until a specific chain length of 40 P_i units ($polyP_{40}$; for GppA)[203] or 100 P_i units ($polyP_{100}$; for polyP glucokinase)[193,204] is released.

Additionally, a bacterial polyP-AMP-phosphotransferase (PAP [EC 2.7.4.B2])[192,205] has been described that apparently metabolizes polyP as a substrate for the synthesis of ADP and ATP via ADP (the latter reaction, ATP formation, proceeds together with an adenylate kinase),[206,207] as well as an enzyme from *P. aeruginosa* that acts both as a polyP:ADP phosphotransferase and an exopolyphosphatase.[208,209] The active site of latter enzyme is assumed to be involved in both enzyme activities.[209]

In contrast to yeast and animals, endopolyphosphatases which cleave polyP in the middle of the chain seem to be absent in bacteria.

Interestingly, the polyP glucokinase from *P. shermanii* can change from a processive to nonprocessive mechanism. The degradation of long chain polyP by this enzyme proceeds via a processive mechanism, while short polyP is hydrolyzed nonprocessively.[193,204] This change of the type of reaction is paralleled by a change of the K_m of the enzyme that first increases slowly with decreasing polymer size but then dramatically increases at a chain length of 30 ($polyP_{30}$). After reaching this size, the enzyme dissociates from the polymer and further degradation proceeds by a nonprocessive mechanism.

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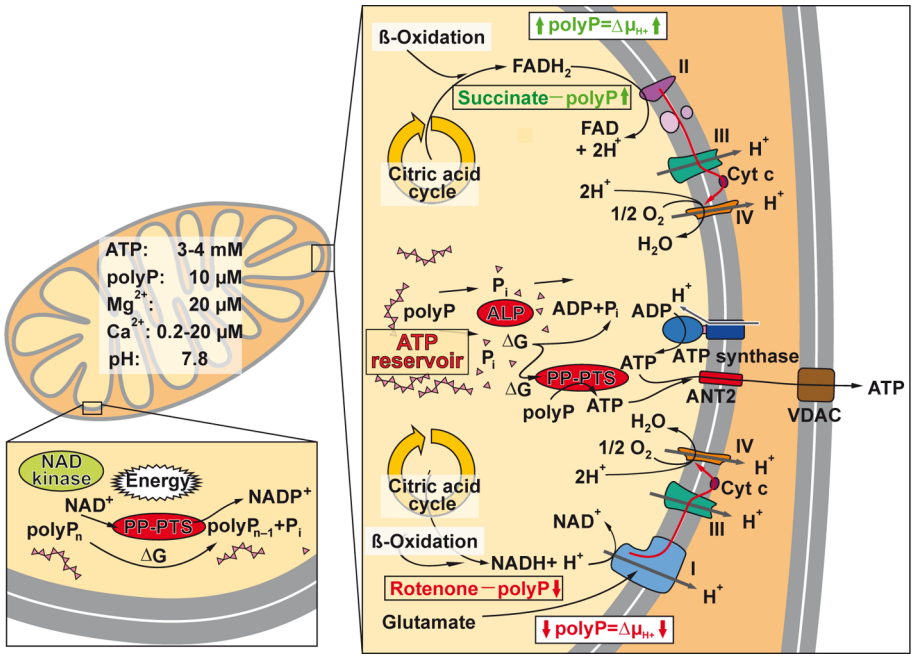

Figure 7. Mitochondria as storage of ATP and ATP buffering organelles. These organelles are the major intracellular ATP generator producing ATP from reducing equivalents during metabolism of glucose and fatty acids via glycolysis (cytosol) and β-oxidation and finally the citric acid cycle (mitochondrion). The reducing equivalents drive the electron transport chain in the inner mitochondrial membrane, which ends in the ATP generator, the ATP synthase. The adenine nucleotide translocase (ANT2) allows the ATP to cross the mitochondrial inner membrane, while the voltage-dependent ion channel (VDAC) is involved in the energetic flux and transport of ATP and ADP across the outer mitochondrial membrane. Within the mitochondrial matrix, polyP is proposed to be metabolized via the NAD kinase (insert), an enzyme that catalyzes the phosphorylation of NAD^+ to $NADP^+$ under consumption of ATP and other nucleoside triphosphates as well as of polyP as a phosphate source. Most likely many of these complex enzymatic reactions, including the ALP and the NAD kinase, act as PP-PTS. A reduced polyP level parallels with a decrease of the mitochondrial membrane potential ($\Delta\mu_H$)/proton gradient (ΔpH^+) at the inner mitochondrial membrane and vice versa. The approximate concentrations of ATP, polyP, Mg^{2+}, and Ca^{2+} as well as the intramitochondrial pH value are given.

6.2. Fungal Enzymes

Some phylogenetic data revealed that eukaryotic fungi share a close relationship with animals.[210,211] In yeasts, polyP accumulates in large amounts inside the vacuole, an acidic, acidocalcisome-like organelle,[212] where cellular compounds are recycled. This vacuole is a dynamic structure that can rapidly modify its morphology. There the polyP polymer accumulates and is additionally found in smaller levels in the cytosol, nucleus, and mitochondria (reviewed in ref 213).

Using the yeast *S. cerevisiae* as a model, most knowledge on polyP metabolism in eukaryotes has been gathered. Among the yeast enzymes involved in polyP metabolism are the cytosolic exopolyphosphatase PPX1,[214,215] the cytosolic endopolyphosphatase DDP1,[216−218] and the vacuolar endopolyphosphatases PPN1,[219−221] which can switch between exo- and endopolyphosphatase activity,[222] and PPN2.[223,224] In addition, a vacuole transporter chaperon complex (Vtc complex) has been identified that synthesizes polyP and acts as a main phosphate storage molecule in yeast. This polymerase uses ATP as a substrate and transfers the γ-phosphate to an acceptor phosphate under formation of a polyP chain.[225] This complex is also involved in the translocation of polyP across the vacuolar membrane (see section 8).

Different reversibly acting PPKs have been identified in other fungal taxa, like in arbuscular mycorrhiza or in the funguslike slime mold *D. discoideum*.[226,227] In addition, PPK-like enzymes have been identified that use other cosubstrates such as the 1,3-diphosphoglycerate:polyphosphate phosphotransferase.[25,228]

The *S. cerevisiae* polyphosphatases have been extensively studied. These enzymes differ in their substrate specificity (preference for long polyP or for short polyP chains), mode of action (processive or nonprocessive), dependence on metal ions, and response to inhibitors;[126,229,230] for a recent review, see ref 213. The PPX1 which degrades long-chain polyP to PP_i in a processive manner under the release of P_i[230] is a member of the DHH family of phosphoesterases,[231] like the mammalian protein h-prune which also acts as an exopolyphosphatase (see below).[232] On the basis of the analysis of the crystal structure, the yeast enzyme is assumed to contain a positively charged tunnel-like structure with multiple arginine and lysine residues, which channels the entering polyP polymer from one end to the catalytic site at the other end of the protein.[233] The existence of such a tunnel-like structure has also been proposed for certain polyP-binding proteins.[234] Also an enzyme related to the yeast PPN1 has been partially purified from rat and bovine brain[220] but not further studied. The DDP1 belongs to the Nudix hydrolase family and seems to be primarily involved in inositol pyrophosphate metabolism but can also act as a polyP-hydrolyzing endopolyphosphatase.[216−218]

6.3. Enzymes from Other Eukaryotes and Animals

In the trypanosomes, the acidocalcisomes have been first discovered and, in turn, used as a model for the understanding of the role and function of these organelles in polyP metabolism also in animals.[235]

6.3.1. Trypanosomes/Unicellular Eukaryotes. Several exopolyphosphates have been isolated, cloned, and expressed

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from the major human pathogenic unicellular eukaryotes *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. In contrast to the yeast enzymes, these exopolyphosphatases preferentially hydrolyze short-chain polyP, such as the exopolyphosphatase from *T. cruzi*,[236] the cytoplasmic PPX1 from *T. brucei* (degradation of $polyP_3$, but not PP_i and long-chain polyP),[237] and the exopolyphosphatase from *L. major*, which is localized in the acidocalcisomes and cytosol.[238] In addition, some members of the Nudix family of enzymes were found to hydrolyze polyP, such as the *T. brucei* Nudix hydrolases TbNH2, an exopolyphosphatase located in peroxisome-like organelles (glycosomes) where glycolysis occurs, and TbNH4, an endo/exopolyphosphatase located in the cytosol and nucleus.[239,240]

6.3.2. Animals. As outlined, until today, an enzyme related to the PPKs has not been identified in higher eukaryotes. It should be granted that, within the cells, in the first place ATP should be considered as the cosubstrate for the enzymatic synthesis of polyP. This energy-carrying nucleotide is assumed to be synthesized intracellularly at two metabolic intersections, either within the mitochondria during the enzyme-controlled reduction/oxidation reactions or in the cytoplasm at the substrate-level phosphorylation steps, like in glycolysis during transfer of a phosphate group to ADP from 1,3-bisphosphoglycerate yielding 3-phosphoglycerate (phosphoglycerate kinase reaction) and from phosphoenolpyruvate producing pyruvate (pyruvate kinase reaction). In these two compartments, mitochondria and cytosol (membrane associated), polyP synthesizing enzymes have been identified.

The mitochondrial ATP-synthase (F_1F_O-ATP synthase [EC3.6.1.34]) has been proposed to be involved in the synthesis of polyP.[159] The authors conclude that polyP synthesis is directly linked with the ATP generation system of the mitochondria and with their energetic state; they propose that a feedback mechanism regulates the levels of polyP and of ATP and reciprocally (Figure 7). It has been suggested, based on the strong evidence that the energy level of the mitochondria is closely correlated with polyP level, that the synthesis of the polymer starts on pyrophosphate[241] that becomes elongated by an unknown polyP-synthesizing enzyme. The level of polyP in mitochondria is ~10 μM[151] and that of ATP 3−4 mM.[242] Enzymatic removal of polyP in mitochondria, via expression of yeast exopolyphosphatase (PPX), results in a decrease of the mitochondrial membrane potential and an impairment of the respiratory chain.[243] This finding can be taken as an indication that polyP might act in the mitochondria as a reservoir for metabolic energy generation in the form of ATP (Figure 7).

In addition, experimental evidence has been presented demonstrating that the plasma membrane associated Ca^{2+}-ATPase (PMCA) from erythrocytes might function as an ATP-polyphosphate transferase and polyphosphate-ADP transferase.[244] The PMCA belongs to the group of PMCA2 [EC 3.6.3.8] since the enzyme forms a phosphate intermediate during the reaction cycle.[245−247] Consequently, it has been postulated that the PMCA2 is associated with polyP allowing also a transient covalent autophosphorylation, followed by a transferase reaction and, by that, the formation of ATP.[244,248] It would be interesting to study this enzyme in further detail.

Among the mammalian enzymes involved in polyP degradation, only one enzyme that corresponds to the yeast PPX1 has been identified, the exopolyphosphatase h-prune which belongs to the DHH family of phosphoesterases.[232] However, this enzyme, in contrast to the yeast PPX1, is a short-chain exopolyphosphatase and inhibited by long polyP chains.[232] The major exopolyphosphatase in mammalian organisms including humans, which also degrades long polyP chains, seems to be the alkaline phosphatase (ALP); see section 7. We propose that this enzyme also acts as a phosphotransferase, as discussed in section 13, like the NAD kinase [EC 2.7.1.23]. The latter enzyme has been traced in mitochondria, mediating $NADP^+$ biosynthesis from NAD^+ (Figure 7);[249,250] this metabolite is required in mitochondria for a series of specific biosynthetic pathways and acts also as an antioxidant.[251] The NAD kinase uses not only ATP but also polyP as phosphate donor for phosphorylation of NAD^+ at the 2′-hydroxyl group of the adenosine ribose moiety of the molecule.[252,249]

Therefore, the NAD kinase, like the ALP, belongs to a group of enzymes capable of using energy-rich polyP instead of nucleotides/ATP as a phosphate donor in phosphotransferase reactions. We propose to operationally classify this group of functionally related enzymes (e.g., ALP and NAD kinase) that catalyze the transfer of energy-rich phosphate from polyP to a substrate (e.g., AMP, ADP, or NAD^+) as a polyP phosphotransferase system (PP-PTS; EC number 2.7) (Figure 6).

7. ALKALINE PHOSPHATASE: ROLE IN POLYP CATABOLISM

The major exopolyphosphatase in animals and humans, which is able to degrade long chain polyP, is the alkaline phosphatase (ALP).[117] This metalloenzyme, which can also act as a phosphotransferase, can therefore be grouped into the polyP phosphotransferase system PP-PTS and is functionally active only as a dimer.[253] It contains two Zn^{2+} ions and one Mg^{2+} ion in each catalytic site of the dimer; these metal ions are involved in the catalytic mechanism of the enzyme.[254] We have demonstrated that the ALP degrades polyP following a processive mechanism.[117] Four isozymes are known, the intestinal ALP, the placental ALP, the germ cell ALP, and the tissue-nonspecific ALP or liver/bone/kidney ALP.[254] The ALP dimers are allosteric enzymes.[253] Studies on the human placental ALP which only requires Zn^{2+} for activity revealed that this isoform, in its fully metalated form, shows the characteristics of a noncooperative allosteric enzyme; both subunits of the ALP dimer function independently, but their catalytic activity is controlled by the conformation of the respective other subunit.[253] On the other hand, the tissue-nonspecific ALP (liver/bone/kidney ALP; also present in brain), which like the intestinal ALP, needs both Zn^{2+} and Mg^{2+}, shows a negative cooperativity with respect to Mg^{2+} binding, which is relevant for the mechanism of polyP degradation, as discussed in section 13. The ALP is characterized by a broad substrate specificity. Besides polyP, the ALP hydrolyzes pyrophosphate, AMP, ADP, ATP, glucose-1-phosphate, glucose-6-phosphate, and β-glycerophosphate.

Apart from the ALP, only two other human proteins have proven to be an exopolyphosphatase. The first protein, h-prune, is characterized by a high sequence homology to PPX but preferably hydrolyzes only short-chain polyP (≤25 P_i residues).[232] The second protein is the osteoclast tartrate-resistant acid phosphatase (TRAP).[255] This exopolyphosphatase hydrolyses, like h-prune, only shorter-chain polyP molecules; it is inhibited by long-chain polyP. On the basis of the facts that the chain length of the acidocalcisomal and

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platelet polyP is around 70−75 P_i units and the mammalian ALP, in contrast to h-prune and TRAP, hydrolyzes both long and short polyP molecules,[117,256] it can be concluded that the ALP plays a predominant role at least in the control of long chain polyP in mammalian/human organisms. The ALP might function in close cooperation with the endopolyphosphatase described by Kumble and Kornberg.[220] This enzyme which corresponds to the yeast PPN1 has been reported also to exist in mammalian tissue; it cleaves long chain polyP to $polyP_{60}$ and has been partially purified from the rat brain and bovine brain.[220] Unfortunately, this enzyme which might be responsible for the occurrence of polyP molecules of distinct, intermediate chain lengths during cell cycle/apoptosis has not been studied further. For example, analysis of the size of polyP in HL-60 cells revealed that both proliferating and non-proliferating cells contain two classes of polyP, long-chain polyP with a narrow size distribution of around 150 P_i residues and medium-chain polyP with 25−45 P_i residues. In apoptotic cells, the long-chain polyP but not the medium-chain polyP disappeared.[257]

With regard to the differential biological effects of different size classes of polyP, the processive mode of action of polyP degradation shown by ALP might be of particular importance. This mechanism allows the degradation of long polyP molecules to monomeric P_i without the intermediate release of polyP molecules of shorter, intermediate chain lengths. In this way, it is prevented that during degradation of long chain polyP shorter polyP molecules are formed with a size that might show different (possibly unwanted) activities. For example, very long polyP polymers, administered extracellularly, with chain length of ≥500, such as those present in microorganisms, have been shown to activate the contact pathway of the blood coagulation cascade, while medium size polyP molecules, with polymer lengths of about 100 phosphate units, such as those released by platelets, accelerate activation of factor V via factor Xa and thrombin and abrogate the anticoagulant function (inhibition of factor Xa) of the tissue factor pathway inhibitor (TFPI); polyP chains with a size of ≥250 are required to enhance fibrin polymerization[122] (reviewed in refs 96 and 258).

The mechanism by which the mammalian polyP degrading enzymes recognize specific chain lengths of polyP is not known. This is in particular intriguing for exopolyphosphatases, like the ALP, because the degradation of long polyP chains begins at the ends of the polymer. In contrast to the *E. coli* enzyme, this, also processive, exopolyphosphatase degrades long-chain polyP, e.g. $polyP_{750-800}$, without formation of any intermediary product.[117] It might be possible that, like seen for the *E. coli* exopolyphosphatase (see above), multiple binding sites on distant portions of the enzyme proteins exist. Both enzymes are functionally active as a dimer. The affinity of the ALP to the polyP substrate increases with increasing chain length of the polymer. Measurements with low and medium chain polyP at pH 9.5 (pH 7.5) and using the enzyme from calf intestine revealed a decrease in the K_m value (in terms of polymer concentration) from 187 μM (218 μM) for $polyP_2$ to 28 μM (0.5 μM) for $polyP_{69-95}$.[117] This increase in affinity is paralleled by a decrease in the maximum rate of the reaction, v_{max}, from 1025×10^{-3} (176×10^{-3}) mol min^{-1} mg^{-1} to 60×10^{-3} (2.5×10^{-3}) mol min^{-1} mg^{-1}, in line with the processive type of reaction catalyzed by the enzyme (increase in reaction rate in the course of the processive degradation of the polyP chain).

8. POLYP METABOLISM IN EUKARYOTIC CELLS: ORGANELLES

There are two intracellular organelles which are rich in polyP, the mitochondria and the acidocalcisomes.[168] Interestingly, within eukaryotic cells, exemplarily demonstrated in the eukaryotic parasite *T. brucei*, these two organelles are in close contact,[259] a finding that is suggestive and indicative toward a functional interaction of the acidocalcisomes with the mitochondria during regulation of cellular bioenergetics.[260]

8.1. Mitochondria

It has been especially the Abramov group[261] that disclosed polyP metabolism being correlated with the polymer chain-length in mitochondria. Short polyP (14 P_i residues) or medium-sized polyP (~70 P_i) increases the respiratory coefficient by activation of complex III and inhibition of complex IV, compared to the control. Simultaneously, these two classes of polyP markedly reduce the oxidative phosphorylation (ADP/O ratio). Long polyP (130 P_i) causes cell death in primary neurons and astrocytes, while medium and short polyP, again, do not elicit this effect. However, it might be considered that any kind of polyP is prone to degradation by ALP, or ALP-like enzymes, a fact which complicates a strong conclusion.[117,262]

A hypothetical polyP synthase has been proposed to be located in the inner membrane of the mitochondria, presumably associated with the mitochondrial ATP synthase (Figure 7).[159] Complex I-linked substrates, like pyruvate, malate and glutamate or the complex II-linked substrate succinate, which stimulate mitochondrial oxidative phosphorylation,[263] are also activators of the mitochondrial polyP synthesis.[159] Inhibition of the complex I system by Rotenone causes a drop of the polyP pool. This fact implies that alterations of the function of these complexes are correlated with the polyP level in the matrix of the mitochondria. This circuit is paralleled with a potential effect of polyP on the proton gradient across the inner mitochondrial membrane (ΔpH^+); at a high potential, polyP concentration increases and vice versa.[168] In turn, two circuits which control the level of polyP in the mitochondria can be formulated, one linked with the individual complexes of the respiratory chain and another linked with the overall proton gradient.

In addition to an ALP-like enzyme, a PPX has been described to be present in the mitochondria of animals (ticks).[264] This enzyme acts as an energy sensor. It has been found that the mitochondrial PPX becomes activated and responds with a higher polyP hydrolysis if the mitochondria are rich in ADP.[264]

These data might suggest that the metabolic cycles of polyP in the mitochondria, perhaps both polyP synthesis and the catabolic pathway of polyP hydrolysis, control the ATP level in these organelles and function as a storage reservoir for ATP. The polyP synthesis requires the energy from ATP (for the formation of the phosphoric acid anhydride bond). Further studies are needed to clarify the metabolism of polyP in mitochondria.

8.2. Acidocalcisomes

As mentioned, the mitochondria are in close apposition[235] or even directly attached to the acidocalcisomes.[260] It has been found that the latter organelles, collectively referred to as acidocalcisomes,[265,266] which occur in both prokaryotic and eukaryotic cells, from bacteria via yeasts to higher vertebrates/

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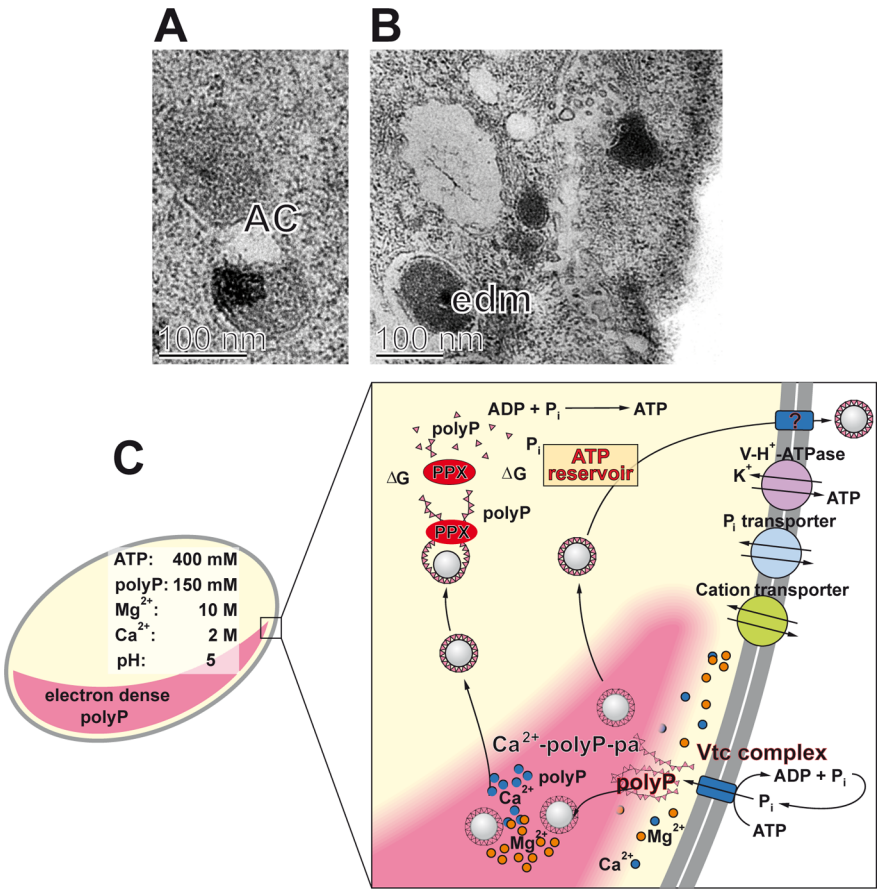

Figure 8. Acidocalcisomes as polyP-synthesizing organelles. (A and B) TEM images of acidocalcisomes (AC) from bone cells. The AC is an organelle which harbors inclusions of electron dense material (edm) that is composed of polyP. (C) The acidocalcisomes are rich in ATP, polyP, Mg^{2+}, and Ca^{2+} and have a low pH of ~5 which is maintained by an H^+-ATPase. The organelle is surrounded by a membrane into which a series of transporters are embedded. Among them is the vacuolar transporter chaperone (Vtc) complex that has been identified in yeast and implicated in polyP synthesis. The polymer is released into the lumen of the organelle. There, polyP is cleaved by the exopolyphosphatase (PPX) at the energy-rich anhydride bonds under release of metabolic energy (ΔG), or, as proposed here, the energy-rich phosphate is transferred to AMP/ADP under formation of ATP by the PP-PTS. Again, the acidocalcisomes would act, like the mitochondria, as an ATP reservoir but with a much higher capacity.

humans, store polyP, accumulate Ca^{2+}, and show an acidic milieu. The acidocalcisomes are round-shaped organelles with an 8 nm thick membrane and have a size of 50−300 nm. They are filled with amorphous polyP that appears as electron-dense deposits[267,268] (Figure 8A,B). These particles can be stained by DAPI, showing a green fluorescence.[128] In the acidocalcisomes, the polyP level reaches millimolar concentrations.[121] Furthermore, a series of proteins has been identified in these organelles that are involved in phosphate and polyP metabolic pathways, like the Vtc complex or a putative phosphate transporter and a putative acid phosphatase.[269] The Ca^{2+} concentration in the acidocalcisomes reaches levels of 2 M, and their matrix pH is about 5.4.[270] This pH value is maintained by an H^+-ATPase, a pump which is different from F_1F_O- as well as the P-type ATPases[271] (Figure 8C). Besides polyP and energy-rich nucleotides, the acidocalcisomes contain pyrophosphate and serotonin.[180,270]

A (putative) transport mechanism of polyP from the mitochondria to the acidocalcisomes and in continuation to the cytosol is not yet known. It is most likely that the polymeric polyP is not exported from the mitochondria to the acidocalcisomes via the mitochondrial ADP/ATP carrier, since this carrier is highly specific for ADP, ATP, and their deoxy-analogues.[272] As an alternative, the export of polyP could occur in nanoparticles. If so, polyP must be complexed with Ca^{2+}, which is present in mitochondria only in micromolar concentrations and is an essential cation functioning during the energy/ATP synthesis. A similar role plays Mg^{2+} which is also present in only small levels with 20−30 μM[273] and is crucially important for the maintenance of the energy homeostasis in mitochondria.[274] Therefore, based on quantitative consideration (next paragraph), it appears to be likely that the main intracellular organelles producing polyP are the acidocalcisomes.

Comparing the polyP level in mitochondria versus acidocalcisomes (~10 μM − 150 mM), the ATP concentration of 3−4 mM versus 400 mM, and the Ca^{2+}/Mg^{2+} level in mitochondria versus acidocalcisomes of ~10 μM − ~2 M/~10 M,[275] it can be assumed that the bulk of polyP, provided by the acidocalcisomes to the cells and the surrounding tissue, is synthesized in the acidocalcisomes (Figure 8C). In turn it can be deduced that polyP acts in the acidocalcisomes like in the mitochondria as a reservoir for the deposition of metabolic energy.[159,168,241,276]

The acidocalcisomes are surrounded by a membrane (Figure 8C) into which a series of transporters and channels is integrated (see ref 266); the existence of some of those channels is still not yet clear. These organelles contain the

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electron-dense material, composed of polyP, that adheres to one side of the membrane of the organelle with the inositol 1,4,5-trisphosphate receptor channel (IP3R; not shown in Figure 8C), required for Ca^{2+} release from the acidocalcisomes, to the mitochondrial Ca^{2+} uniporter.[260] Among the other transporters/channels within the acidocalcisome membrane are the vacuolar H^+-ATPase and the vacuolar H^+-pyrophosphatase and other transporters, which import Ca^{2+} and P_i and additionally Zn^{2+}, Fe^{2+}, and polyamines. These pumps keep the pH of the acidocalcisomes around 5.[277] Strong evidence has been worked out that in trypanosomes and yeast it is the Vtc complex that mediates polyP synthesis.[225] In the yeast *S. cerevisiae*, the Vtc complex consists of four subunits, one catalytic (Vtc4) and three accessory (Vtc1, Vtc2, and Vtc3).[278] The synthesis of polyP from ATP depends on the presence of the electrochemical gradient across the acidocalcisome membrane and requires the formation of a tunnel-like structure by the central domain of Vtc4, which is assumed to be involved in the translocation of the polyP chain into the lumen of the vacuole.[225,279] This tunnel-like structure with the polyP chain has been analyzed by X-ray crystallography of the catalytic domain in the presence of ATP.[213,225] As presently assumed, the polyP formation driven by Vtc occurs at the cytoplasmic side of the complex in close association with the acidocalcisome membrane. It remains open from where the large amounts of ATP are coming from. As mentioned above, the acidocalcisomes are very rich in ATP (400 mM). The polyP formed from ATP then accumulates in the lumen of acidocalcisomes and is not present in a freely diffusible form.[280] The formation of polyP particles is strongly favored because of the high concentration of Ca^{2+} (2 M) and Mg^{2+} (10 M) present in the acidocalcisomes.[256] It is agreed that polyP released to the lumen of the acidocalcisomes is prone to hydrolysis by the surrounding exopolyphosphatase [PPX] (see refs 266 and 281), which is opening the energy-rich anhydride bond under the release of metabolic energy (ΔG). Again, on this step metabolic energy is released from the polymer in addition to P_i which might contribute to the high level of ATP within the acidocalcisomes (Figure 8C). This catalysis might occur via an enzyme grouped, like the ALP proposed here, to the class of enzymes classified to the phosphotransferase system (PP-PTS), and polyP could act as a large reservoir for ATP production in the acidocalcisomes.

In addition to its biocatalytic function as a polyP polymerase, the yeast Vtc complex has been implicated in multiple processes such as membrane trafficking, membrane fusion, and microautophagy.[282,283] The polyP in the Vtc has been reported to activate fusion with other vacuoles under stimulation of SNAREs (soluble NSF attachment protein receptors). The latter process, activation of SNAREs requires zippering supported by the release of the vesicular-fusion protein SEC18 and addition of ATP.[284] Most likely adjacent to the Vtc transmembrane regions within the membranes of the acidocalcisomes, they release their granula.[279]

9. (POTENTIAL) ATP TRANSFER BETWEEN MITOCHONDRIA AND ACIDOCALCISOMES

The concentration gradients for ATP and polyP from the mitochondria to the acidocalcisomes are substantial with amounts consisting of several orders of magnitude (Figure 7 and Figure 8). Certainly, this comparison does not necessarily mean that the primary place of intracellular ATP synthesis is in the acidocalcisomes. It might indicate that ATP and perhaps also polyP are actively transported from the mitochondria to the acidocalcisomes (Figure 9).

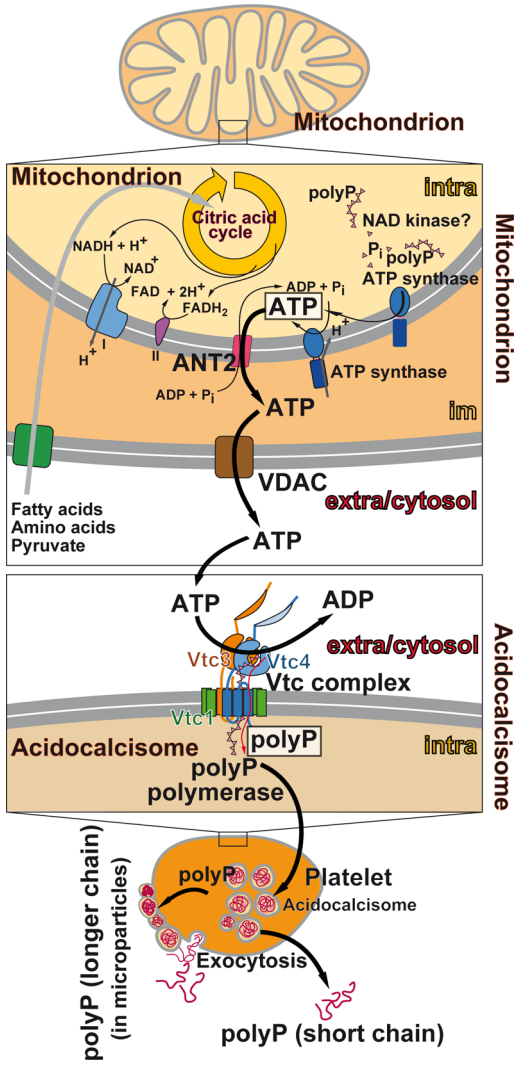

Figure 9. Most probable synthesis route of polyP in eukaryotic cells. The mitochondria take up fatty acids, amino acids, and pyruvate which fuel the citric acid cycle. Subsequently, the reduced coenzymes are channeled into the respiratory chain resulting in ATP synthesis by using the membrane potential to drive the ATP synthase. ATP is transported via ANT2 from the matrix to the intermembrane space and from there through the VDAC to the extramitochondrial cytosol. In the cytosol, ATP reaches the Vtc complex, at the acidocalcisomes, and there the polyP polymerase forms polyP and translocates the polymer into the acidocalcisomes. Subsequently, the polyP rich organelles, abundantly present in blood platelets, release polyP into the cytosol and then via exocytosis into the extracellular space as either short chain polyP or as longer chain polyP, packed into Ca^{2+} rich particles.

It is suggested, based on the existing close apposition of acidocalcisomes to the mitochondrial outer membrane, that a transfer or even a shuttle of ions or molecules between the two organelles exists, like postulated for Ca^{2+} from the acidocalcisomes to the mitochondria through the voltage-dependent anion channels (VDACs).[260] It would be helpful for an understanding of the bioenergetical interaction between the two organelles if ATP is channeled from the mitochondria via their VDACs to the acidocalcisomes either directly or after

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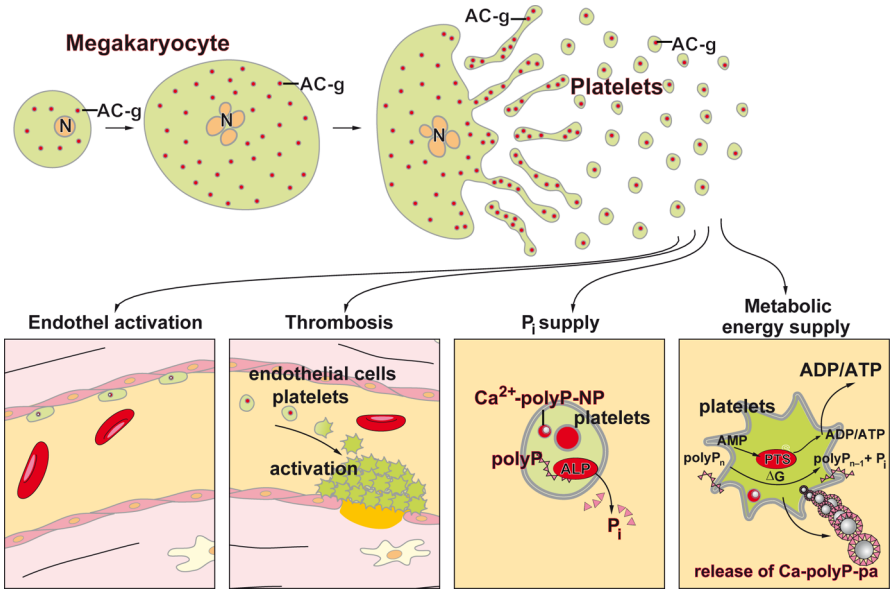

Figure 10. Formation of polyP nanoparticles (polyP-NP) in blood platelets. The platelets are released into the bloodstream by precursor cells, the megakaryocytes, residing within or originating from the bone marrow. The megakaryocytes undergo a series of remodeling events, resulting in the release/budding of thousands of platelets from a single megakaryocyte. Within the megakaryocytes, and especially in the platelets acidocalcisomes, granules (AC-g) accumulate that are rich in polyP complexed with Ca^{2+} or Mg^{2+}. Prominent (patho)physiological roles of the platelets are their interactions with the coagulation system, the leukocytes, and the activated endothelium; their role in hemostasis and thrombosis; supply of orthophosphate, as a result of the ALP-catalyzed hydrolysis of Ca-polyP-NP; and, as suggested here, the generation of metabolically synthesized ATP via the PP-PTS system, either before or after the release of polyP, most likely in the form of Ca-polyP particles.

bridging a small cytoplasmic gap. The VDACs are located in the mitochondrial outer membrane and are key regulators of the import of the respiratory substrates, ADP, and P_i, into mitochondria and simultaneously of the release of mitochondrial ATP to the cytosol (Figure 9). As observed, a blockade of these transport channels results in an inhibition of a series of cell functions, like cell proliferation.[285] The flux of ATP through VDAC saturates only a concentration of 100 mM, a value which is much higher than the overall concentration of ATP in the mitochondria which is 3−4 mM.[286]

Interestingly, the VDAC can be blocked by the binding of tubulin to the channel. Experiments using carboxy-terminal tail peptides of the tubulin dimer revealed that only the detyrosinated but not the tyrosinated peptides close the channel.[287] The post-translational modification of tubulin by tyrosination is catalyzed by an ATP-dependent tubulin-tyrosine ligase [EC 6.3.2.25] that adds a tyrosine residue at the carboxyl end of α-tubulin.[288] It might be intriguing to study if this enzyme that might regulate the passage of molecules through the channel causes channel closure at high cytosolic ATP levels and opening (ATP efflux) at low levels of the nucleotide.

It has been briefly noted that within the VDAC, polyP can interact with the binding site of NADH.[289] Thereafter, it has been discussed if polyP can cross the mitochondrial outer membrane through VDAC channel from inside to outside. However, the available experiments did not support this view.[290] On the basis of the few experimental evidence, which is first of all indirect, it is most likely that polyP within the acidocalcisomes is synthesized de novo through the Vtc polyP polymerase, at least in the yeast model.[280]

In conclusion and according to the currently existing data, it appears that the bulk of polyP is synthesized in the acidocalcisomes and distributed from there to the cytoplasm and then to the extracellular space. This view is supported by the finding that ionized polyP, if present in the cytosol, is toxic if the expression of cytosolic PPX1 is suppressed.[279] This adverse effect is most likely caused by the property of polyP to be a strong chelating and/or sequestering agent for metallic ions.[291] It is reported in section 10 that in acidocalcisomes polyP exists as Ca^{2+} or Mg^{2+} salts and packed there into amorphous nano/microparticles. These particles are, due to their high zeta potential (−34 mV; at pH 7.4), less prone to ALP hydrolysis.[87] This high value is found at pH 11 and a calcium (as $CaCl_2$) to phosphorus (Na-polyP) weight ratio of >2. If the pH is lowered to 7 or even to 4, the zeta potential drops to ∼8 mV.

On the basis of the given experimental data, the most likely route along which polyP is synthesized from the mitochondrial ATP is summarized in Figure 9. ATP is synthesized in the mitochondria, tightly linked to the respiratory chain, and released from the matrix to the cytosol via ANT2 and VDAC. There, ATP generates via the Vtc complex in the acidocalcisomal membrane, polyP.[213] Within the acidocalcisome polyP is deposited as amorphous polyP material (reviewed in refs 235 and 292). Packed in those organelles within the cells, polyP is released, for example, from the blood platelets via exocytosis (see ref 293).

The released polyP occurs in two forms as shorter polymers [60−100 P_i residues] that promote coagulation, and the longer polymers [>100 P_i] that are packed into microparticles, and initiate coagulation.[180,294] The size of the particles is around 500 nm (Z-average) or ∼200 nm (SEM). The polyP molecules exist in these particles in an amorphous state,[292] surely caused by the high stoichiometric surplus of Ca^{2+} over polyP.[256] Associated with the outer cell membrane, the microparticles are assembled with other components of the blood clotting in polar caps, after platelet activation.[295]

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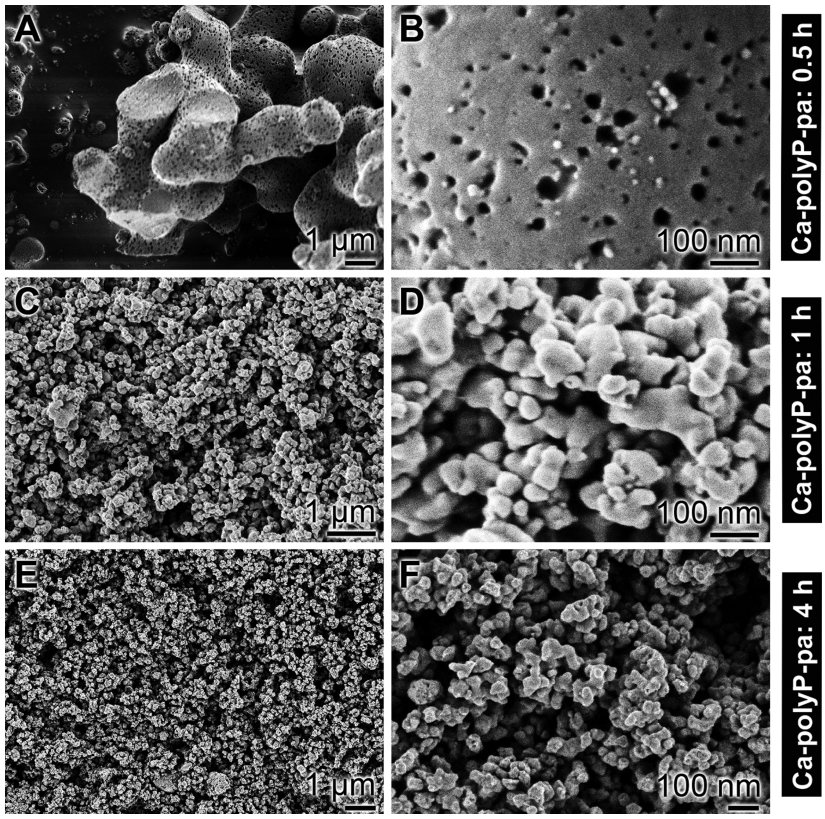

Figure 11. Biomimetically fabricated amorphous Ca-polyP microparticles (Ca-polyP-pa); SEM; (A and B) Particles formed after a short incubation period of 0.5 h and a longer reaction period (C and D) of 1 h and (E and F) of 4 h. Adapted with permission from ref 30. Copyright 2018 The Royal Society of Chemistry.

10. POLYP NANO(MICRO)PARTICLES: PHYSIOLOGICAL FORMATION AND BIOMIMETIC SYNTHESIS

It is conceivable to fabricate polyP for any kind of biomedical application in the same way, with a similar size and chain length, like the cells are preparing it. The chain length of polyP in metazoan cells is physiologically between 20 and 200 P_i units.[96,97] The highest amounts of polyP are found in the blood platelets (thrombocytes),[121] mast cells,[181] and in mineralizing bone cells of the vertebrate skeleton.[262] There, the polymer exists in the form of amorphous particles together with the divalent cations. The platelets are nucleus-free cell fragments, with a life-span of about 7−10 days, that originate from megakaryocytes.[296,297] They are released within the bone marrow or during lung passage.[298] During their maturation to the platelets, the megakaryocytes underwent an interlaced series of remodeling events; thousands of platelets are formed from a single megakaryocyte.[296,299] Subsequently, the platelets circulate in the blood and primarily function as regulators of hemostasis (initiated by endothelial activation [attachment-adhesion-aggregation/signaling]) and thrombosis (formation of a blood clot with a central core [fibrin rich] and a loosely packed shell).[300] After enzymatic hydrolysis of polyP with the ALP, the produced P_i residues contribute to the maintenance of the phosphate homeostasis[301] and, bioenergetically, the polyP, released from platelets, also serves as a supply of metabolic energy[28,29] (Figure 10). In the megakaryocytes, dense granules are formed which are embedded into acidocalcisomes,[302] filled with polyP of a chain length of 70−75 P_i units and other molecules comprising energy-rich bonds.[120,121] These organelles are subsequently directed to the platelets during their maturation process. Mast cells are filled with polyP entrapped into acidocalcisomes as well.[303] These cells have a longer half-life with ∼40 d.[304]

10.1. Synthesis within the Cells

As determined, and basically as expected, a major portion of polyP in the platelets is packed to nano/microparticles (size of around 200 nm) that are amorphous and exposed membrane-associated on their cell surface.[180,305] The platelet polyP is present as medium-sized chains (∼100 P_i units) in the dense granules of these cells. The anionic polyP readily binds to cations, consisting of only single atoms, like Ca^{2+},[256] or to longer chain polycations, as shown for synthetic polyethylene glycol linked to charged tertiary amine groups.[306] As outlined above (section 8), the acidocalcisomes contain ∼150 mM polyP and 2 M Ca^{2+}/10 M Mg^{2+}. In turn, a large stoichiometric surplus of the cation over the polymeric anion (in terms of P_i units) exists in the acidocalcisomes (Figure 8), and the overall pH value in acidocalcisomes adjusts to ∼5.[277] In contrast, the polyP synthesis at the Vtc complex proceeds at a pH of 7 or even higher, as might be present at the cytosolic side of the organelles[280] (Figure 8). The pH environment in the acidocalcisomes shifts rapidly after alkaline stress, resulting in an increased mobilization of polyP.[307] Consequently, our group prepared the Ca^{2+}-polyP salt from $CaCl_2$ and sodium-polyP (Na-polyP) at an excess of Ca^{2+} at pH 10, the third equivalence point of phosphoric acid.[10,256]

10.2. Biomimetics

Applying these reaction conditions, nano/microparticles are formed from Ca^{2+} and Na-polyP (Ca-polyP-MP), having a size

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of 100−300 nm[256] (Figure 11). Importantly, these particles, obtained at a stoichiometric ratio between Ca^{2+} and polyP (based on P_i) of ≥ 2, are amorphous. Only particles in the amorphous phase are prone to a considerable dissolution process, compared to those in the crystalline state.[308] During preparation of the particles, the pH was kept constant at 10 in the pH-Stat. After termination of the reaction, the particles were washed extensively with water and ethanol; they remain stable in the dry state. If suspended in nonbuffered water, the pH in the environment dropped down to a pH of ∼5 without dissolution of the particles.

The size of the biomimetically fabricated particles can be controlled, perhaps like in the acidocalcisomes, by two parameters, first, by the duration of the addition of the $CaCl_2$ solution to the Na-polyP solution and, second, by the chain length of the Na-polyP, present at the beginning of the reaction. In contrast to longer chain Ca-polyP-MP, Ca-polyP samples, prepared with $CaCl_2$ from polymers with a chain length of ≤3, form crystalline Ca-polyP particles (Ca-polyP-pa).[30] SEM (scanning electron microscopy) inspections of the amorphous polyP particles revealed that the size of the globular to irregular-ellipsoid particles, formed during a short reaction period of 30 min (Ca-polyP-pa: 0.5 h), is large and measures ∼1.2 μm (Figure 11A,B). These particles have a porous interior space transversed by channels of ∼40 nm (Figure 11B). An extension of the reaction time during the preparation of the particles to 1 h (Ca-polyP-pa: 1 h) results in much smaller globular particles with ∼60 nm (Figure 11C,D). A further prolongation to 4 h (Ca-polyP-pa: 4 h) causes a further reduction of the size of the particles to ∼25 nm (Figure 11E,F). Decreasing the chain length of the polyP reactant likewise causes a reduction of the size of the particles.[30]

Since in physiological acidocalcisomes the size of the polyP-containing electron-dense particles is around 50−300 nm, they are amorphous and have a chain length of ∼70 P_i units,[180,305] we have adjusted the size of the particles for all our studies between 100 and 300 nm and used as a reactant polyP with a chain length of ∼40 P_i units.[256] The amorphous state of the particles has been verified by XRD (X-ray powder diffraction).[256]

The FTIR recordings show the characteristic signals for polyP in the Ca-polyP-pa,[30] such as the asymmetric stretching vibration of $(PO_2)^-$ at ∼1259 cm^{-1}, the asymmetric vibration of $(PO_3)^{2-}$ at ∼1103 cm^{-1}, the asymmetric vibration of (P−O−P) at ∼865 cm^{-1}, as well as the symmetric vibration of (P−O−P) at 755 cm^{-1} (Figure 12). It is apparent that the intensity of the $(PO_2)^-$ decreases with the duration of the reaction, a sign that the packaging of the particles increases with time. EDX analyses revealed that the Ca/P atomic ratio within the Ca-polyP microparticles is 0.62, reflecting that every ionic phosphate group is bound to the divalent Ca^{2+} in a substoichiometric way (based on phosphate).[309]

11. APPLICATIONS OF BIOMIMETIC POLYP PARTICLES

Amorphous polyP particles have been fabricated with the cations Mg^{2+}, Ca^{2+}, Sr^{2+}, Zn^{2+}, and Fe^{2+}/Fe^{3+}. The Mg^{2+}-polyP complex was prepared from Na-polyP and $MgCl_2$ and found to form heterodisperse particles with an average size of 250 nm.[310] The Na-polyP reactant could be partially substituted by the natural polyanionic polymer hyaluronic acid resulting in a product that comprises similar biomechanical properties like cartilage. Interestingly, this composite material was able to dissolve small calcium phosphate bone splinters, present in synovial fluid, and is prone to ALP hydrolytic degradation.[156]

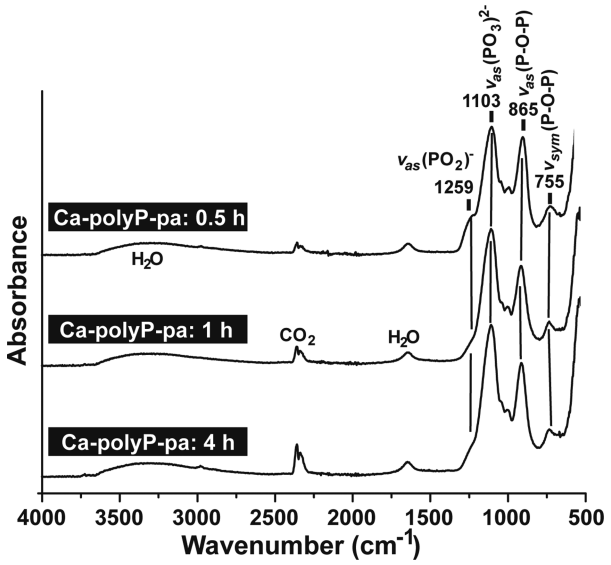

Figure 12. FTIR spectral analysis of Ca-polyP microparticles, prepared during a short (0.5 h) incubation period and after the longer (1 and 4 h) fabrication time. Characteristic signals are highlighted, like the asymmetric stretching vibration of $(PO_2)^-$ at ∼1259 cm^{-1}, the asymmetric vibration of $(PO_3)^{2-}$ at ∼1103 cm^{-1}, as well as the two signals for P−O−P at ∼865 and 755 cm^{-1}.

11.1. Morphogenetic Activity

The Mg^{2+}-polyP particles were found to elicit morphogenetic activity since they enhance growth of chondrocytes and induce gene expression in these cells, such as the expression of *ALP*, *aggrecan*, and several *collagen* genes. In addition, Ca^{2+}-polyP particles have been prepared from $CaCl_2$ and Na-polyP resulting in the fabrication of 100−300 nm particles that can be degraded by ALP.[256] Again, these particles were able to induce a series of genes involved in bone formation (see ref 30). Animal studies with normal and diabetic mice revealed that the Ca^{2+}-polyP particles like the Mg^{2+}-polyP particles strongly accelerate wound healing even in diabetic animals.[311] Strontium has been proven to enhance bone formation and improve bone microarchitecture in osteoporosis.[312] Considering this fact Sr^{2+}-polyP particles have been prepared from $SrCl_2$ and Na-polyP having a size of around 340 nm. Besides their in vitro morphogenetic activity, these particles strongly induced the regeneration process of bone defects in vivo.[313] Zinc is an essential ion that is crucial for maintaining cell membrane integrity and cell intermediary metabolism. Zinc deficiency causes delayed wound healing and immune dysfunction.[314] Therefore, Zn^{2+}-polyP particles have been prepared from Na-polyP and $ZnSO_4$ (unpublished). The ∼250 nm particles were found to accelerate migration of human endothelial cells in the in vitro wound healing model.

The polyanion polyP offers another option toward a biomedical application in host−guest chemistry. It has been shown that polyP can be partially substituted by other anions like zoledronic acid,[315] hyaluronic acid,[156] or adenine arabinoside monophosphate (araAMP) (unpublished). With the use of this technology, complexes could be fabricated that might be applicable for drug delivery in which the components are held together by noncovalent bonds without losing their biological activities. Advantageous is the fact that the host polyP nano/

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microparticles also display their beneficial functional properties, e.g., eliciting morphogenetic activity, in addition to the biomedical effect of the guest as inhibitor of tumor cell proliferation,[315] stimulus of cartilage repair,[156] or inhibitor of herpesvirus infection.[316]

11.2. Coacervation

Recently, it could be demonstrated that polyP particles do not undergo dissolution in water or culture medium unless they come into contact with peptides or proteins.[87] During this process, the amorphous Ca^{2+}-polyP particles, characterized by a high zeta potential, disintegrate and form a coacervate phase. This coacervate formed from the Ca^{2+}-polyP particles is the functionally active form of the biomaterial and characterized by the property to be biodegradable; in vitro it becomes disintegrated with time.

12. INTRACELLULAR ENERGY: FROM POLYP TO ATP

It is the rule that all metabolically active tissues produce heat with the adipose tissue as the most prominent example.[317] Most of the ATP is synthesized in mitochondria, tightly coupled to the electron transport chain that moves electrons from an electron donor to the terminal electron acceptor (O_2) via a series of redox reactions. The degree of utilization of the energy of the formed proton gradient, the proton motive force, that is converted into biochemically utilizable energy in ATP during the reaction from ADP is not hundred percent; a portion of the energy generated during oxidation is lost as heat.[318,319]

12.1. Intracellular Generation of Energy As Heat

There are only a few tissues in which the energy produced during certain exergonic chemical processes is almost completely released as heat; it is primarily the brown adipose tissue that acts as a heat generator, while the white tissue functions as an energy storage. This uniqueness of the brown adipose tissue is due to the presence of the brown fat-specific uncoupling protein, thermogenin, that allows the dissipation of the electrochemical/chemical energy of the proton gradient formed at the inner mitochondrial membrane as heat and, by this, the uncoupling of the oxidative phosphorylation/ATP production.[320] A second, enzymatic pathway that has been identified to expend energy proceeds through futile creatine cycling by a coordinated upregulation of genes controlling creatine metabolism in beige adipocytes.[321,322] Thermogenin or the creatine cycling enzymes residing at the mitochondrial membrane(s) promote thermogenesis by dissipating the proton-motive force and/or increasing the rate of substrate flux through the mitochondrial respiratory chain.

12.2. Metabolic Energy: ATP

Most commonly ATP is used as a primary energy carrier that is storing and transferring the energy of the mitochondrial transmembrane electrochemical potential (ECP) into the energy of a chemical bond, a process that is mediated by the F_1/F_O-like ATP synthases. These enzymes contain two motor sectors: (i) the F_O couples proton translocation to rotation, and F_1 couples rotation to ATP synthesis.[323] The rotation, the cyclic conformational changes in the catalytic sites within the F_1 unit, is driving ATP synthesis and release. During this process, the Gibbs free energy of the ECP is first converted into the mechanical energy of the rotation of the centrally located γ-subunit and then into the chemical energy-rich phosphoanhydride bond. With the uncoupling protein UCP-1 (thermogenin), the electrochemical potential is completely and irreversibly converted to heat, possibly resulting in an entropy change,[324] while with the ATP cycling via the ATP/ADP carrier between the F_1/F_O-like ATP synthases and the creatine cycling the energy is only partially dissipated and the rest is restored into ATP/creatine phosphate. Basically this partial reusage of chemical energy from ATP to the formation of phosphoanhydride and phosphate ester linkages describes the energy shuttle mediated by the large group of phosphotransferases [EC 2.7] enzymes.

Usually the phosphotransferases, like the hexokinase, use 1 mol of ATP per 1 mol of substrate (glucose) to synthesize 1 mol of phosphorylated substrate (glucose-6-phosphate) and 1 mol of ADP. In this reaction, a sum of ΔG^0 of -30.5 kJ mol^{-1} is released during hydrolysis of ATP to ADP and P_i. From this sum of free energy (ΔG^0), a portion of 13.7 kJ mol^{-1} is reused and stored in the phosphorylated product.[325] The remaining energy $\Delta G^0 \approx -17$ kJ mol^{-1} is dissipated. This means that during the phosphotransferase reaction a major portion of the chemical energy originally present in the phosphoanhydride bond of ATP is not stored as metabolic energy. As outlined later, the experimental data available strongly suggest that, both in the extracellular space and intracellularly, polyP acts as a phosphate donor for the generation of ADP or ATP.[148,149] In mammalian organisms, the reaction during which ΔG^0 is released is primarily catalyzed by the enzyme ALP. Assuming that the phosphorylation of AMP to ADP or ADP to ATP is also catalyzed by a phosphotransferase, it is likely that also during this reaction a portion of the energy stored in the (terminal) phosphoanhydride bond of polyP undergoes dissipation, most likely in the form of heat. Consequently, more than one phosphoanhydride bond in the polyP would be needed to be invested for the formation of an energy-rich bond in the nucleotide. Moreover, such a reaction might require an intermediate prior to the formation of the energy-rich phosphoanhydride bond. A strong candidate for such an intermediate has been recently identified in studies on myosin. The myosin light chain kinase mediates the reversible phosphorylation of calmodulin.[326] This enzyme has been found to hydrolyze ATP via formation of a stable intermediate with the γ-phosphate of ATP in the dissociated metaphosphate ($P_\gamma O_3^-$) state.[327,328] Hence, we propose that in a close interaction of the two subunits of the membrane-bound ALP homodimer, the formed metaphosphate intermediates can react either with AMP under formation of ADP which is subsequently converted into ATP by adenylate kinase (ADK) reaction or with water under release of orthophosphate (see section 13).

13. POLYP, ALP, AND ADK: AN EXTRACELLULAR ATP GENERATOR COMPLEX

The ALPs are ubiquitous membrane-bound glycoproteins.[329] In vertebrates, the enzyme is attached, as an ecto-enzyme, to the plasma membrane by a GPI anchor.[330] The formation of the functionally active ALP dimer at the cell membrane has been reported to depend on the redox state of the cell (NAD^+/NADH ratio).[331,332] Previously it has been shown that the ALP degrades polyP through a processive mechanism,[117,262] i.e., without dissociation of the inorganic polymer from the enzyme after each catalytic cycle until degradation is completed (see section 7). The ALP has been considered as catalytically promiscuous;[333,334] the enzyme has broad substrate specificity and accepts both polyP and AMP as substrate.[117,335,336]

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13.1. Cell Biological Evidence for a Role of ALP and ADK in ATP Generation

Cell biological studies revealed that inhibitors of ALP and ADK severely block migration behavior and metabolic activity of some cells, especially of fibroblasts and epi/endothelial cells.[10,149,337] An illustrative example is the migration process of endothelial cells during the initial stage of vascularization (Figure 13). During this migration process, the irregularly scattered cells onto the culture substrate (Figure 13A) join to 100 μm large ring-shaped cell circles during a surprisingly short incubation period in vitro, of about 4 to 8 h (Figure 13B,C). In the presence of Ca-polyP-MP, the organized tube-like arrangement comprising cell migration and pattern formation is strongly enhanced (Figure 13D,E). This increase can be significantly prevented by adding levamisole (LEV), an established inhibitor of ALP[338] (Figure 13F,G). In addition, the accelerated process of tube-like formation can be severely suppressed by the addition of the natural ADK inhibitor P,^{1}P^5-di(adenosine-5′)pentaphosphate [Ap_5A][339] (Figure 13H,I). On the basis of previously gathered data demonstrating that the levels of ADP and ATP increase following addition of polyP to cells in vitro, a more direct study was conducted to examine whether ATP is involved in this enhanced migration and accelerated tube-like pattern formation. The polyP-supplemented cell culture assays were coincubated with apyrase, an enzyme that hydrolyses nucleoside triphosphates to their corresponding di- and mononucleotides under P_i release.[336] During this coincubation study, polyP with apyrase, the tube-like organization pattern of the cells was almost completely blocked (Figure 13J,K). These cell biological data were completed and corroborated by analytical data measuring the ATP level in the culture medium in the absence or presence of polyP (Figure 14A). Upon addition of polyP to the culture system, an over 3-fold increase in the ATP pool is measured. Addition of both the ALP inhibitor (levamisole) and the ADK inhibitor (Ap_5A) strongly reduced the polyP-mediated extracellular ATP level. Interestingly, the phosphate trimeric Ca-polyP$_3$-MP particles did not cause a significant change in the ATP level measured in controls (without polyP). A quantitative assessment of the effect of polyP on tube formation in the absence or presence of polyP is shown in Figure 14B. The strong increase in this pattern formation in assays with polyP is significantly reduced after coincubation with levamisole and Ap_5A. The removal of ATP from the system with apyrase blocked the polyP stimulatory effect almost completely.

The results of the tube-like formation assays were confirmed in assays measuring the migration capability of the endothelial cells (Figure 15). For this series of experiments, the in vitro scratch assay was applied.[340] Endothelial cells were seeded onto an ECM substrate. After incubation for 12 h, a "scratch" was performed in the cell monolayer, and the migration rate of the cells into the scratch area was monitored. Microscopic analysis revealed that after an incubation period of 12 h, cells in both the controls (without polyP) and in the polyP-treated cultures migrated into the initially removed cell strips. However, the density of the in-migrated cells within the previously cell-free strips was significantly higher in the polyP-containing assays (Figure 15C,D) as compared to the controls (Figure 15A,B).

These experimental data strongly suggest that after enzymatic hydrolysis of polyP by ALP and the consecutive ADK reaction, an increase in ATP level in the extracellular space of the cultures occurs. The following theoretical consideration supports this empirical observation.

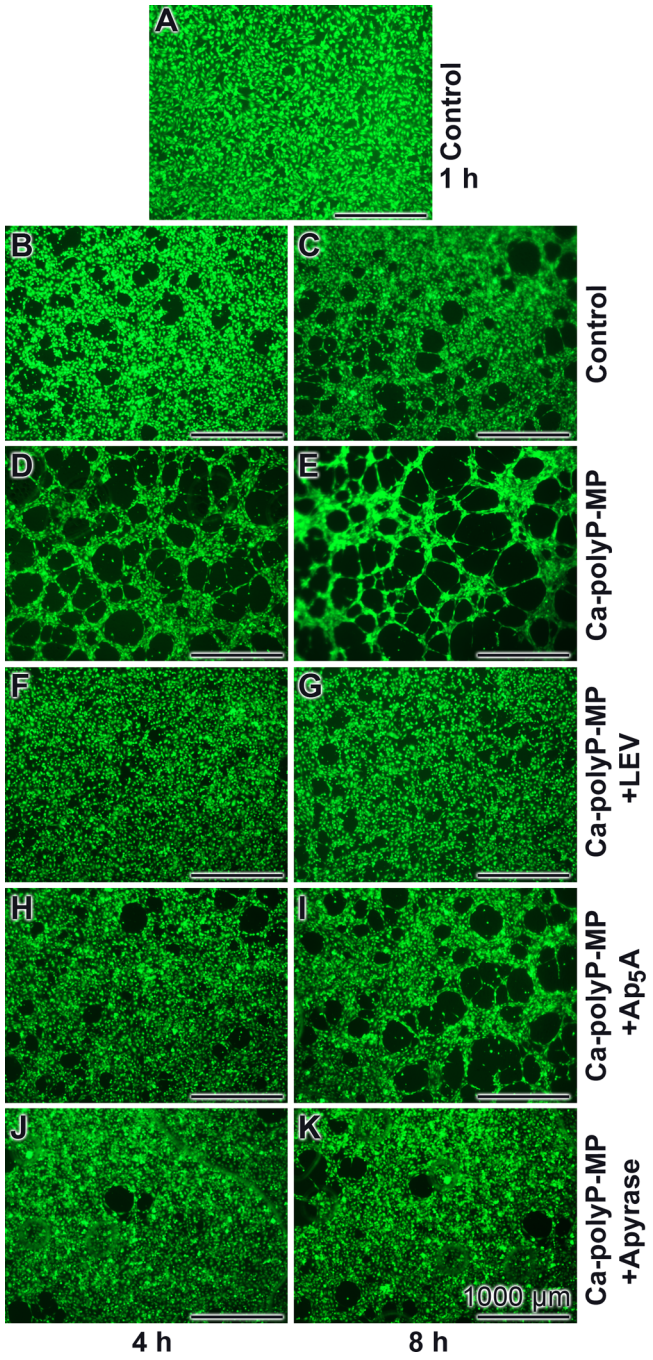

Figure 13. Migration and pattern formation (tube-like formation assay) of endothelial cells in vitro during an incubation period of 4 and 8 h, respectively. The cells were stained with calcein and visualized by fluorescence microscopy. (A–C) Control assay in the absence of polyP and inhibitor. (D and E) Organization of the cells in the presence of 50 μg/mL of Ca-polyP-MP. In addition, coincubation experiments with 1 mM LEV, an ALP inhibitor (F and G), or with 40 μM of the ADK inhibitor Ap_5A (H and I) were performed. Finally, the cultures were incubated with Ca-polyP-MP and 10 U/ml apyrase (J and K). Reprinted with permission from ref 10. Copyright 2018 Portland Press Limited on behalf of the Biochemical Society.

13.2. Model of Concerted ALP-ADK Action

As summarized in Figure 16, the ALP-mediated phosphoryl transfer reaction from polyP to AMP could, in principle,

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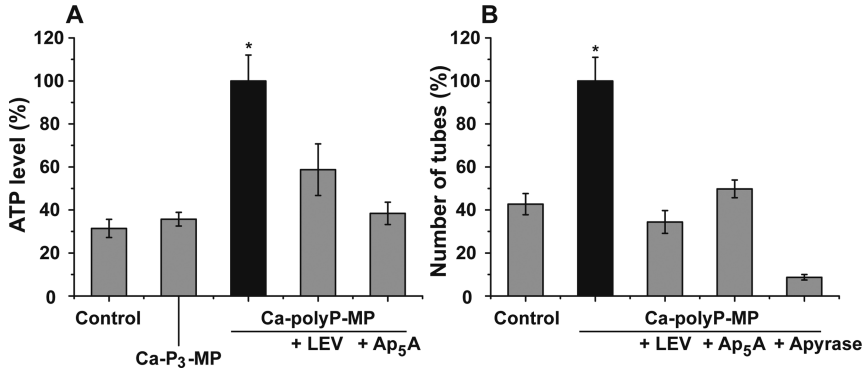

Figure 14. (A) Quantitative assessment of the extracellular ATP level in endothelial cell culture in vitro, in the absence of polyP (control) or after addition of 50 μg/mL of Ca^{2+}-containing microparticles, prepared from Na-tripolyphosphate (Ca–P_3-MP) or from Na-polyP (40 phosphate units; Ca-polyP-MP) and $CaCl_2$. The level of ATP in the assays with Ca-polyP-MP was set to 100% [corresponding to 600 pmol/10^6 cells]. After coincubation with either levamisole (LEV) or Ap_5A, inhibitors of ALP or ADK, the ATP level drops significantly. (B) Reduction of the polyP-caused tubelike pattern formation after coaddition of levamisole (LEV) or Ap_5A to values close to that measured in the control (without polyP). A complete removal of ATP from the culture system with the enzyme apyrase almost completely stopped the tubelike pattern formation. Adapted with permission from ref 10. Copyright 2018 Portland Press Ltd..

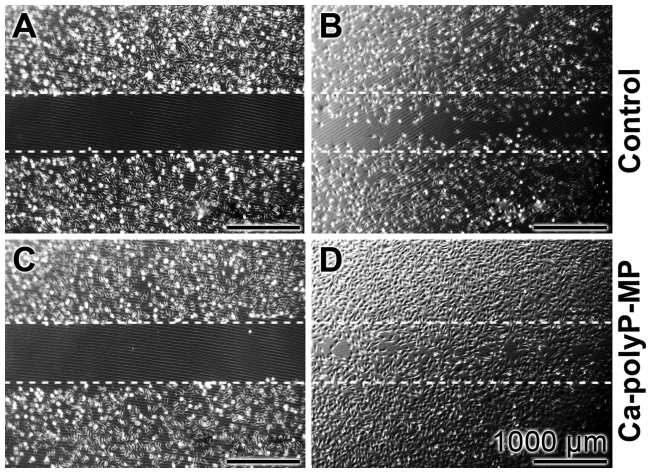

Figure 15. Analysis of endothelial cell migration onto ECM extract substratum by the in vitro scratch assay. The assays in A and B were free of polyP, while in C and D, 50 μg/mL of Ca-polyP-MP were added. The dotted lines mark the areas lacking cells at the beginning of the experiments; into these zones, the cells migrated in. The images were taken at 0 h (A,C) and 12 h (B,D) after scratch formation.

proceed through one of three different mechanisms: first, a S_N2-type mechanism via a pentavalent transition state with simultaneous bond cleavage and new bond formation; second, a dissociative (elimination-addition) mechanism, characterized by the formation of a metaphosphate intermediate after cleavage of the leaving P_i group; and third, an associative (addition–elimination) mechanism during which a pentavalent phosphorane intermediate is formed by nucleophilic attack, followed by the release of the leaving group from polyP.[341] In the first (concerted) mechanism, no intermediate is formed, while the dissociative and associative mechanisms are two-step processes that do involve an intermediate. Both mechanisms (dissociative and associative) are stepwise S_N1-type processes, in contrast to the concerted mechanism, in which bond-breakage and new bond formation occur simultaneously. These reactions can also be described using a More O'Ferrall–Jencks diagram.[342] In view of the results reported for the myosin light chain kinase (see section 12), as well as the results reported for model systems,[342] we assume that the ALP reaction proceeds through a dissociative mechanism, involving the formation of a metaphosphate intermediate.

13.2.1. Mg^{2+} Ions. Mg^{2+} has a decisive role in the allosteric activation of the ALP; it enhances the reaction rate and binds to the ALP dimer with negative cooperativity.[343,344] It is assumed that the particular ALP subunit, containing Mg^{2+}, has the higher affinity for the substrate than the one without a bound Mg^{2+}; consequently, both subunits exhibit an unequal affinity for the substrate/product.[345] Here, we present a model that might allow an explanation of the phosphoryl-transfer from polyP to AMP, catalyzed by ALP. This model is based on, first, the findings that the functionally active ALP exists as an asymmetric homodimer,[344,346] consisting of two ALP subunits that likely undergo alternating conformational changes, and changes in affinity to the substrate molecule, during the catalytic cycle,[345] and, second, the assumption that the enzyme acts through a dissociative mechanism, involving the formation of a metaphosphate intermediate. In this model, depicted in Figure 17, we propose that the Mg^{2+} containing high affinity ALP subunit binds polyP. The product of the catalytic reaction, ADP, formed by phosphoryl-transfer from polyP to AMP, remains bound to this subunit and is released as soon as a conformational change occurs by binding of Mg^{2+} to the second low affinity subunit. The thereby activated second subunit (now present in the Mg^{2+} containing high affinity form) then binds the partially degraded (by one P_i) polyP product and performs the next cleavage reaction, and so forth. During this stepwise phosphoryl-transfer reactions, the degradation of the polyP proceeds.

13.2.2. Catalytic Reaction of ALP. In our model, the catalytic reaction involves the cleavage of the O–P bond at the phosphorus located at the terminal position of $polyP_n$ (n = number of P_i units) by one ALP molecule of the dimeric enzyme under formation of the metaphosphate intermediate (Figure 17). This metaphosphate intermediate is bound to the bimetallo Zn^{2+}-center of active site of the ALP.[347] Only after the release of the leaving group (polyP shortened by one P_i unit), the nucleophilic attack of the 2-fold negatively charged AMP at the enzyme-bound metaphosphate intermediate occurs (negatively charged but partially neutralized by interaction with positively charged functional groups in the active site) under formation and release of ADP from the

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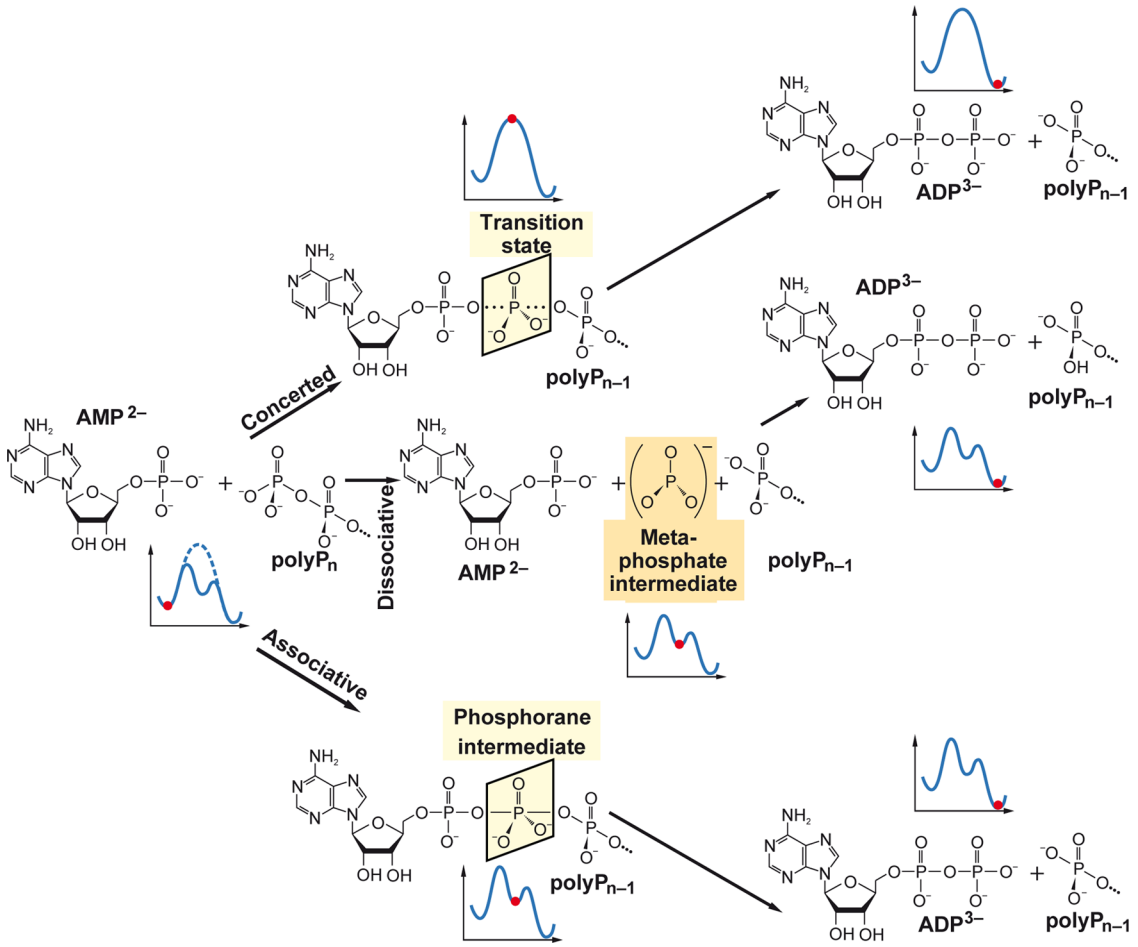

Figure 16. Three possible mechanisms (concerted, dissociative, and associative) for the phosphoryl transfer reaction catalyzed by ALP. Comparing with model systems, the dissociative mechanism (formation of a metaphosphate intermediate; reaction scheme in the middle) is most likely. The concerted pathway (no intermediate, only a transition state is passed; upper reaction scheme) and the associative pathway with the formation of a phosphorane intermediate (lower reaction scheme) are less likely. The energy vs reaction coordinate plots given as inserts indicate the progress of the reaction (concerted pathway: one transition state; and dissociative or associate pathway: two transition states).

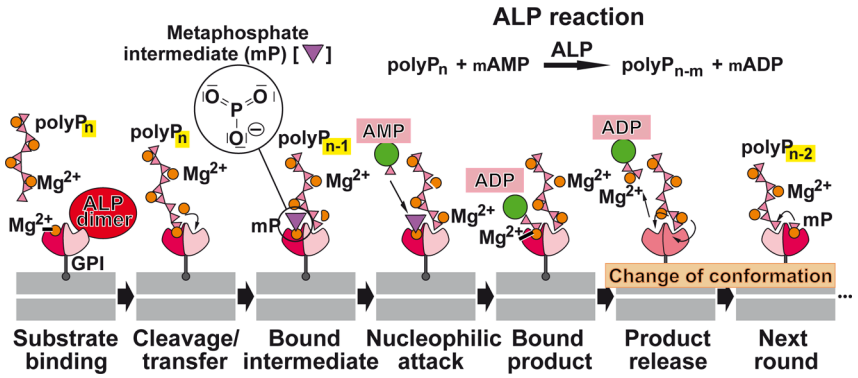

Figure 17. Proposed interaction of the ALP subunits during polyP degradation and ADP formation by membrane-bound ALP dimer. (Left to right) The ALP dimer consisting of a high-affinity subunit (red; containing a bound Mg^{2+}) and a low-affinity subunit (pink; without Mg^{2+}) is attached to the cell membrane via the GPI anchor of each subunit. After binding of the substrate, $polyP_n$ (n = number of P_i residues) to the high-affinity subunit and cleavage of the terminal P_i of the polyP chain, the resulting $polyP_{n-1}$ is transferred to the low-affinity subunit, facilitated by the close proximity of the catalytic sites of both subunits. The cleaved terminal P_i, as a metaphosphate (mP) intermediate, noncovalently bound at the catalytic site of the high-affinity subunit becomes attacked by the second, AMP substrate. The ADP product formed by nucleophilic attack of AMP at the phosphorus of the metaphosphate intermediate remains tightly bound to the high-affinity subunit until a conformational change into the low-affinity form occurs, most likely induced by binding of Mg^{2+} to the low-affinity ALP subunit. Thereby the ADP is released, most likely as a Mg^{2+}-ADP complex. The $polyP_{n-1}$, bound to the second, now activated (by binding of Mg^{2+}) high-affinity unit, then performs the next catalytic cycle (cleavage of $polyP_{n-1}$ into $polyP_{n-2}$ and enzyme-bound metaphosphate intermediate; release and transfer of $polyP_{n-2}$ to the first subunit; formation of ADP by nucleophilic attack of AMP; and release of ADP after conformational change induced by Mg^{2+} binding to the other, first subunit).

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enzyme. Simultaneously, the shortened polyP translocates to the second ALP molecule of the ALP dimer, where it is cleaved again under formation of an enzyme bound metaphosphate intermediate that is then transferred to a second AMP molecule, resulting in the formation and release of a further ADP. These steps are repeated until the polyP molecule is completely degraded. Consequently, the degradation of polyP follows an apparently processive mechanism that involves (i) an interchange of the ALP subunit conformations and (ii) the transfer of the progressively shortened polyP molecule from one subunit to the other and vice versa during each catalytic cycle. Thereby, the action of the subunits is not synchronous but time-lagged to each other. Of course, it must be taken into account that water which is present in excess amounts might compete with AMP in the ALP-catalyzed phosphoryl-transfer reaction. However, primarily the water hydroxide ions (OH^-) act as a powerful nucleophile, and their concentration at neutral pH (with 10^{-7} M) is comparably low, in the same range or even lower than the concentration of AMP. Nevertheless, a possible coupling of both reactions, the strongly exergonic hydrolysis of the phosphoanhydride bond and the phosphoryl-transfer reaction, cannot be excluded, e.g., via alternating hydrolysis and transphosphorylation steps by the two ALP subunits.

13.2.3. Coupled ALP-ADK Reaction. As experimentally shown, exposure of cells in vitro, like SaOS-2 osteogenic sarcoma cells, to polyP elicits in those cells a 2- to 3-fold increase of the ADP/ATP level both in the intra- and the extracellular (medium) space.[148,149] If the system is treated with levamisole (inhibitor of the ALP) and with Ap_5A (inhibitor of the ADK) this increased nucleotide level was almost completely abolished.[10] This finding suggested that the two enzymes in the cellular system are involved in the polyP-mediated generation of ADP/ATP.

Since ADP produced in the ALP reaction can subsequently be used as a substrate for the ADK in a cooperative reaction, the energy stored in polyP can be transformed into metabolically useful energy in the form of ATP.[149] The ADK which is exposed, like the ALP, to the extracellular space at the outer cell membrane[149] catalyzes the reversible interconversion of all three adenine nucleotides. The ATP generated in this transphosphorylation reaction, by the concerted action of the two enzymes, ALP and ADK (both enzymes are cell-membrane-bound), is formed extracellularly (Figure 18). Among the different isoforms of ADK known, the AK1β which is secreted into the extracellular space[348,349] is the most likely isoform involved in this combined ALP-ADK reaction.

The application of only the ADK inhibitor Ap_5A (without levamisole) enabled a dissection of both enzyme activities of the ALP/ADK system. Cells exposed to polyP without this inhibitor showed an upregulation of the extracellular ATP with a high ATP/ADP ratio, while cells coincubated with polyP and Ap_5A showed a relative increase in extracellular ADP (shift of the ATP/ADP ratio in favor of ADP).[149] From these results, it can be concluded that the energy-rich phosphate liberated during the ALP-mediated cleavage of polyP is first transferred to AMP under formation of ADP which is subsequently interconverted to AMP and ATP through the ADK reaction. The final product, ATP, can then be used, among others, as a substrate in kinase reactions (Figure 18). Alternatively, the AMP molecule together with the ATP can undergo a series of subsequent catalytic cycles catalyzed by neighboring ADK molecules to channel the energy to more distant places along the membrane.

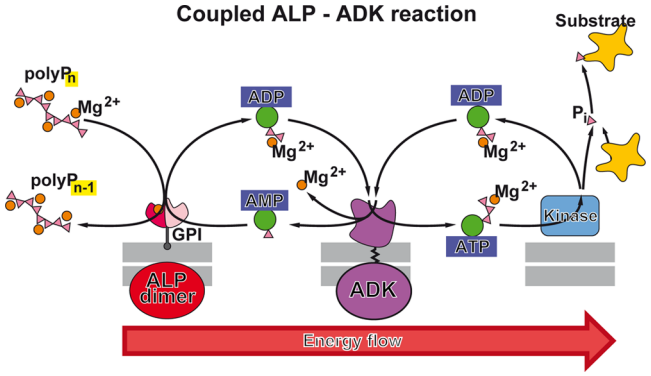

Figure 18. Energy channelling from membrane-bound ALP dimer via membrane-bound ADK to energy-consuming processes during ATP formation from polyP. The Mg^{2+}-ADP complex released from the ALP dimer during each catalytic cycle, together with a second Mg^{2+}-ADP, is converted by ADK into AMP and Mg^{2+}-ATP which can be utilized for a variety of energy-consuming reactions in the extracellular space, e.g., as a P_i-donor for kinases. The concerted action of both enzymes (ALP and ADK) also allows the produced AMP to enter the next catalytic cycle of ALP via nucleophilic attack at the polyP metaphosphate intermediate bound at the catalytic site of the enzyme. It should be noted that only ADP and ATP but not AMP are complexed with a divalent cation (one Mg^{2+} ion each). Consequently, one Mg^{2+} is released during the ADK reaction, which could bind to the low-affinity ALP subunit to induce the conformational change of the high-affinity subunit to release the bound ADP product (see Figure 17).

In contrast to the ALP, the phosphoryl transfer reaction catalyzed by the latter enzyme, the ADK, occurs via the concerted pathway, in which a negatively charged oxygen on AMP attacks, as a nucleophile, the terminal γ-P_i of ATP; bond formation to the nucleophile and bond cleavage to the leaving group proceed simultaneously. Only a single transition state with the pentavalently coordinated γ-P_i is formed that dissociates under the release of two ADP molecules.[350]

In the ADK reaction only ADP and ATP but not AMP is complexed with one Mg^{2+} ion each. Therefore, during the ADK equilibrium reaction (2 Mg^{2+}-ADP ↔ AMP + Mg^{2+}-ATP + Mg^{2+}), one additional Mg^{2+} ion will be needed to obtain two Mg^{2+}-ADP molecules from AMP and Mg^{2+}-ATP (Figure 18). However, during polyP degradation/formation of Mg^{2+}-ADP, only 0.5 Mg^{2+} ion is released per one phosphate unit of the polyP molecule, because, in the Mg^{2+}-polyP, (maximally) one Mg^{2+} ion is bound to two P_i units of the polyP chain (maximum Mg:P ratio: 0.5). Consequently, the system will become depleted of free Mg^{2+} ions in the course of the reaction if not substituted by Mg^{2+} from other sources. It might be possible that the Mg^{2+} balance is restored by alternating hydrolysis- and phosphoryl-transfer reactions that could occur during degradation of Mg^{2+}-polyP.

14. POLYP TRANSPORT AND ENERGY CHANNELING IN THE EXTRACELLULAR SPACE

As described in section 2, the extracellular space consists both of a fluid phase and a fibrillar ECM that provides structural support for the tissue.[351] It is obvious that the structure and composition of the ECM can influence transport, stability, and degradation of polyP or polyP particles released into the extracellular space. The (charged) structural fibrous proteins of

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the ECM, collagen and, in particular, proteoglycans and glycosaminoglycans bind a large volume of water but can also interact electrostatically with polyanionic polyP or polyP particles. This could either facilitate or impair the transport and distribution of polyP in the extracellular space.

14.1. Potential Transport through the ECM

The potential transport ways of polyP through the ECM are not yet known. Principally, the extracellular conveyor system for polyP could be based on either a free polyP/polyP-nano/microparticle transport mechanism or a spreading of the polymer encapsulated into extracellular vesicles.[351] Focusing on the first possibility, a transport of polyP could occur either by (i) free diffusion (following a concentration gradient), (ii) hindered diffusion (due to transient binding/immobilization of the molecules to ECM components), or (iii) facilitated diffusion (interaction with ECM components that facilitate the diffusion). Like the cell interior,[352,353] the extracellular medium has a comparably high viscosity and its physical properties do not correspond to those of an ideal solution. This might even affect the diffusion of small molecules. In addition, the diffusion especially of larger molecules, of macromolecules/polymers, like polyP and polyP particles, might be hindered by steric restrictions or binding to ECM components. Generally speaking, the movement of particles through the ECM is triggered by Brownian random motion, a process that is influenced by steric, electrostatic, or hydrodynamic interactions.[351] It has been shown that the transportability of the matrix is negatively correlated with the concentration of their fibrous elements, in particular glycosaminoglycans.[354] Furthermore, the Brownian motion of particles is significantly impaired by electrostatic interactions between charged particles and charged components of the ECM.[355]

According to a model proposed by Stylianopoulos et al.,[355] the effect of repulsive electrostatic interactions on the diffusion of nanoparticles in the ECM will only become significant at fiber diameters equivalent to the Debye length (a measure of a electrostatic effect of charged particles in a solution). Hence, electrostatic interactions should be relevant for glycosaminoglycans (negatively charged) with a size of a few nanometers but less relevant for collagen fibers (positively charged) with a size of up to a few micrometers. Moreover, neutral nanoparticles should diffuse faster than charged particles. Therefore, it can be expected that the diffusion of polyP-particles through the ECM can be influenced and modulated by changing the zeta potential of the particles,[356] as described.[87]

14.2. Cell-Based Transport

It is reasonable to assume that the prevalent way of transport of polyP in the extracellular space likely occurs in form of platelet-packaged particles.[357] Recently, it has been discovered that polyP is also present in exosomes. Exosomes are extracellular vesicles with a size of 30−100 nm that have attracted increasing interest as mediators of cell-to-cell communication.[358] These vesicles are continuously released from cells into the extracellular fluid via exocytosis[359] and can contain polyP, besides proteins, nucleic acids, and signaling molecules, as identified in so-called prostasomes that are secreted from prostate cancer cells and expose long chain polyP on their surface.[360] The hydrodynamic size of exosomes can be markedly higher compared to the size of the membrane vesicles due to the formation of a proteinaceous "corona" which causes a retardation of their mobility.[361] Exceptionally, polyP released from cells (*D. discoideum*) has also been reported to induce cell aggregation and to inhibit cytokinesis in an autocrine negative feedback loop.[188,362]

In addition to the transport of polyP in the extracellular space, the energy flow through the ECM in form of high-energy phosphate, released from polyP, is essential for the proper functioning of an extracellular energy supply system. As described in section 13, the ADKs play a decisive role in the phosphotransfer from polyP to adenine nucleotides. These enzymes are thought to form a phosphotransfer network both intra- and extracellularly that regulates a variety of energy-dependent processes and signaling events which are essential for cell growth and function, as well as differentiation.[363]

The mechanisms and advantages of the intra-cellular energy channeling has been intensively discussed.[364] Because the diffusion of ATP and other energy carrying nucleoside triphosphates (NTP) through the cytosol is limited, it has been proposed that cellular energy metabolism involves multiple "microcompartments" where the production sites of the nucleotides are in close proximity to the sites of NTP consumption.[364] This model enables a direct transfer of energy from the NTP-generating system to the NTP-consuming system.

On the basis of these considerations, we assume that polyP, together with the membrane-bound ALP and ADK, and an ATP consuming reaction can form such an energy transfer or energy channeling system in the extra-cellular space (Figure 18). Due to its processive mode of action, the ALP is able to produce large amounts of energy on the spot, and, in concert with the ADK, large amounts of ATP. The metabolic energy, in the form of ATP, could then be used by energy-consuming reactions, most likely occurring at or close to the cellular membrane. The advantage of this system: the close coupling of the two reactions at a specific site allows for a fast adaptation of ATP generation (via the ALP/ADK system using high-energy P_i from polyP) to the energy/ATP demand of those processes that utilize ATP in particular at rapidly changing or high turnover rates. Intracellularly, such enzymatic phosphotransfer networks can even couple spatially separated ATP-producing and ATP-consuming processes, as reviewed in Dzeja and Terzic.[363]

It should be mentioned that the site of polyP degradation/consumption within the interstitium/extracellular fluid or the cytosol is spatially separated from the site of polyP synthesis/storage within the platelet acidocalcisomes. This seems to be reasonable because in this way the formation of a futile cycle is avoided, i.e., a metabolic cycle that dissipates energy by hydrolysis of polyP without any meaningful anabolic reaction.[365] The driving force of such a cycle would be the strongly exergonic hydrolytic cleavage of the polyP phosphoanhydride bonds. However, it might be possible that, e.g., in case of low availability of proteins/molecules acting as phosphoreceptors, polyP degradation can switch from ALP/ADK-mediated ATP generation to ALP-mediated hydrolysis under release of large amounts of energy in the form of heat. Under these conditions, polyP might play some role in extracellular thermogenesis, in particular in bradytrophic tissue with low cell numbers and vascularization.

15. CONCLUSION

It might be surprising that the importance of polyP, a purely inorganic biopolymer, as one of the oldest energy-delivering molecules in living systems has not been recognized for a long

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time. All the more so since there are no other molecules in living beings that can store as much energy in the form of energy-rich bonds as polyP. Compared to ATP, which is composed of both an inorganic and an organic part, polyP has a uniform, comparably simple structure. The discovery of the enzymes, enzyme systems, and mechanisms that enable one to convert the energy stored in the energy-rich phosphoanhydride bonds of that molecule into metabolic energy in the form of ATP has enhanced the understanding of the basic biochemical processes involved in energy storage and release. In particular, these discoveries make it possible to elucidate an aspect of energy metabolism that has hitherto not been considered or has received only little attention: energy supply and storage in the extracellular space. This extracellular space occupies a much larger volume in animals and humans than the intracellular space. For example, many tissues, such as cartilage and bone, consist of a huge extracellular matrix in which only a few cells are embedded. And this matrix does not contain the mitochondria which serve as energy factories of the cells.

The ECM, however, is rich in enzymes that metabolize extracellular nucleotides and nucleosides, most of them are involved in the regulation of purinergic signaling.[366] Such a function implies that the concentration of the nucleotides is low and that they act only within a delimited region within the ECM, close to the surface of those cells that expose the respective nucleotide-sensitive receptors. Indeed, the concentration of ATP in the extracellular space only amounts to 10^{-9} to 10^{-7} M; only in the pericellular space, ATP levels from 10^{-6} to 10^{-5} M are reached. Therefore, in dependence on the adenylate energy charge (AEC)[367] in the extracellular space, the levels of AMP can exceed those of the water OH^- ions, making this nucleotide (AMP^{2-}) an efficient nucleophile competing with water in the ALP-mediated phosphoryl-transfer reaction. The main factors that determine AEC in the intra-cellular space are (i) the F_1F_O ATP synthase reaction as the main cellular process that synthesizes ATP from ADP, (ii) the ADK reaction which catalyzes the interconversion of the three adenine nucleotides (ATP, ADP, and AMP), (iii) the enzymatic cleavage of the β,γ-phosphoanhydride bond in ATP releasing ADP like in phosphotransferase (kinase) or hydrolase (ATPase) reactions, and (iv) the enzymatic cleavage of α,β-phosphoanhydride bond in ATP yielding AMP like in many anabolic synthetase processes.[368] In the extra-cellular compartment (interstitium or ECM), the phosphoryl-transfer from polyP, catalyzed by the cell membrane-bound ALP/ADK system, has to be added, which can be assumed to play a dominant role in determining AEC in the extracellular space as described in this review. In addition, a series of further enzymes are known to be involved in ecto-enzymatic interconversion reactions that control the level of the purinergic agonists like ATP or ADP, such as the nucleoside triphosphate diphosphohydrolases, the nucleotide pyrophosphatase/phosphodiesterases, and the ecto-5′-nucleotidase.[366] Besides these degrading enzymes, the ATP regenerating nucleoside diphosphate kinases might be mentioned.[367] Some of those enzymes are highly specific for nucleotides, while others degrade a variety of substrate molecules, like the ALP which hydrolyzes both ATP and the phosphoanhydride bonds in polyP.

In the model, proposed in this review, the ALP directly transfers the energy-rich phosphates from polyP, through a metaphosphate intermediate, to AMP which is subsequently converted to ATP via an ADK reaction. This reaction seems to be possible due to the broad substrate specificity of the ALP which accepts both polyP and AMP as a substrate. The ATP then transfers the phosphate to the final phosphate acceptor molecule. In this case, a highly exergonic reaction (hydrolysis of polyP) is linked with an endergonic reaction (formation of the phosphorylated substrate) via a shared intermediate product. Such a reaction scheme in which the product of the first (exergonic) reaction is used as a substrate for a second (endergonic) reaction is a typical example for a coupled reaction used by many reactions in biological systems.[369]

On the basis of our data, the ALP, like the PPX, which both release P_i processively from the termini of the substrate, acts as exopolyphosphatase. Evidence has been presented that this enzyme can also act as a phosphotransferase. If it is considered that the metabolic energy, which is released by cleavage of the P−O−P bond in the polyP molecule, is reused for ADP/ATP formation, the term polyP:AMP(ADP) phosphotransferase system (PP-PTS) is justified.

It will be an interesting task to elucidate the mechanism(s) of recognition of the diverse, possibly supramolecular structures of the complexes/particles formed by polyP and different counterions by the various proteins (enzymes and polyP-binding proteins) that interact with the polymer. Recently, in addition to the polyP-metabolizing enzymes described in this article, several polyP-binding proteins characterized by a CHAD (conserved histidine α-helical domain) motif have been identified, which are associated with the surface of the bacterial volutin granules.[370] Later this group of proteins carrying the conserved CHAD domain has also been identified in archaea and eukaryotes (in the plant *Ricinus communis*).[371] Structural analysis revealed that the CHAD domains are formed by helical bundles and a central pore surrounded by highly conserved basic amino acids. PolyP molecules but not nucleic acids are bound with high affinity (dissociation constants in the nanomicromolar range).[371] The investigation of the potential function of these structurally interesting proteins in regulation of polyP metabolism, especially in higher eukaryotes, deserves further attention.

The elucidation of the function of polyP in extracellular energy metabolism is also of medical importance, especially for diseases of tissues that are composed of only a few cells and a large extracellular matrix and have poor vascularization. Thus, the discovery of the energy-supplying function of polyP has led to the development of first new approaches in regenerative medicine based on polyP, especially for the treatment of bone and cartilage diseases, but also for wound healing disorders.[30,311] Even the application of polyP as a major component of a bioink, suitable for 3D printing of implant materials in the absence or presence of cells included, has been successfully shown.[372]

Nevertheless, many questions remain unanswered, for example, about the regulatory mechanisms that control the formation and degradation of polyP, its transport over greater distances via the bloodstream, the formation of the polyP-loaded platelets from the megakaryocytes, the release of polyP from the platelets, or its possible chaperone function and role in regulation of autophagy. PolyP is also a component of many materials used in bone replacement. However, in these cases, crystalline polyP is mostly used which, unlike the amorphous polyP and polyP MP described in this review, is not biologically active and cannot serve as an energy source.[315] It can be expected that polyP, an inorganic polymer with a crucial function in biological systems, not only under

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physiological but also pathological conditions, will gain increasing attention besides the universal biochemical energy carrier ATP.

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Notes

The authors declare no competing financial interest.

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Heinz C. Schröder is a chemist and physician and completed his doctorate in both disciplines with distinction. In 1982−1983, he did a Liebig fellowship at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, and in 1988, he did a fellowship at the National Cancer Center Research Institute, Tokyo, Japan. Since 1985, he has been a professor at Mainz University/University Medical Center. His main scientific works include the description of polyphosphate in bone, as well as the disclosure of enzymes involved in polyadenylate and interferon 2-5A metabolism and of the role of HIV-Tat. His present research interests include the mechanisms of formation and application of biogenic nanoparticles. He has authored or coauthored more than 500 scientific articles (h-index: 59 [ISI-WOS]) and has received several awards. Together with Prof. Dr. W.E.G. Müller, he has been involved either as a coordinator (ENVRAD, UVTOX, BIOTOXmarin, BIO-LITHO, and BIOMINTEC) or partner in several large EU projects.

Xiaohong Wang is a chemist and material scientist. In 2005, she became a professor in inorganic chemistry. She has long-standing expertise in the development and characterization of regenerative-active materials and the molecular processes underlying their biological/morphogenetic activity. Her achievements include the elucidation of the mechanism of hardening of biogenetically formed silica and of the enzymatic formation of calcium carbonate "bio-seeds" in bone mineralization. Since 2006, she has had close collaboration with the group of W.E.G. Müller and she joined his team in Mainz in 2009. Her scientific work comprises over 300 publications (h-index: 34 [ISI-WOS]). In addition, she has coordinated/participated, as a PI, in several EU-funded projects, such as CoreShell, SPECIAL, MarBioTec, or the ongoing H2020-InnovaConcrete. In addition, she is the scientific coordinator of the German-Chinese "Joint Center" on Bioinspired Materials.

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