INORGANIC POLYPHOSPHATE AND F_0F_1-ATP SYNTHASE OF MAMMALIAN MITOCHONDRIA

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Abstract

Inorganic polyphosphate is a polymer which plays multiple important roles in east and bacteria. In higher organisms the role of polyP has been intensively studied in last decades and involvement of this polymer in signal transduction, cell death mechanisms, energy production and many other processes were demonstrated. In contrast to east and bacteria, where enzymes responsible for synthesis and hydrolysis of polyP were identified, in mammalian cells polyP clearly plays important role in physiology and pathology but enzymes responsible for synthesis of polyP or consumption of this polymer are still not identified. Here, we discuss the role of mitochondrial F_0-F_1-ATP synthase in polyP synthesis with results which confirm this proposal. We also discuss the role of other enzymes which may play important role in polyP metabolism.

Abbreviations

PolyP – inorganic polyphosphate
$\Delta\Psi_m$ – mitochondrial membrane potential
PPK - polyphosphate kinase
PPX – exopolyphosphatase
PTP – Permeability Transition Pore
ATP - Adenosine-5'-triphosphate
ADP - Adenosine-5'-diphosphate
AMP - Adenosine-5'-monophosphate
P_i – inorganic phosphate
ALP - Alkaline phosphatase
AK - Adenylate Kinase
H-prune - Human Metastasis Regulator Protein
TRAP - tartrate-resistant acid phosphatase

INTRODUCTION

Inorganic polyphosphates (polyPs) are the ancient homopolymers, which consist from several to thousands orthophosphates residues (Figure 1). PolyP found in all living creatures, however, the role of this polymer the physiology and pathophysiology of some

organisms including mammalians is still disputable. Thus, while its role in microorganisms is well studied and the enzymes responsible for the polyP metabolism discovered decades ago, investigation of polyP metabolism and functions in mammalian cells is still cutting edge topic. Despite the fact that polyPs were firstly isolated from yeast in the end on 19th century, the main information regarding its functions in microorganisms and unicellular eukaryotes started to appear in the second part of 20th century. The group of A. Kornberg, made a big breakthrough in the polyP studies after isolation, purification and cloning of the polyP kinase (PPK) from *E. Coli* and exopolyphosphatases (PPX) from *E. Coli* [1, 2] and *Saccharomyces cerevisiae* [3]. PolyP kinase is responsible for reversible conversion of the terminal (γ) phosphate of ATP to polyP and vice versa. Exopolyphosphatases hydrolyze the terminal residues of polyP to P_i, however, while the *E. Coli* PPX prefers long chain polyPs, the exopolyphosphatase isolated from *Saccharomyces cerevisiae* (sc PPX1), can hydrolyze polyPs of different chain length. Moreover, scPPX1 is 40 times more effective in its activity compared to *E. coli* PPX [4].This knowledge helped a lot in creation of genetic tools, which could regulate the level of polyPs in different cells. Many functions of polyP in mammalian cells were discovered by transfection of different mammalian cells with scPPX1 or PPK.

Figure 1.

BODY

Recently we attempted to prove the hypothesis, which we proposed in 2010 [5], that mitochondrial F_0F_1 ATP synthase is one of the enzymes responsible for the synthesis of inorganic polyphosphates in mammalian cells [6].

Mitochondrial F_0F_1 ATP synthase is an enzyme, which uses proton gradient ($\Delta\Psi_m$), generated by the activity of the electron transport chain, for the synthesis of ATP. This unique enzyme can work in both directions - ATP synthesis, using the $\Delta\Psi_m$; and in reverse mode – using the energy of ATP hydrolysis for pumping protons from mitochondrial matrix to intermembrane space, when $\Delta\Psi_m$ is decreased (Figure 2 A and B) [7]. It is well known that mitochondria produce majority of cellular ATP, however decrease of $\Delta\Psi_m$ can lead to the activation of cell death pathways, so during different pathologies, when the $\Delta\Psi_m$ decreases, mitochondria start to consume cellular ATP for the $\Delta\Psi_m$ maintenance [8]. To prove that mitochondrial F_0F_1 ATP synthase is involved into the polyP metabolism we applied 4 different methods, two of which showed that this enzyme is able to synthesize polyP and two others that it can hydrolyze it.

One of the best method to assume the quality of isolated mitochondria is to measure how they consume oxygen for oxidative phosphorylation purposes. We measured mitochondrial respiration with the help of Clark electrode. In our experiments we evaluated different stages of mitochondrial respiration by Chance (Figure 2 C) [9]: **V_2** – "passive" mitochondrial respiration, when there are substrates for electron transport chain (glutamate and malate) and PO^{4-} in the system but no ADP (substrate for oxidative phosphorylation); **V_3** – mitochondrial respiration in the presence of ADP, stage when oxidative phosphorylation take place; **V_4** – the stage when all added ADP converted into ATP and mitochondrial respiration rate reverting close to V_2 level. This parameter might be altered if the quality of mitochondrial isolation is not very good or during different pathological conditions; **V_{CCCP} –** the mitochondrial respiration rate in the presence of uncoupler CCCP. Usually it is the maximal respiration rate as far as phosphorylation processes do not limit the work of electron transport chain. This parameter should be higher than V_3 or on the same level with it (Figure 2). **RC** – respiratory coefficient - V_3/V_4 ratio. This parameter shows the coupling of oxidation and phosphorylation processes during mitochondrial respiration and varying from 3 to 15 in healthy mitochondria;

ADP/O - the ratio of added ADP (converted by mitochondria to ATP) to the amount of oxygen used during the oxidative phosphorylation process (V_3). This parameter shows the efficiency of mitochondrial oxidative phosphorylation.

Figure 2. Scheme of function of mitochondrial respiratory chain

We found that addition of polyP to the isolated mitochondria increased the mitochondrial ADP independent respiration (V_2). In contrast, presence of polyP supressed ADP dependent respiration and almost eliminated V_4 (the effect was concentration dependent). Usually, these results indicate mild uncoupling of mitochondria and for us it was understandable as far as we previously showed that polyP in physiological concentration might modulate permeability of mitochondrial membranes throw activation of mitochondrial permeability transition pore (PTP) [10]. However, addition of CCCP increased the respiration rate in the presence of polyP the same as in control experiments, which indicated that in the presence of polyP mitochondria are in fully coupled state. To exclude the effect of polyP on mitochondrial PTP we performed experiments with inhibitor of PTP Cyclosporine A (CsA). The application of 1 μM of CsA didn't change the respiration rate of control mitochondria and the effect of polyP on mitochondrial respiration (**figure 2 D**). From the control experiments, it's perfectly seen that addition of ADP - a substrate for oxidative phosphorylation and F_0F_1 ATP synthase

increases respiration rate (V_3) (Figure 2 C). It is happening because F_0F_1 starts to synthase ATP from ADP and P_i and, as we know, it requires the energy of $\Delta\Psi_m$ for this process. Protons from intermembrane space flow throw F_0 into mitochondrial matrix; its energy helps F_0F_1 to change the conformation and create more favourable environment for ATP synthesis (Figure 2 A). However, at the same time this event lowering $\Delta\Psi_m$, which is the signal for electron transport chain (I-IV complexes) to work faster. For the experiments we add certain amount of ADP, so when F_0F_1 convert all added ADP into ATP the oxygen consumption decreasing and we can see V_4 state. If the amount of added ADP will be in 5-10 times higher, the V_3 state will take majority of oxygen in the respiration chamber and we will not see the transition from V_3 to V_4.

Addition of polyP increases mitochondrial respiration, the same as we can see after application of ADP, but the rate of respiration in the presence of ADP is much higher than in the presence of polyP. By classical scenario, F_0F_1 ATP synthase adds one P_i to the ADP and after that ATP leaves active site of the enzyme. In case of polyP we do not know how many inorganic phosphates the enzyme can add to the polymer, so the effect of polyP on oxygen consumption might be longer.

To verify that polyP might be produced by F_0F_1 ATP synthase we monitored the level of polyP in isolated mitochondria with the help of DAPI [11]. In 2010 Pavlov et.al. showed that modulation of the activity of respiratory chain by different inhibitors and substrates can change the level of polyP in real time [5]. In our experiments, we also showed that addition of glutamate and malate or succinate (substrates for I and II respiratory complexes, accordingly) caused rise in the level of polyP, while inhibition of respiration by rotenone and oligomycin stopped polyP production. Measuring the level of ATP in the same conditions will show the same results – concentration rise in the presence of substrates for oxidative phosphorylation and downfall in the presence of inhibitors. Taking into account that in microorganisms ATP might be used for polyP production by polyphosphate kinase and vice versa, it was always a question if the rise of polyP level in mitochondria depends on previous production of ATP. Therefore, in our experiments, when we monitored the level of polyP in mitochondria, we performed two series of experiments: we added ATP at the begging of the experiment, to the system without substrates for oxidative phosphorylation; and at the end of the experiment, to the system with substrates for oxidative phosphorylation, but when the polyP production was stopped by oligomycin

In both cases, the addition of ATP didn't lead to the rise of polyP level, so we concluded that there is no intermediate enzyme in mitochondria, which uses ATP production during oxidative phosphorylation for polyP synthesis.

As we mentioned before when $\Delta\Psi_m$ is decreasing F_0F_1 ATP synthase starts to consume ATP. There are many ways how to measure this activity, the main point is to decrease or eliminate $\Delta\Psi_m$ to switch the activity of the enzyme into hydrolysing mode. Most of the detection methods are based on the release of P_i from ATP during the hydrolysis, but in our case because of the nature of polyP these methods couldn't be used as far as polyP signal would interfere with P_i signal. When P. Mitchell tried to prove his chemiosmotic theory, the main idea of which was that during electron transport mitochondria generate proton gradient in intermembrane space (Figure 2 A and B) and afterwards uses it for phosphorylation purposes, he applied elegant method for detection of ATP hydrolysis by oligomycin sensitive mitochondrial ATP synthase [12]. This method is based on the idea that ATP hydrolysis at pH values close to neutral leads to the appearance of protons and acidulation of the incubation media, which is detectable via pH electrode. For this series of experiments, we used sub-mitochondrial particles. We showed that in the presence of polyP there was significant increase in acidulation rate, which wasn't dependent on the presence of ATP and was blocked by oligomycin. However as far as we used sub-mitochondrial particles with many other mitochondrial enzymes in the system present, in the next series of experiments we lysed mitochondria, and immunocaptured mitochondrial F_0F_1 – ATPase in the microplate well via specific antibodies – so we created a system which contained just F_0F_1 – ATPase, without other mitochondrial enzymes. Our experiments showed that F_0F_1 – ATPase could hydrolyze polyP in oligomycin dependent manner.

Docking experiments. To understand weather polyP can bind to the catalytic sites of ATP synthase we performed docking experiments. For this purposes we used bovine F_0F_1 ATP synthase monomer in first rotational state (6ZPO) from protein data bank. The structure of this protein was determined by electron cryo-microscopy by John Walker's group and was published in 2020 [13]. For docking experiments we used polyP 14 (SpolyP), which has 14 residues of inorganic phosphate in the chain. The structure of SpolyP was created and minimized with the help of ChemOffice tools and converted into pdb format with the help of OpenBabel software [14]. The F_0F_1 ATP synthase (6ZPO) file was downloaded from protein data bank website in pdb format and was prepared for docking in UCSF Chimera [15]. Docking experiments were performed with help of AutoDock Vina [16]. For the first round of docking, we decided to cover all F_1 region and

to see all possible sites where polyP can bind to the enzyme. Our results showed that SpolyP selectively binds to the ADP binding site of F_1 region with Autodock Vina binding score -9.3 (Figure 3 A, table 1). During the preparation of the enzyme for docking experiments, we deleted all ligands that were attached to the ATP synthase. After the docking with polyP we merged our docking results with original structure of ATP synthase (6ZPO), which contained all ligands. It indicates that polyP binds exactly to ADP binding site (figure 3 B). In the second round of docking, we narrowed the grid box and focused it to ADP binding site, which we had found in previous round. It slightly increased the binding energy values to -9.6 (table 1). To understand that our calculations are adequate we performed docking experiments with ADP molecule. Ones again we merged our docking experiments with original structure of ATP synthase and saw that our docked molecule (figure 3 C, red colour) binds to the same site and at the same conformation as a ligand in the original ATP synthase (figure 3 C, green colour).

Ligand	$\Delta G_{binding}$ (kcal/mol)
SpolyP (F_1 region)	-9.3
SpolyP (ADP binding site)	-9.6
ADP (ADP binding site)	-9.2

Mammalian enzymes of polyP metabolism. Despite the fact that metabolism of polyP in mammalian cells is still an open question, there are many reports, which show that some of the mammalian enzymes can use polyP as a substrate for phosphorylation or can phosphorylate polyP itself (elongate it). We want to describe some of these enzymes.

Endopolyphosphatase. In 1996 group of A. Kornberg showed that mammalian tissues has endopolyphosphatase activity [17]. In this research, vacuolar endopolyphosphatase from *Saccharomyces cerevisiae* was extracted, purified and its activity regarding polyP was tested. Moreover, in this research crude extracts from mammalian brain, heart, kidney, lungs and liver was tested on endopolyphosphatase activity, the highest endopolyphosphatase activity was present in brain tissues [17]. Endopolyphosphatases are the class of enzymes that also called polyP depolymerases, they cleave long chain polyphosphates into short and medium chains. These type of enzymes were found in many microorganisms and unicellular eukaryotes. Despite the fact that mammalian tissues showed endopolyphosphatase activity, alignment of PPN1

sequence of *S. cerevisiae* with mammalian genome haven't showed any similarities. Considering the fact that in this particular research the group worked with crude extracts, no other research groups succeeded to extract and purify the endopolyphosphatase from mammalian tissues and whole genome alignment haven't showed any similarities between mammalian genes and PPN1 of *S. cerevisiae* we believe it is rather hasty to conclude that mammals have endopolyphosphatases. However, considering the recent report, that bacterial long chain polyphosphates can disturb multiple functions of mammalian cells [18] we believe that mammalian cells should have an enzymes which could cleave long chain polyphosphates but probably it is not endopolyphosphatases but another enzymes with endopolyphosphatase activity.

PMCA of erythrocytes. Reusch et. al. in 1997 showed that plasma membrane calcium pump (PMCA) isolated from human erythrocytes is associated with two homopolymers – inorganic polyphosphates and poly-3-hydroxybutyrate (PHB) [19]. This tandem of polymers was also found in other transporting systems. First of all these polymers can itself form voltage activated calcium channels in the plasma membrane of different bacteria [20]. Further studies had shown that this complex does not have any proteins, which is currently the only case of an ion channel of "non-protein" origin [21]. In 2005, similar complex was extracted from mammalian mitochondria. Incorporation of this complex into planar lipid bilayer showed that it possess biophysical and electrophysiological properties of mitochondrial Permeability Transition Pore (PTP) [22]. In the period from 2007 to 2009, several studies revealed that polyP and PHB play crucial role in functioning of KcsA [23, 24], TRPA1 [25] and TRPM8 [26] channels, in KcsA and TRPM8, polyP, along with PHB, plays role as a structural component of the channels. In 2016 group of E. Pavlov showed that polyP and PHB has an association with subunit c of F_0F_1 ATP synthase, which is believed a main structural part of mitochondrial PTP [27, 28].

Furthermore, Reusch et. al. showed in their research that PMCA of human erythrocytes can transfer orthophosphate residues from ATP to polyP and vice versa, hereby showing polyphosphate kinase activity [19]. PMCA belongs to the P-type calcium transport ATPases, which uses the energy of ATP molecules to transfer Ca^{2+} from cytoplasm outside the cell. Interestingly, another calcium ATPase - SERCA, despite the structural and functional similarity to PMCA, doesn't have nor polyphosphate kinase activity nor can use polyP as energy source for its own activity [5].

ALP. Alkaline phosphatase (ALP) is an enzyme dephosphorylating different compounds. It shows its highest activity at alkaline pH values and under physiological

conditions is not very selective and can dephosphorylate different substrates including ATP, ADP, AMP and many other organic and inorganic substrates which contain orthophosphate residues [29]. ALP present in all living forms starting from bacteria to mammals. In humans it present in all tissues. In 2001 it was shown that mammalian intestinal alkaline phosphatase has exopolyphosphatase activity and "digest" polyPs with polymerization rate up to 800 residues. Interestingly, ALP didn't require Mg^{2+} or other divalent atoms for its activity, while other known exopolyphosphotases are highly dependent on it. However, in the presence of EDTA exopolyphosphatase activity of ALP was inhibited, which shows that some replaceable metals are crucial for the activity of the enzyme. As far as ALP contains Zn^{2+} and Mg^{2+} ions in its structure, results with EDTA looks logical.

In two other papers published in 2015 and 2017 were shown that treatment of SaOS-2 cells with amorphous Ca^{2+} polyphosphate nanoparticles [30] and polyP (sodium salt) [31] increased the number of mitochondria in the cells and caused translocation of SaOS-2 cell's alkaline phosphatase to the cell surface [30]. Further studies reviled that polyP treatment causes release of matrix vesicles, which contain both alkaline phosphatase (ALP) and **Adenylate Kinase** (AK) into the extracellular space [31]. Interestingly, it was shown that pre-incubation of SaOS-2 cells with polyP increased the level of extracellular ADP and ATP. PolyP dependent ADP increase was even higher in the presence of AK inhibitor A(5′)P5(5′)A. However, A(5′)P5(5′)A almost totally inhibited polyP dependent ATP increase. SaOS-2 cells can release intracellular ATP via exocytosis, however polyP dependent ATP increase wasn't dependent on the presence of exocytosis inhibitors N-ethylmaleimide and brefeldin A, therefore authors concluded that the rise in ATP level is not due to the ATP release from the cells. The authors propose that the energy stored in polyP's phosphoanhydride bonds might be used for enzymatic ATP synthesis via ALP and AK system, where ALP is responsible for polyP dephosphorylation and AK for using the energy and orthophosphate residues from polyP degradation for nucleotide phosphorylation. AK is an enzyme, which can catalyse reaction: **ADP+ADP ↔ ATP + AMP**, in the presence of Mg^{2+}, this reaction can go in two directions, depending on the substrates availability. According to the proposed schemes authors propose that the ALP degrade polyP and uses its energy for ADP synthesis from AMP [31, 32]. However, there is no scientific evidence that ALP when dephosphorylating different substrates can phosphorylate nucleotides (AMP or ADP).

H-prune. In 2008 Tammenkoski et.al. demonstrated that Human Metastasis Regulator Protein (H-Prune) has polyphosphatase activity and can efficiently hydrolyze

short chain polyP (3 orthophosphates in the chain) [33]. In the same research, it was shown that long-chain polyPs (more than 25 orthophosphates in the chain) not only couldn't be hydrolyzed by h-prune but also act as inhibitors and prevent hydrolysis of short chain polyP by h-prune [32],

TRAP. Another enzyme, which has polyphosphatase activity, is tartrate-resistant acid phosphatase (TRAP) [34]. TRAP is abundantly express in osteoclasts and play an important role in bone formation. This enzyme contain iron atoms in its catalytic center, which can generate reactive oxygen species via Fenton reaction. In this research, authors purified TRAP from osteoblasts via coprecipitation with anti-TRAP antibodies. Experiments showed that TRAP can degrade polyP and this degradation wasn't dependent on ROS generation via Fenton reaction. TRAP could degrade polyPs with different chain length, ranging from 40 to 750 residues; however, the speed of degradation was much higher for short and medium chain polyPs than for long chain polyPs (degradation of $polyP_{750}$ was almost not detectable even after 24 hours of incubation). Moreover, it was found that low concentrations of polyP could inhibit phosphatase activity of TRAP, inhibiting activity of polyP increased with the chain length of the polymer. IC_{50} for $polyP_{750}$ was ≈ 0.663 μM, for $polyP_{300}$ ≈ 4.4 μM, for $polyP_{40}$ ≈ 47.6 μM and for $polyP_{15}$ ≈ 846 μM. Finally, authors showed that polyP inhibited bone resorption activity of osteoclasts, based on which authors proposed that polyP might be a key molecule that regulates TRAP-mediated osteoclast bone resorption [34].

NAD kinase. In 2012 Ohashi et.al. for the first time identified and characterized human mitochondrial NAD kinase [35]. NAD kinase is the sole $NADP^+$ - biosynthetic enzyme known to catalyse phosphorylation of NAD^+ to yield $NADP^+$, using ATP or inorganic polyphosphate as the phosphoryl donor. This enzyme is present in all living forms, however its activity can be different in bacteria and humans. For example, NAD kinase of some bacteria can use both ATP and polyP for NAD^+ phosphorylation, while in mammals it uses only ATP. During characterization of newly found enzyme authors showed that it can use both ATP and polyP (hexametaphosphate and tetrapolyphosphate) for NAD^+ phosphorylation [35].

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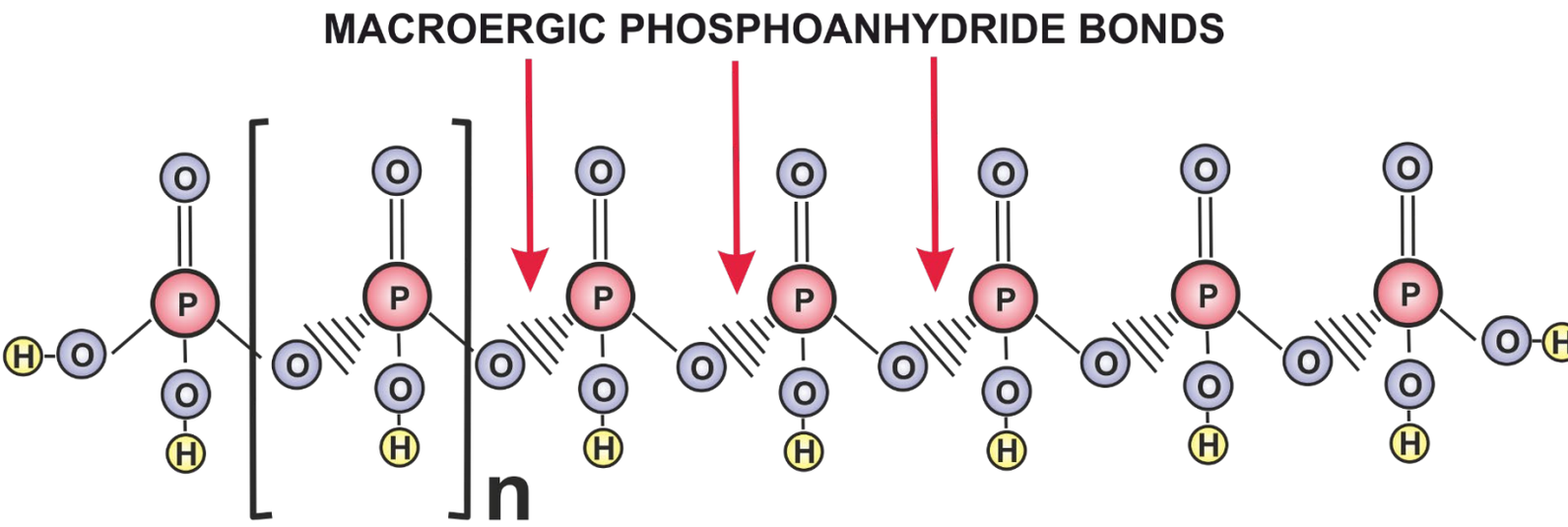
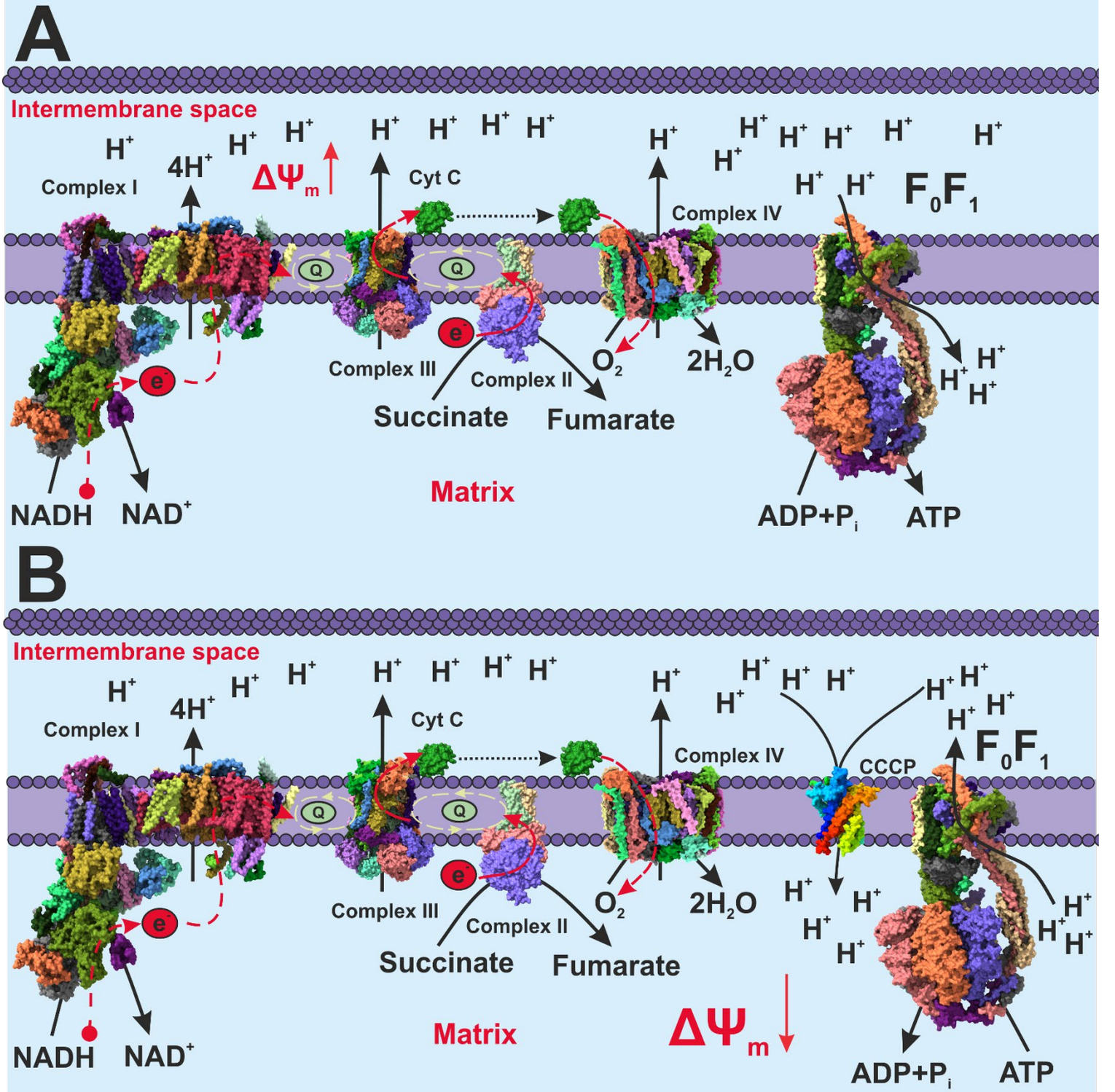
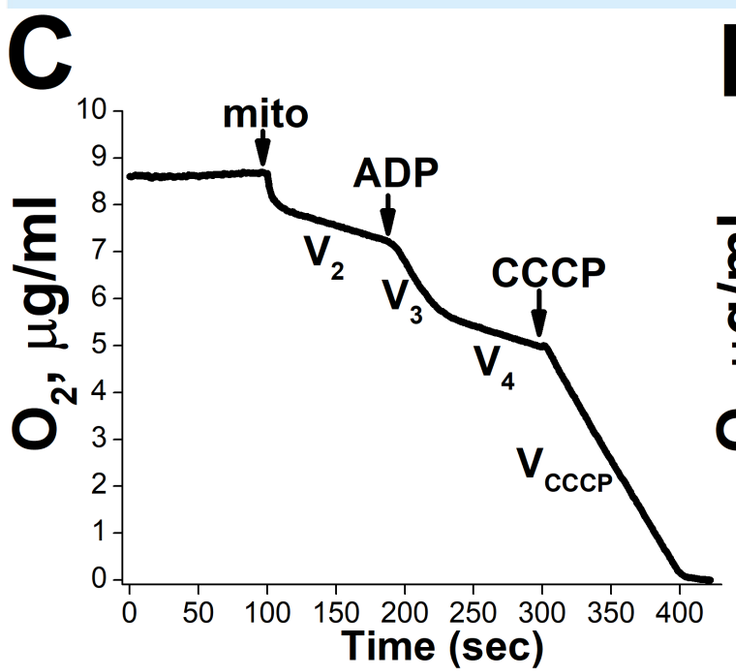
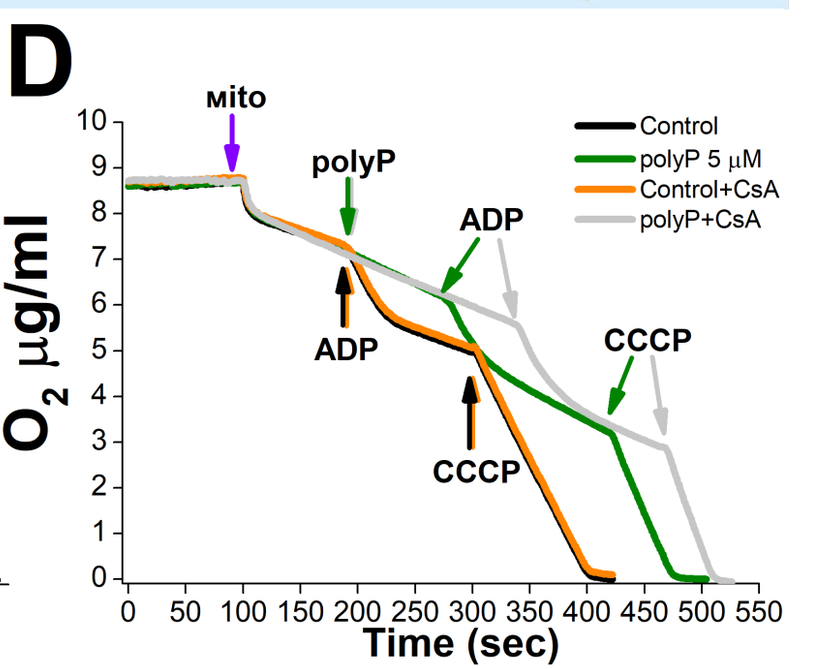


Figure 1. Structure of inorganic polyphosphates

Figure 2. Scheme of function of respiratory chain. A and **B.** Coupled and uncoupled states of oxidative phosphorylation; **C.** States of mitochondrial respiration by Chance; **D.** Classical inhibitor of PTP CsA doesn't prevent effect of polyP on mitochondrial oxidative phosphorylation.


A
Intermembrane space
H+
4H+
Complex I
ΔΨm
Cyt C
Complex IV
F0F1
Q
e-
Complex III
Complex II
O2
2H2O
Succinate
Fumarate
NADH
NAD+
Matrix
ADP+Pi
ATP
B
Intermembrane space
CCCP
ΔΨm


C
mito
ADP
V2
V3
CCCP
V4
VCCCP
O2, μg/ml
Time (sec)


D
mito
polyP
ADP
CCCP
Control
polyP 5 μM
Control+CsA
polyP+CsA
O2 μg/ml
Time (sec)

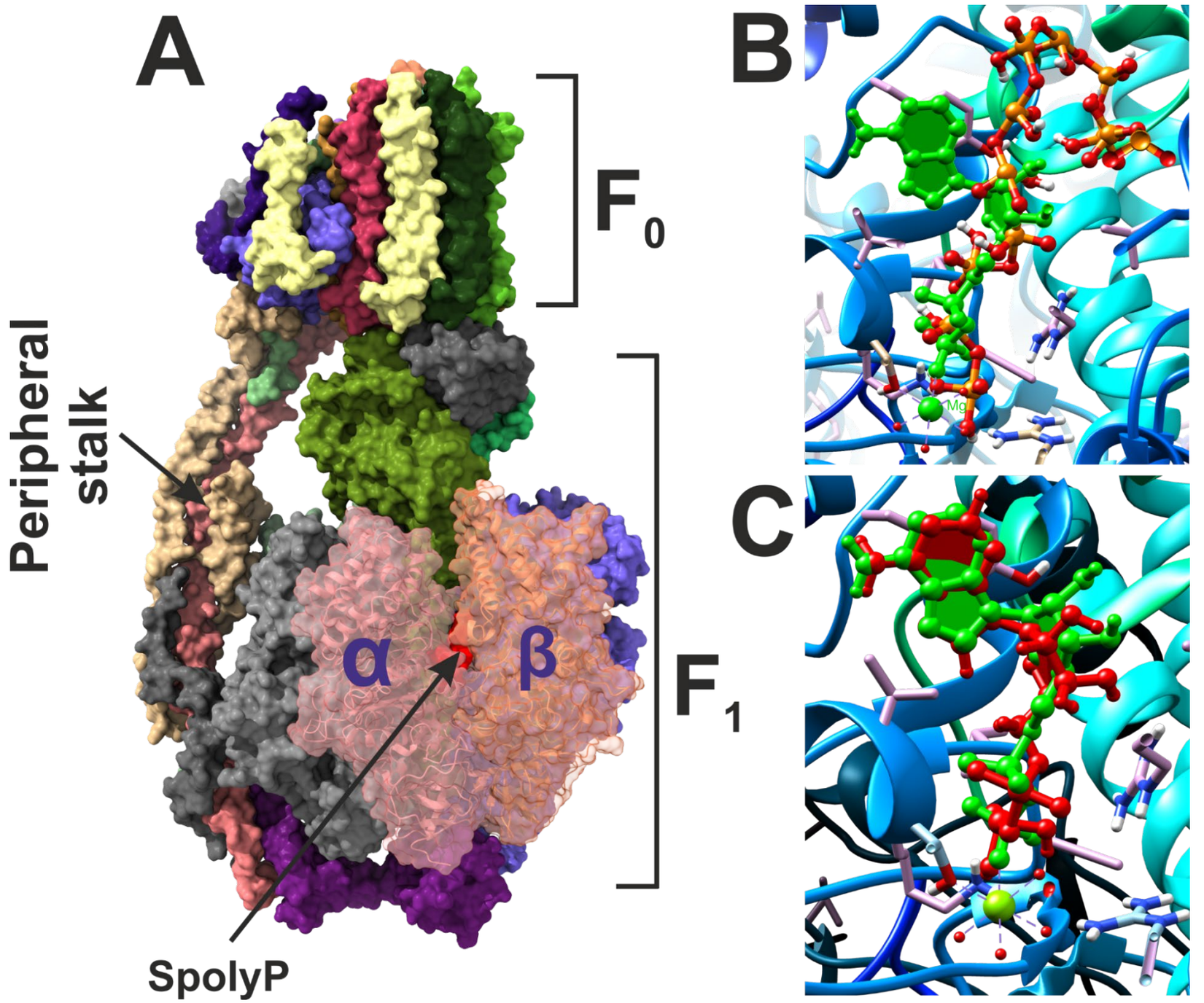


Figure 3. Inorganic polyphosphate binds to the ADP binding site of F_1. A. Position of the docked polyP on F_0F_1 ATP synthase; **B.** PolyP (red-orange molecule) binds exactly to the ADP binding site of F_1 - comparison of docked polyP position with the position of ADP (green molecule) – ligand that was present in the original F_0F_1 ATP synthase molecule from protein data bank (6ZPO); **C.** Position comparison of the docked ADP (red molecule) and ADP which was present in the original F_0F_1 ATP synthase molecule.

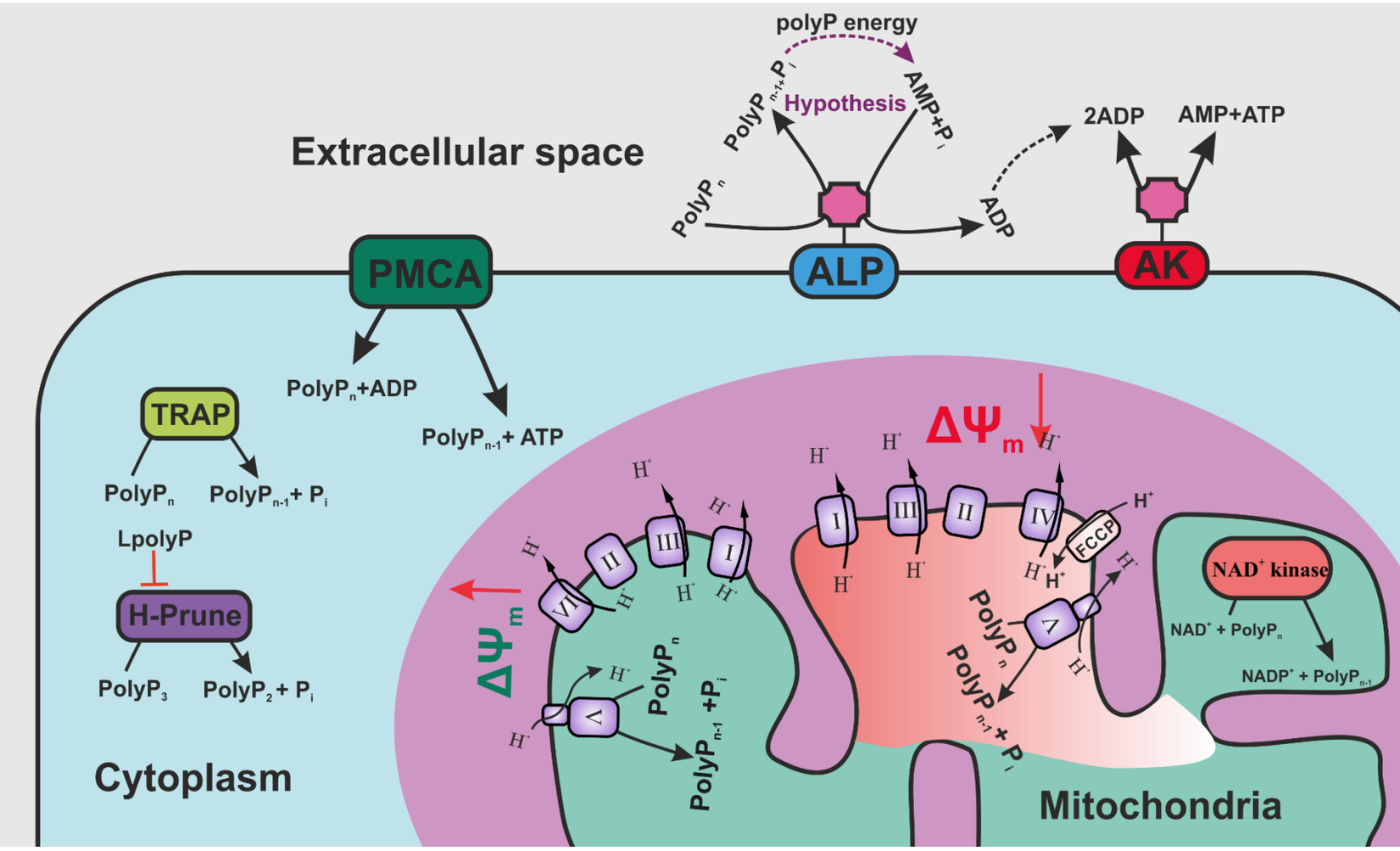


Figure 4. Mammalian enzymes, which can produce inorganic polyphosphate or use it as a substrate.