

POLYAMINES IN MODULATING PROTEIN AGGREGATION

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Abstract: Polyamines are ubiquitous aliphatic polycations with multiple molecular and cellular functions. They were first identified by Leeuwenhoek in 1678. Since then many investigations had been done to understand the physiological significance of these molecules. Being cationic at physiologic pH, they interact with various biomolecules including DNA, RNA, proteins, and help in many cellular functions. Apart from their vast number of physiological functions, they are also implicated in modulation of protein aggregation or amyloid formation. It is now important to combine and analyze all of the findings on polyamine-induced aggregation, come to a conclusion, and relate the phenomenon of this protein aggregation to the physiology of the cellular function. Through this review, we had tried to cover almost all the investigations that had been done to-date, to explore the roles of polyamines in aggregation of various proteins. We have also incorporated future research avenues that might be of interest to many cellular biologist and protein chemists.

Keywords: Polyamine; Protein aggregation; Alpha-synuclein; Tubulin; Glucose dehydrogenase.

Introduction

The polyamines, spermidine (spd) and spermine (Spm) and their precursor putrescine (Put) are ubiquitous low molecular weight aliphatic polycations with multiple cellular functions. The polyamine content in the cell is regulated through the complex control of synthesis, catabolism, uptake and excretion. The complete details of polyamines, and their metabolism, have earlier been reviewed by Pegg and McCann (1982), Janne and colleagues (1991), Tabor and Tabor (1984), Pegg *et al.* (1982), Seiler (1990), Seiler and Heby (1998). Polyamines are essential for normal cell growth (Cohen, 1998). Their uptake and biosynthesis gets stimulated during cellular responses to proliferative stimuli (Kakinuma *et al.*, 1988). Studies by the Merrell group and others, using specific ODC inhibitors (Mammont *et al.*, 1978; Prakash *et al.*, 1978; Newton *et al.*, 1977) or genetic approaches (Steglich *et al.*, 1982) to

manipulate levels of endogenous polyamines, confirmed that amines derived from ornithine are essential for mammalian cell viability, and high levels are necessary for optimal mammalian cell growth. On the other hand, when polyamine contents are increased, there is an induction of the degradation and excretion systems, accompanied by inhibition of polyamine uptake and biosynthesis.

The cationic nature of the polyamines at physiological pH enables them to interact with essential molecules such as nucleic acids, phospholipids and certain proteins, which accounts for many of their physiological functions (Blagbrough *et al.*, 1997). For instance, they modulate protein synthesis at several different levels including acceleration of protein synthesis, assembly of 30S ribosomal subunits, and Ile-tRNA formation (Igarashi *et al.*, 1988). They also serve as intracellular messengers affecting the activity of G proteins, protein kinase C, and phospholipases (Eichberg *et al.*, 1981; Sechi *et al.*, 1978; Bueb *et al.*, 1992) etc. Polyamines also cause DNA condensation, and induce conformational transition in polynucleotides from the right

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handed B-form to the left handed Z-form (Thomas *et al.*, 1991; Bancroft *et al.*, 1994; Hasan *et al.*, 1995; Chica *et al.*, 2001). They have been found to act as promoters of programmed ribosomal frameshifting during translation. Changes in polyamine levels have been associated with ageing and various diseases such as Cancer, Pancreatitis, Parkinson's disease, Alzheimer's disease, Inflammation, etc (Minois *et al.*, 2011; Moinard *et al.*, 2005). Other than these functions, polyamines are also implicated in biology of protein aggregation and its modulation thereof. It is now important to combine and analyze all of the findings on polyamine-induced aggregation, come to a conclusion, and relate the phenomenon of protein aggregation to the physiology of the cellular function. The review article is meant to address these issues.

Polyamine Promotes Protein Aggregation

There are various factors such as temperature, pH, protein concentrations, etc. that alter the protein conformations and lead it to the process of aggregation (Fink, 1988; Carrio and Villaverde, 2002). Polyamines are sought to be the molecules that might prevent aggregation of proteins *in vivo*. However, their increased levels in various amyloidogenic diseases and many *in vitro* investigations have implicated their role as promoters of protein aggregation. There are various proteins like Alpha-synuclein, Tubulin, Spectrin, Fibrinogen etc. whose aggregation seem to alleviate in presence of polyamines. Many of these proteins on getting aggregated lead to pathological conditions. Also, in plants there are certain proteins, for example, Light harvesting complex, whose aggregation is affected by polyamines. All the aggregation behavior and mechanisms are explained below.

Alpha-Synuclein aggregation

Alpha-synuclein (α -Syn) is an abundant presynaptic protein, the aggregation of which is implicated as a causative factor in several neurodegenerative diseases, collectively called synucleopathies including Parkinson's disease (Tong *et al.*, 2010). A recent report has proposed the physiological function of α -Syn is to act as a cellular ferrireductase, responsible for reducing

iron (III) to bio available iron (II) (Davies *et al.*, 2004). Structurally α -Syn can be divided into three distinct domains: (i) the N-terminal amphipathic domain, which comprises the first 60 amino acids; (ii) the hydrophobic NAC (non-amyloid component) region, which spans residues 61–95; and (iii) the C-terminal domain spanning residues 96–140, which is negatively charged at neutral pH (Bisaglia *et al.*, 2009). It is natively unfolded in its monomeric state, but can adopt α -helical (Eliezer *et al.*, 2004) or α -sheet secondary structure (Serpell *et al.*, 2000; Glenner *et al.*, 2001) upon binding to the membrane or aggregation, respectively. α -Syn aggregation is greatly facilitated by metal ions (Uversky *et al.*, 2001b), dopamine (Lataweik *et al.*, 2010), double-stranded DNA (Hedge *et al.*, 2007), low pH values (Hoyer *et al.*, 2007; Hashimoto *et al.*, 1998), elevated temperature (Hashimoto *et al.*, 1998), and C-terminal truncation (Serpell *et al.*, 2000).

Almost a decade back using Circular dichroism and fluorometric thioflavin T kinetic studies, biogenic polyamines were also demonstrated to accelerate the aggregation and fibrillization of α -Syn (Antony *et al.*, 2003). Various other experimental approaches have also been undertaken to clearly understand the mechanism of polyamine and α -Syn interaction. Characterization of complexes of natural and synthetic polyamines with α -Syn by NMR has shown that polyamines increase the extent of nucleation by ~ 10 and the rate of monomer addition ~ 40 -fold. A recent study underlines the role of polyamine induced α -Syn aggregation in the pathogenesis of PD. Using functional MRI, differentially affected brainstem regions were identified, and gene expression analysis of these regions revealed disease-related decrease in the polyamine catabolic enzyme SSAT leading to imbalanced high polyamine levels and hence enhanced α -Syn toxicity (Lewandowski *et al.*, 2010). Fink and associates observed that not only polyamines, but also various polycations like polylysine, polyarginine, and polyethyleneimine also modulate α -Syn aggregation (Goers *et al.*, 2003). Moreover, it was observed that the extent of accelerating aggregation effect depends on the nature of the polymer, its length, and concentration.

Alpha-synuclein aggregation process appears to be branched; with one pathway leading to fibrils and another to oligomeric intermediates that ultimately forms amorphous deposits (Uversky *et al.*, 2009). Previously, it was reported that in the presence of metal ions, the kinetics of α -Syn fibrillation was consistent with a nucleation-dependent mechanism (Wood *et al.*, 1999; Conway *et al.*, 2000), with the critical early stage of the structural transformation of the protein involving a partially folded intermediate (Uversky *et al.*, 2001a). However, the precise mechanism of how polyamines promote the aggregation of α -Syn is not well understood. To date at least three possible mechanisms of polyamine induced α -syn aggregation have been suggested. The first model articulate that spermine binding reduces the net charge of α -Syn (from -10 to -6) and the concomitant conformational change reduces the size of the protein by a factor 2 leading to a highly compact structure (Grabenaus *et al.*, 2008). The increase in proportion of such compact conformations induces faster aggregation. On the other hand, the NMR analysis and molecular dynamic simulations suggests that polyamine interacts with α -Syn's polyamine binding site at the acidic C-terminal region comprising amino acids 109-140 (Fernandez *et al.*, 2004) leading to formation of more extended conformation and release of long-range contacts between the C-terminal region and NAC and the N-terminal parts of the α -Syn molecule (Bertoncini *et al.*, 2005). The third model has been proposed by utilizing a Novel experimental approach that directly probes interactions between aggregation-prone (misfolded) states of proteins (McAllister *et al.*, 2005; Malkowa *et al.*, 2008; Lyubchenko *et al.*, 2010). According to this model, presence of spermidine at concentrations comparable to physiological ones affects the very first steps of α -Syn assembly, i.e, induction of conformational changes in α -Syn producing a misfolded state of the α -Syn monomer with a higher propensity for self-assembly (Krasnoslobodtey *et al.*, 2010).

It is now clear that polyamines play an important role in α -Syn aggregation, which is a causative factor for many synucleiopathies. Reduction of polyamine levels inside affected areas of brain to an extent that reduce α -Syn

aggregation could be considered as a treatment for these diseases. This could be achieved by certain compounds that could reduce the levels of polyamines by either elevating polyamine catabolism or inhibiting polyamines synthesis so that α -Syn aggregation is effectively reduced. But, on a cellular level it has to be made sure that decreased polyamines level are maintained inside the cell, as cell uptakes polyamines from the external medium very rapidly.

Microtubule Assembly and Tubulin Aggregation

Microtubules (MT) are cylindrical hollow polymers composed of tubulins or α -tubulin/ β -tubulin heterodimer (Desai and mitchison, 1997), which are highly negatively charged (20-30e-) (Priel *et al.*, 2004; Sacket *et al.*, 1985; Tuszyński *et al.*, 2005), at both of its C-terminal ends. These tubulin dimers polymerize end to end in protofilaments, which are the building block for the microtubule structure. MTs are implicated in major organizational tasks in eukaryotic cells, which include organizing the cytoplasm, polarity establishment, morphology and cell division. Nakano and his co-workers in 1982 had shown that polyamines induce the disassembly of reconstituted microtubules in vitro (Iwata *et al.*, 1982). Thereafter, a large number of studies have indicated that polycations, including biogenic polyamines, promote the assembly or aggregation of tubulin (Anderson *et al.*, 1985; Dowden and Shay, 1981). In agreement to these observations, studies on many cell lines - CHO (Pohjanpelto *et al.*, 1981), HeLa and epithelial NRK cells (Savarin *et al.*, 2010) - had shown that absence of polyamines in these cells resulted in disruption of MT network. All these evidences also indicate that polyamines at physiological concentrations, were necessary for promoting tubulin aggregation and hence microtubule assembly. The mechanism of polyamine induced aggregation of tubulin is proposed to be due to the sharing (bridging) of polyamines between the highly negatively charged C-terminal ends of tubulin leading to an attraction between two tubulin heterodimers (Mechulam *et al.*, 2009). This would, therefore, increase the lifetime of encounter between an incoming tubulin, and the growing nucleus, that would enable adopting the correct orientation by facilitated diffusion. In a

recent advancement, spermine near its physiologic ionic strength does promote the bundling of microtubules while preserving MT dynamics (Loic *et al.*, 2011). This MT-polyamine relationship opens up many therapeutic potentialities. Since, it is known that impairment of microtubule system could be detrimental to the cancer cells (Jun and Paraskevi, 2005; Jordan and Wilson, 2004; Paula *et al.*, 2003), and can lead to increase alpha-synuclein aggregation and toxicity (Raquel *et al.*, 2009). It would, therefore, be interesting to see the effect of MT and polyamine targeting drugs for treating these pathological conditions. Such drugs have already been proposed as an interesting combination therapy to induce apoptosis of breast cancer cells (Nair *et al.*, 2007).

Spectrin Aggregation

Spectrin, a major rod-shaped membrane skeletal protein is one of those proteins whose aggregate formation in nature has a physiological significance. Spectrin consists of intertwined alpha and beta chains that form heterodimers. These heterodimers then associate to form head to head tetramers and higher oligomers (Shotton *et al.*, 1979; Goodman *et al.*, 1998). The preferred state of spectrin aggregation in the skeletal network is tetrameric, and it can be reversibly transformed to dimeric and tetrameric forms on the membrane (Farmer *et al.*, 1985). The aggregation of spectrin is known to affect the stability of human erythrocyte membrane-skeletal network (Ipsaro *et al.*, 2010). Polyamines being cationic in nature at physiological pH were expected to interact with spectrin, as it is rich in electronegative charges. Shindler and his co-workers explored that addition of Polyamines increases the aggregation of spectrin (Schindler *et al.*, 1980). Using electron spin resonance studies, in erythrocytes also, spermine was reported to induce spectrin aggregation; which lead to reduction in the ability of transmembrane glycoproteins to diffuse in bilayer, and decrease membrane deformability by stabilization of the skeleton network (Wyse and Butterfield, 1988).

LHC II Aggregation

The light-harvesting complex of plants is an array of protein and chlorophyll molecules embedded

in the thylakoid membrane which transfer light energy to one chlorophyll a molecule at the reaction center of a photosystem. In addition, the plant light harvesting complex of Photosystem II (LHC II) also plays photoprotective role by increasing thermal dissipation of excess absorbed light energy in the photosystem, in the phenomenon referred to as non-photochemical quenching (NPQ) (Horton *et al.*, 1994). It is believed that NPQ is controlled by the structural organisation of LHCII. Xanthophyll cycle, i.e, the conversion of the violaxanthin into zeaxanthin alters LHCII aggregation, and hence, allows adoption of the quenched state being a key feature of the LHCII model for high energy quenching (Ruban *et al.*, 1994; Peter *et al.*, 2005). Interestingly, all the three polyamines (putrescine, spermidine, spermine) are found in plants LHC II (Navakoudis *et al.*, 2007) and are already known to cause significant alterations in the secondary structure of PS II (Bograh *et al.*, 1997). It was also observed in one interesting study that polyamines stimulated quenching in LHC II (Tsiavos *et al.*, 2012). However, the observed quenching becomes larger as the cationic charge of the amine increases (Put < Spd < Spm). Furthermore, spermine treatment also induces aggregation of LHC II. Therefore, it seems likely that aggregation might be induced by either nonspecific electrostatic interactions or there is more specific binding of Spm and Spd to LHC II. The same research group using Raman spectra also showed that polyamine induced changes at LHC II mimics to a great extent the effect of protons (i.e. acidification of the suspension). Similar to polyamines, LHC II aggregation can also be induced by addition of mono-bi-trivalent cations, lowering the pH, removing the detergent, adding zeaxanthin, increasing pressure and crystallization.

Fibrinogen Aggregation

Fibrinogen and fibronectin are glycoproteins present in plasma and extracellular matrix, respectively. They have previously been identified as major constituents of heparin-precipitable fraction of plasma (Fyrand and Solum, 1976; Stathakis *et al.*, 1977). Fibrinogen plays a major role in blood clotting (Blomback *et al.*, 1978), whereas fibronectin is involved in cell adhesion, growth, migration, differentiation, etc.

(Pankov and Yamada, 2002). Almost three decades back, aggregation of these two proteins from human plasma by utilization of polyamines was reported (Matti *et al.*, 1980). But the aggregation-inducing capacity of polyamines was relatively weak, as the ionic strength of the incubation buffer had to be reduced considerably under iso-osmotic level for significant aggregation to take place. The aggregation inducing capacity of polyamines was found to increase with increasing chain length. Spermine was most effective aggregation inducer, while putrescine did not lead to aggregation in the incubation conditions used. It was pointed out that aggregation of plasma and serum proteins by polyamines is based on electrostatic interactions. In another study, it was seen that polyamines, at a concentration of 1 - 5 mM and at a low ionic strength, induced a rapid, extensive polymerization of fibronectin into filamentous structures (Vuento *et al.*, 1980). Similarly to the earlier report, polyamine polymerization inducing effect was found to increase in the order putrescine < spermidine < spermine (Vuento *et al.*, 1980).

Polyglutamine Protein Aggregation

Polyglutamine-repeat diseases result from the expression of mutant proteins containing an expanded polyglutamine domain (Feigin and Zgaljardic, 2002; Zoghbi and Botas, 2002). In a very systematic study carried out to understand the pathophysiology of polyglutamine-repeat diseases using a stable neuronal cell line, spermine synthesis was seen to be alleviated in cells expressing pathological-length polyglutamine (poly Q) proteins (Colton *et al.*, 2004). On further analysis, activation of the polyamine synthetic pathway was found to be associated with increased aggregation of polyQ proteins in situ and promotion of pathological-length polyQ protein aggregation in vitro. In addition, the specific suicide inhibitor of ODC, DFMO (Weeks *et al.*, 1982), was found to reduce the level of SSAT mRNA, augment cellular aggregates and increase cell death in cells expressing non-pathological-length polyglutamine proteins. Based on these observations, a novel mechanism was proposed that the pathological length polyQ proteins

promote their own aggregation and cell death by increasing the polyamine production pathway.

Alpha-Chymotrypsin

α -Chymotrypsin, a serine protease is a three-chain protein connected by five inter- and intra-chain disulfide bonds. This protein is folded into two anti-parallel β -barrel domains consisting of a Greek key motif followed by an anti-parallel hairpin motif. Only two short lengths of α -helix are present in α -chymotrypsin, one in each domain (Wright, 1973). α -chymotrypsin was reported to be driven toward amyloid aggregation by addition of 2,2,2-trifluoroethanol (TFE) (Pallares *et al.*, 2004; Ghaleh *et al.*, 2007), and thermal stress (Ghaleh *et al.*, 2008). TFE is a solvent known to stabilize partially folded proteins and to promote amyloid formation in several models (Srisailam *et al.*, 2003; Chiti *et al.*, 2000). Surprisingly, polyamines displayed promoting effect on TFE-induced formation of non-native α helices in α -chymotrypsin and hence in amyloid formation (Ghaleha *et al.*, 2008). The authors argued that these observations might be due to the kosmotropic properties of Polyamines. Interestingly, amyloid formation was not observed in case when polyamines were added in the thermal-induced aggregation of α -chymotrypsin. Never-the-less, polyamines imparts almost opposite effects on aggregation of the same protein in the presence of different aggregation inducing agents.

Polyamines Suppress Protein Aggregation

Polyamines also have a role to play in disaggregation. It is believed that polyamine can be used as an additive in solution to avoid thermal aggregation of proteins. However, research in this direction has been confined to very selective proteins.

Glutamate Dehydrogenase Aggregation

Glutamate dehydrogenase is an allosteric mitochondrial enzyme that catalyzes the reversible NAD(P)⁺ linked oxidative deamination of L-glutamate into α -ketoglutarate and ammonia (Grabowska *et al.*, 2011). Aggregation plays an important role in the allosteric control of Glutamate dehydrogenase by allowing the

allosteric transition of the enzyme from inactive to active form to occur (Cohen *et al.*, 1975). Sabbaghiana *et al.* (2011) recently pointed out that putrescine and spermidine suppressed the thermal aggregation of Glutamate dehydrogenase in a concentration dependent manner (Marjan *et al.*, 2011). Interestingly, both polyamines also afforded protection in relation to retention of enzymatic activity, while spermidine was found more effective in protecting secondary structural changes. Using docking, Gorgani and his associates suggested that putrescine interact with Glutamate dehydrogenase by binding at positions in the vicinity of the ADP binding position, which is in accordance with their capacity to control aggregation.

Lysozyme Aggregation

Lysozyme, an abundant bacteriocidal protein found in human secretory fluids is extensively used as a model protein for several studies (Cisani *et al.*, 1984; Singh and Singh 2004). Certain naturally occurring human lysozyme variants are also known to form amyloid fibrils. In search for additives that can reduce the heat-induced aggregation; Takagi and his co-workers reported suppression of thermal aggregation and inactivation of lysozyme by spermine and spermidine (Koudou *et al.*, 2003; Okanojo *et al.*, 2004; Kentaro *et al.*, 2003). It was proposed that polyamines prevent aggregation by formation of ion pairs with local negative charges which effectively increase the net charge of the protein, leading to increased electrostatic repulsion and reduction of intermolecular interactions, including disulphide exchanges. The indispensable feature in the structure of polyamines for their function as an aggregation suppressor is the presence of multiple amines (Okanojo *et al.*, 2005). Although, Polyamine instigates a marked increase in solubilization of aggregation-prone molecules but they also bring about slight destabilization of native structure of lysozyme (Kudou *et al.*, 2003).

Future Perspectives

It is known that polyamines are basic requirement not only for regulating cellular function but also maintaining macromolecules structure and

function. Higher level of polyamines in the cell therefore, may result in formation of toxic aggregates or amyloids to many specific proteins. Importantly, polyamine-induced aggregation of proteins is not a generalized phenomenon but confined to many specific proteins. Protein specific aggregation by polyamines implicates that most proteins might have evolved against the polyamine selection pressure and therefore, are not observed to be aggregated. The aggregated protein seen might be examples of proteins that were not evolved with polyamines. Future research on this dimension might unveil many important biological functions. Never-the-less the observed aggregation phenomenon due to polyamine levels implicates the role of polyamines in many patho-biology of proteins and hence the diseases thereof. To come to these conclusions, it is important to investigate the in vivo insights that explain the physiological significances of the polyamine-induced aggregates and its involvement in proper protein folding.

Nevertheless polyamines can also suppress or inhibit aggregate formation suggesting their efficacies against many amyloidogenic protein aggregates that lead to neurological diseases should be investigated. Less toxic polyamine compounds that are now available in the market might be good therapeutics for many of such amyloidosis.

Abbreviations

Put, Putrescine; Spd, spermidine; Spm, Spermine; α -Syn, Alpha-Synuclein; NMR, Nuclear Magnetic Resonance; MT, Microtubule; NPQ, Non-Photochemical Quenching; NADP, Nicotinamide adenine dinucleotide phosphate; ADP, Adenine dinucleotide Phosphate;

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