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Review

Interactions of natural polyamines with mammalian proteins

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Abstract

The ubiquitously expressed natural polyamines putrescine, spermidine, and spermine are small, flexible cationic compounds that exert pleiotropic actions on various regulatory systems and, accordingly, are essentially involved in diverse life functions. These roles of polyamines result from their capability to interact with negatively charged regions of all major classes of biomolecules, which might act in response by changing their structures and functions. The present review deals with polyamine-protein interactions, thereby focusing on mammalian proteins. We discuss the various modes in which polyamines can interact with proteins, describe major types of affected functions illustrated by representative examples of involved proteins, and support information with respective structural evidence from elucidated three-dimensional structures. A specific focus is put on polyamine interactions at protein surfaces that can modulate the aggregation of proteins to organized structural networks as well as to toxic aggregates and, moreover, can play a role in important transient protein-protein interactions.

Keywords: aggregation; cytochrome P450; ion channels; polyamines; protein kinases; protein-protein interactions; redox chains.

Introduction

The major mammalian polyamines putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_4NH(CH_2)_3NH_2$], and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] are small, flexible cationic compounds (Figure 1) that are ubiquitously expressed and essentially involved in function and dysfunction of living cells. Tuned by a complex system of biosynthesis, interconversion, metabolism, uptake, and efflux, polyamines display rapidly changing levels that are especially high in proliferative cells, reaching millimolar concentrations (1–7). As polyamines bear (almost) fully protonated amino groups at physiological pH (Figure 1) (8), they are capable of binding to negatively charged regions of natural macromolecules, such as nucleic acids and proteins and also to smaller molecules, such as phospholipids or nucleotides, thereby massively affecting their structures and functions (9, 10). Accordingly, polyamines were found to modulate numerous regulatory processes in model systems that range from cellular signaling to the control of gene expression and translation and offer explanations for the obvious involvement of polyamines in the regulation of cell growth, differentiation, and death [reviewed in (1, 11–14)]. The broad actions of polyamines on essential life functions are reflected by their effects on global gene expression. A recent study in a yeast mutant, an eukaryotic system comprising a three times smaller genome than mammalians, showed a significant regulation of some 10% of the genome in (direct or indirect) response to spermidine or spermine (15). The essential requirement for polyamines and balanced levels of polyamines is underlined from studying knockouts that lack distinct genes involved in polyamine synthesis and are not viable (10).

In a seminal paper on polyamines in 1984, Tabor and Tabor (11) stated that 'Their specific function is still obscure, but their ubiquitous distribution, their high concentration in cells, and the increase in the concentrations found in rapidly growing tissues have stimulated many investigations on these compounds.' Today, 27 years later and after countless studies on all aspects of polyamine actions throughout all kingdoms of life, details on the mechanisms by which these actions are carried out and even on their principal targets are still far from being understood.

In particular, there exists little information on which impact polyamine interactions with proteins might have on the function of cellular and supracellular structures. Most reviews on polyamines focus on nucleic acids as their primary targets based on studies that had shown a preferential distribution to these sites. As a plausible distribution to protein sites had not been detected, proteins are commonly considered minor targets (see next section) (16–18). In the present paper, we challenge this opinion by critically reviewing (although disperse) findings on polyamine-protein interactions, focusing on mammalian proteins and – wherever possible – on structural evidence from three-dimensional (3D) analyses. Based on this information, we discuss the various modes in which polyamines can interact with proteins, describe some representative examples, and speculate on their relevance in the living cell. A particular aim of this review is to attract the attention of the reader to the potential that polyamines might have in modulating important protein-protein interactions and thereby cellular (signaling) networks.

Targets of polyamine actions

Potential targets of polyamines identified with isolated cellular (or extracellular) components are difficult to validate *in*

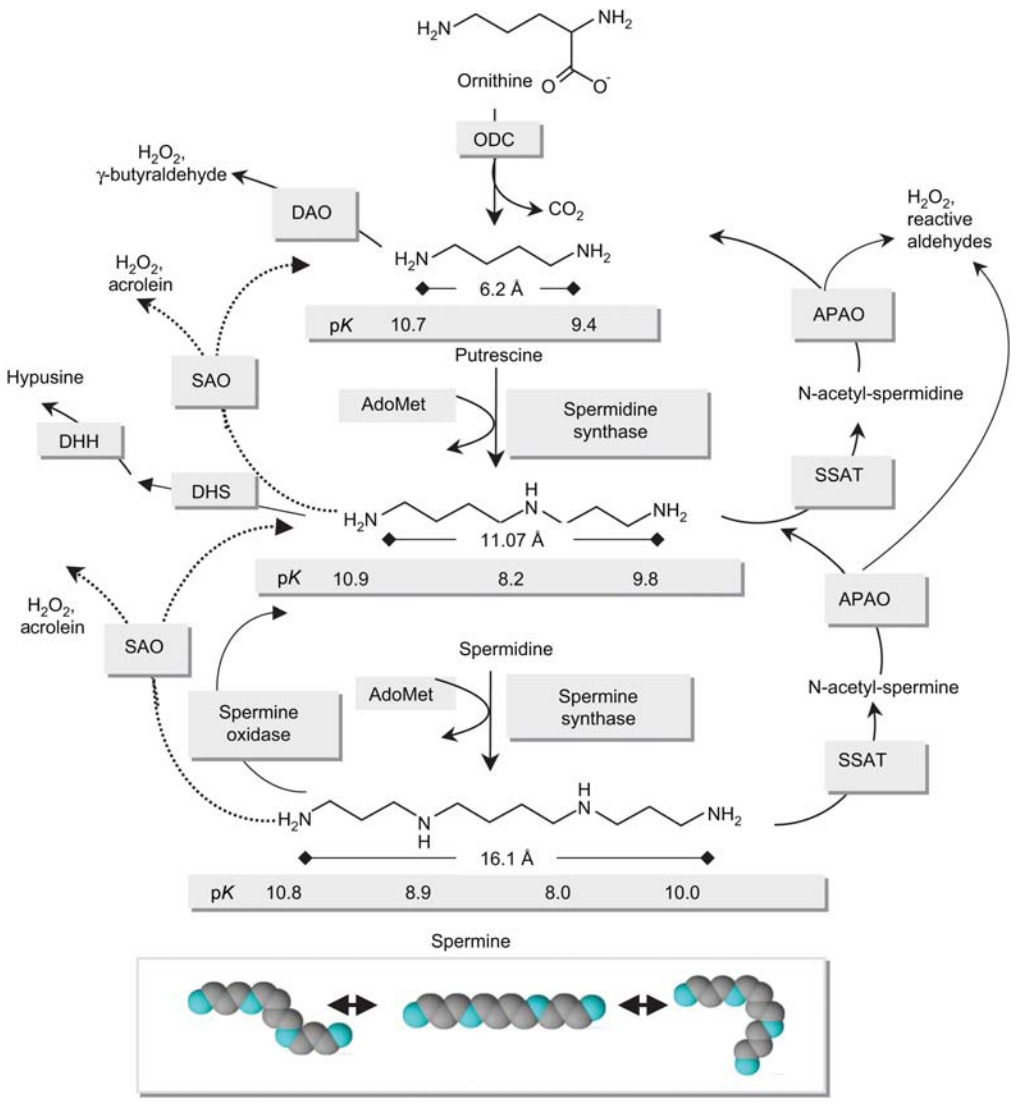

Figure 1 The major mammalian polyamines putrescine, spermidine, and spermine.
A simplified scheme of their biosynthesis, interconversion, metabolism, major products, and the involved enzymes (dotted arrow: low activity in humans). ODC, ornithine decarboxylase; spermidine synthase, spermine synthase; SSAT, spermidine/spermine-N^1-acetyltransferase; APAO, acetylpolyamine oxidase; DAO, diamine oxidase; SAO, serum amine oxidase; DHS, deoxyhypusine synthase; DHH, deoxyhypusine hydroxylase. p*K*a values of amino groups are taken from (8). The ACD/3D Viewer software (www.acdlabs.com) was used for determining distances between terminal nitrogens in the linear molecules and illustrating the flexibility of spermine by depicting three of its almost 'infinite' conformations.

vivo because: (i) many of the reported interactions with polyamines are relatively weak and other inorganic and organic cations present in excess over polyamines might effectively compete for a supposed target; and (ii) as a host of additional targets exists, polyamines rapidly redistribute when cells are ruptured for analytical procedures. Nevertheless, attempts have been made to assess the intracellular distribution of polyamines.

Watanabe et al. (17) chose an indirect method based on the cellular concentration of major classes of acidic (macro)molecules and on the binding constants of polyamines to these classes, which they had determined for each class (using two types of DNA and RNA, rat liver microsomes as predictive sample for phospholipids, and ATP). They estimated that binding of spermine and spermidine in rat liver would be mostly to RNA (up to 85% of total polyamine) and significantly less to DNA (5.7% of total polyamine), phospholipids, and ATP (4.5% and 2.3% of total polyamine, respectively). The authors stated that cytosolic proteins would not substantially interact with polyamines without giving further evidence or considering a potential binding to acidic proteins. This reference is still found in recent publications (16).

Using fast-freezing fixation techniques and specific anti-polyamine antibodies, polyamines were immunolocalized in HeLa cells at the electron microscopic level (18). This study localized the major part of intracellular polyamines in the nucleus (63%) with a six times higher concentration in the

dense chromatin than in the active chromatin. In the cytosol, polyamines were mainly associated with ribosomes; some binding was observed with the plasma membrane.

The preferential distribution to DNA and RNA forms in living cells corroborated the suggested role of polyamines in the maintenance of the chromatin conformation as well as in many aspects of gene expression and translation. However, there is still little information on the relevance of polyamine interactions with proteins in intact cells. As even acidic proteins exhibit a far lower density of electronegative charges than nucleic acids with their phosphate backbones, polyamine interactions with proteins possibly have been below the detection threshold in distribution studies.

How do polyamines interact with partnering molecules?

Polyamines are not just analogous to 'simple' monovalent and divalent inorganic cations as they interact not only by electrostatic forces but also involve hydrogen bonding and hydrophobic interactions from their hydrocarbon parts. A most peculiar property of polyamines is the overall flexibility of their linear alkyl chains that enables them to adapt to a wide variety of partner molecules in a more or less specific way. Bound to multiple subsites of one partner or even of neighboring partners, polyamine conformations can vary from the totally extended form, in which, e.g., spermine can span some 16 Å between N^1 and N^{12}, and bended forms with distances between N^1 and N^{12} reduced to a few Å (Figure 1). With regard to DNA as a central target, polyamines bind to the major and minor grooves of the double helix (mainly via interactions with N^7 of purines and O^2 of thymine) and in parallel with the backbone phosphate groups (19). There, the 3- and 4-valent polyamines can act like clamps that hold together distant sites of the same strand or bridge between the two strands of the double helix, causing conformational changes and condensation of DNA (in its isolated and also in its chromatin form); the condensed form protects against damaging agents, however, also affects the accessibility of target sequences, e.g., for transcription factors (20–22). Binding of polyamines to DNA in the nucleus has been shown to be preferentially to the condensed, transcriptionally inactive chromatin (18).

Considering interactions with proteins, polyamines can be accommodated in binding pockets serving as substrates, e.g., of enzymes involved in polyamine synthesis and metabolism, or as modulators of receptors, ion channels, transporters, and others. Moreover, polyamines can bind to negatively charged patches on the protein surfaces, neutralizing ('masking') these sites locally. Interactions can involve acidic groups on the same patch, but also more distant groups on the same protein or on a different one ('bridging'). Bridging can lead to conformational changes (collapse, size reduction, etc.). By masking and bridging sites at protein surfaces, polyamines can induce (reversible) protein aggregation. In this context, we want to point to an important role that polyamines might play as modulators of protein-protein interactions when bound to sites at protein interfaces (23).

In addition to reversible interactions, polyamines can be covalently linked to surfaces by transglutaminase action (thereby adding positive charge to the modified proteins that possibly alters their function) and sequentially undergo crosslinking to other proteins.

Figure 2 summarizes the repertoire of modes by which polyamines can interact with proteins; it will be discussed in detail in the following sections, illustrating individual types of polyamine interactions by representative examples.

Specific binding in protein pockets

Substrate binding

The manner in which polyamines bind to various enzymes involved in their biosynthesis and metabolism (depicted in Figure 1) has been elucidated from crystal structure analyses. Specific accommodation is achieved by the geometry of the substrate pockets and their lining with subsites capable of interacting with different parts of the polyamine molecules. Accordingly, polyamine binding is stabilized by a combin-

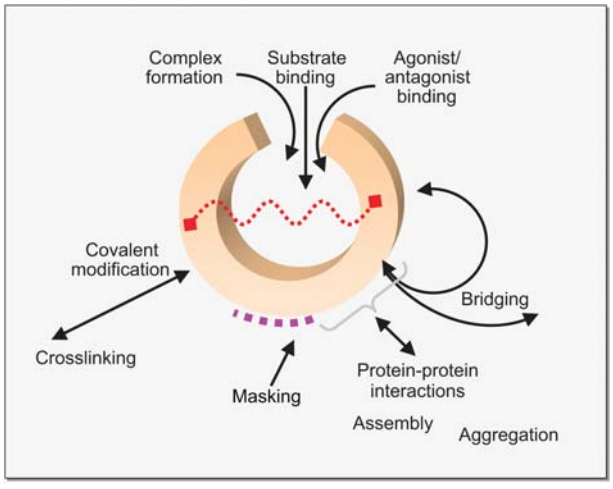

Figure 2 The various modes of polyamine interactions with proteins.
Polyamines can be recognized as substrates or modulators in binding pockets of respective proteins (enzymes, receptors, ion channels, etc.). In addition, polyamines associate with negatively charged patches on protein surfaces (Figure 4), neutralizing ('masking') these locally (purple dots). Interactions could involve acidic groups on the same patch, but also more distant sites on the same protein or on a different one ('bridging'). By masking and bridging sites on protein surfaces, polyamines can induce (reversible) protein aggregation and, particularly, at protein interfaces, polyamines might substantially affect protein-protein interactions. In addition to reversible interactions, polyamines can be covalently linked to surfaces by transglutaminase action and sequentially undergo crosslinking (Figure 3). Polyamine binding can lead to conformational changes (collapse, size reduction, etc., indicated by a dotted red line).

ation of dispersion forces, hydrophobic contacts, hydrogen bonding, and ionic interactions.

Spermidine and spermine synthase Spermidine and spermine are produced in sequential steps by two specific enzymes (Figure 1) that transfer aminopropyl groups from decarboxylated *S*-adenosylmethionine (dcAdoMet) first to putrescine (spermidine synthase) and thereafter to spermidine (spermine synthase). Crystal structures of both human enzymes as ternary complexes with dcAdoMet (or its product 5′-methylthioadenosine) and the respective amine revealed how the substrates are positioned in the active sites and gave insight into the mechanism of the reaction (24, 25). Both enzymes are homodimers with independent active sites in each subunit, however, requiring dimerization (involving the N-terminal domain) for catalysis. The similar active sites of both enzymes accommodate dcAdoMet in a negatively charged binding pocket linked to a narrow channel in which the amine substrate is located exposing the N^1-amino group towards the aminopropyl group (25). Appropriate positioning of the substrates for the aminopropyl transfer is achieved by interactions of their amino groups with acidic residues and additional hydrophobic interactions with the alkane parts (24, 25). Spermidine synthase differs from spermine synthase by its smaller substrate pocket that recognizes putrescine but is not able to accommodate the larger spermidine.

Spermidine/spermine N^1-acetyltransferase (SSAT) This highly inducible cytosolic enzyme plays a key role in reducing intracellular polyamine levels as acetylation of spermidine and spermine leads to products that are either secreted from cells or oxidized by N^1-acetyl-polyamine oxidase [reviewed in (26)]. The crystal structure of the human enzyme shows a dimer with two functionally different channels on opposite sites, each of them lined with residues from both dimers (27). In the ternary complex with polyamine and acetyl-CoA, both channels accommodate acetyl-CoA. The polyamine (analog) binds in a stretched conformation only in channel 1, interacting with five acidic residues (Asp, Glu) and interspersed hydrophobic residues. Thereby, the N^1 residue comes close to the pantetheine moiety of CoA from which a direct transfer of the acetyl group to the primary N^1 of the polyamines takes place (27). As revealed from the crystal structure of the highly homologous mouse SSAT, a tyrosine residue is positioned in such a way that it could serve as a catalytic acid and protonate the sulfur of CoA, and a glutamic acid abstracting a proton from N^1 as a general base (28).

Polyamine oxidases Polyamine biosynthesis is not a unidirectional process, but can be reversed by polyamine oxidases. The peroxisomal acetylpolyamine oxidase (APAO), a constitutively expressed flavoprotein, recognizes N^1-acetylspermine and N^1-acetyl-spermidine – but not spermine or spermidine – as substrates and cleaves them generating 3-acetamidopropanal, H_2O_2, and the smaller polyamines spermidine and putrescine, respectively (29). A different enzyme, the highly inducible spermine oxidase (SMO), also a flavoprotein, prefers spermine as substrate and produces spermidine, 3-aminopropanal, and H_2O_2 (30). Generation of H_2O_2 and aldehydes, particularly by SMO, results in cytotoxicity but also might be the mechanism by which polyamine analogs act on tumor cells in a beneficial way (31).

Both mammalian APAO and SMO have not yet been crystallized. Some crude information on the positioning of polyamines in these enzymes might be derived from the crystal structures of polyamine oxidase from Zea maize (mPAO) (32) and spermine oxidase (Fms1) from yeast (33). Although both enzymes show low sequence homology (approximately 20%), their active sites are at the interface of the FAD-binding domain and the substrate binding domain and spermine binds in a tunnel that is U-shaped and deep in mPAO and more shallow in Fms1. However, the mode of substrate binding differs substantially: whereas all amino groups of spermine are bound to mPAO through hydrogen bonds (N1-Glu170, N5-Tyr169, N10-Glu62, and N14-Tyr439) (32), only the N1 amino group interacts through two hydrogen bonds with Fms1 (to Asp94, Asn195) while the middle part of spermine is stabilized by hydrophobic interactions [W174-spermine (C6-9)-W65 sandwich] (33). The differing orientation of spermine towards the cofactor FAD results in oxidation at C11 by Fms1 to give spermidine and 3-aminopropanal (similar to mammalian SMO), but at C9 by mPAO producing 3-(aminopropyl) 4-aminobutyraldehyde and 1,3-diaminopropane (33).

A second class of polyamine oxidases containing tightly bound copper and 2,4,5-trihydroxyphenylalaninequinone (TPQ) as cofactor (abbreviated as Cu/TPQ-AO) catalyzes the oxidative deamination of primary amines to the corresponding aldehyde and H_2O_2. Diamine oxidase (DAO), a homodimeric glycoprotein found in a wide array of human tissues (highest activities in kidney, intestine, placenta, and various tumor tissues) has relatively broad substrate specificity. It recognizes histamine, putrescine, and cadaverine as the preferred substrates and exhibits much lower/negligible activities towards the longer polyamines (34, 35). Oxidation of putrescine is the terminal step in polyamine catabolism leading to γ-aminobutyraldehyde that can be converted to the neurotransmitter γ-aminobutyric acid (GABA) by a specific aldehyde dehydrogenase (35). In numerous tumor tissues, elevation of ornithine decarboxylase and thereby increased putrescine levels are accompanied by elevation of DAO resulting in high formation of GABA that might counteract proliferation (35).

Serum amine oxidase is a different Cu/TPQ-AO that is present in bovine serum (and in serum of several other mammals), but absent in human serum except during pregnancy and various pathological conditions (e.g., some types of cancer, diabetes, vascular disorders, and others). Oxidation of the primary amines of spermidine and spermine by the enzyme can generate cytotoxic species because serum (unlike cells) might not contain sufficient aldehyde dehydrogenase to convert the reactive aldehydes to the corresponding acids. These aldehydes can undergo spontaneous β-elimination to give acrolein and the respective shortened polyamine (Figure 1). A most recent, comprehensive review on

Cu/TPQ-AOs and the potential implications of their cytotoxic products in cancer treatment is given in (36).

Deoxyhypusine synthase (DHS) Hypusine is an unusual amino acid that has been exclusively detected in the eukaryotic initiation factor 5A (eIF5A) and proven to be indispensible in protein synthesis (37). Hypusine is formed by post-translational modification of eIF5A by two enzymes. DHS reduces spermidine in a NADH-dependent process, cleaves spermidine, and transfers the 4-aminobutyl moiety from a first intermediate at its Lys329 to the ε-amino group of Lys50 in an exposed loop at the N-terminal site of eIF5A. Subsequently, this covalent intermediate is hydroxylated by deoxyhypusine hydroxylase (38).

The crystal structure of the ternary complex DHS-nicotinamide adenine dinucleotide (NAD)-polyamine analog (N^1-guanyl-1,7-diaminoheptane) reveals a homotetramer that is a dimer of dimers and each dimer comprises two independent antiparallel active sites at its interface lined with residues from both monomers (39). NAD-binding is required to enable spermidine binding. Thereby, the geometry of the active site pocket is altered to form a narrow deep tunnel with negatively charged residues at its entrance and the bottom that perfectly anchor the primary amino groups of spermidine in its extended form (39). A further (highly conserved) Glu137 is in close contact to the C-4 of the nicotinamide moiety of NAD as well as to the position of the secondary amino group of spermidine, suggesting an important role of this residue in the catalytic reaction.

Binding as modulators

Complex formation A novel mode of polyamine-protein interactions was recently revealed in a carbonic anhydrase-spermine adduct (40). Carbonic anhydrases (CAs) are zinc enzymes that play an essential role in pH homeostasis as they catalyze the hydration of carbon dioxide to HCO_3^- and H^+. Spermine and spermidine were shown to be strong inhibitors of various human carbonic anhydrase (hCA) isoforms, in particular, of the transmembrane form hCA-IV (spermine: K_i 10 nM; spermidine: K_i 112 nM), the mitochondrial forms hCA-VA and VB (spermine: K_i 0.835 μM; spermidine: K_i 1.33 μM), and the dominant cytosolic form hCA-II (spermine: K_i 84 μM; spermidine: K_i 1.11 μM) (40).

The crystal structure of the cytosolic form hCA II localized spermine deeply in the active site adopting a coiled conformation and anchored by hydrogen bonding to the fourth (non-protein) ligand of zinc (a water molecule/hydroxy ion). A network of further hydrogen bonds and van der Waals interactions (to Thr199, Thr200, and Pro201) stabilizes the interaction (40).

Modulation of ion channel functions Endogenous compounds that block or stimulate conduction in ion channels trigger vital physiological processes, including neurotransmission, visual signal transduction, cardiac electrical signaling, fluid secretion in the kidney and gastrointestinal tract, and many more; respective structures and processes have served as important targets of multiple new therapeutics and vast literature exist on this topic (41, 42). At this time, more than 55 inherited 'channelopathies' have been identified, around 13% of all marketed drugs act on ion channels (42); many more respective compounds are in development. Polyamines might belong to the potent group of endogenous modulators, as they are capable of affecting several different types of voltage gated or ligand gated ion channels at physiological conditions, entering from the outside and/or inside of the plasma membrane. Of some of these channels or of their purified domains, crystallographic data allow a closer inspection of polyamine binding (that might likewise be representative for other channels).

Intracellular polyamines have been shown to modify inward rectifying channels including glutamate receptors (AMPA and kainate types) and inward rectifier K^+(Kir)-channels, extracellular polyamines affect the *N*-methyl-D-aspartate (NMDA) receptor (43). For the low concentrations required to modulate several channel functions, polyamine effects should also prevail under (patho)physiological conditions in the presence of competing monovalent and divalent cations.

K^+ Inward rectifying channels Kir channels comprise a gene family encoding seven subfamilies of pore-forming homotetrameric membrane proteins that selectively conduct K^+ and are present on excitable and non-excitable cells. Kir channels play important physiological roles, particularly in the control of the resting membrane potential and electrolyte balance, in the regulation of neuronal and cardiac electrical activity, and in pancreatic function (44). At a membrane potential that is negative to the potassium equilibrium potential, Kir channels pass K^+ through a highly specific pore in the inward direction, whereas little or no efflux takes place at the opposite driving force (45, 46). The flow direction is controlled by intracellular polyamines, especially spermine and spermidine, which enter the pore and occlude intracellular ion access at (sub)micromolar concentrations (46). Putrescine shows a weaker effect, comparable to that of Mg^{2+}. Crystal structure analyses of chicken Kir2.2. (90% homology to human Kir2) and of a microbial KirBac3.1 reveal molecular details of the transmembrane and intracellular parts of the channel, the (iris-like) structural reorganization during K^+ flux, and the nature and mechanism of polyamine binding (47, 48). Two distinct types of polyamine binding pockets were identified: a closed pocket at the intracellular interface of two subunits that first harbors polyamines and, after reorientation of the cytoplasmic sites, releases the polyamines to enter deep into the pathway of K^+ permeation and plug it (48). In both pockets, polyamines bind in extended form and interact with multiple sites exerting electrostatic as well as van der Waals interactions (48).

Ionotropic glutamate receptors Glutamate, the chemical excitatory transmitter in the central nervous system, activates three types of ionotropic channels named after their agonists: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) type receptors, the kainate type receptors, and the *N*-methyl-D-aspartate (NMDA) receptors. These

ligand gated ion channels function in their native forms as heterotetramers that have a common architecture: each subunit comprises a large extracellular amino terminal domain that is involved in receptor assembly, a ligand binding domain (LBD) that binds agonists and antagonists and activates gating, a transmembrane domain forming the ion channel, and a cytoplasmic C-terminal domain involved in receptor regulation (49, 50). The organization of these modules has been suggested from crystal structure analyses of isolated N-terminal domains and LBDs of different receptor subtypes, and it was recently proven upon solving the crystal structure of the full (homotetrameric) AMPA GluA2 receptor (49).

On activation by glutamate binding to the LBD, the transmembrane ion channel of the AMPA receptors (GluA1–4) opens, the kainate receptors (GluK1–5) additionally require monovalent cations and anions for activation (51). AMPA and kainate receptors are permeable to sodium, potassium, and calcium and within the transmembrane ion channel they possess binding sites for blocking by polyamines. A particular strong voltage-dependent block by spermine has been reported for GluK3-containing kainate receptors owing to its very strong binding (K_D around 40 nM), whereas spermine binding to GluK2 was 100-fold weaker (50, 51). Binding was mainly determined by a conserved Q590, a negatively charged E594, and several hydrophobic components (50).

NMDA receptors (GluN1, GluN2A-D, GluN3A-B) function in a more complex way. They consist of two GluN1 and GluN2 (or GluN3) subunits, require glutamate binding to GluN2 and glycine binding to GluN1 (or GluN3) for activation, and are modulated by various ligands including protons, Zn^{2+}, and spermine that bind to the extracellular regulatory domains of the subunits (52). Polyamines, particularly spermine, can increase receptor currents, but also block currents. Stimulation by spermine was shown to be due to binding to the extracellular regulatory domains; binding studies with the purified regulatory domains GluN1-R, GluN2B-R, and GluN2A-R revealed discrete binding sites of spermine with K_d values in the micromolar range (19, 33, and 140 μM, respectively) that are distinct from the sites at which the antagonist ifenprodil bound with high affinity (52). Binding sites of spermine that result in a voltage-dependent block of the channel were identified by mutation studies in the outer vestibule of the channel pore, partially overlapping with the binding site of extracellular Mg^{2+}(16, 53).

Other ion channels Other cation channels were found to respond to polyamines in an analogous way. The melastatins TRPM4 and TRPM5, members of the transient receptor potential (TRP) superfamily, were blocked by intracellular spermine (at 35–61 μM), as well as TRPM7, in which spermine also permeated the pore (54, 55). The homologous nociceptive receptor TRPV1 (from the vanilloid subfamily), activated by various stimuli such as heat, touch, low pH, extracellular cations, capsaicin, was also stimulated by low concentrations (5 μM) of spermine (and spermidine) that permeated the pore (54, 56).

Polyamines were further shown to block cGMP-gated channels that are non-selective cation channels and open upon binding of the nucleotide (57). Examples such as the olfactory channel (58) and the retinal channel (59) bound polyamines from both the extracellular and intracellular sites. In the retinal channel, extracellular spermine at 3 μM was a permeant blocker, whereas intracellular spermine blocked in a more complex way, i.e., a permeant and non-permeant mode (59).

Binding to transporters

Transport across membranes Owing to their multiple charges, polyamines are hydrophilic and cannot permeate membranes by passive diffusion, but require transporters. From functional studies at least 15 different polyamine transport systems into mammalian cells have been suggested; however, with the exception of a recently discovered cation-Cl^- cotransporter (CCC9a), their identities are unknown (60). CCC9a, a glycoprotein expressed on epithelial and non-polarized cells, transported spermidine slightly faster than spermine (K_m values of both in the low μM range) and putrescine much slower (60). In addition, it appears probable that cation channels such as TRPV1 and other TRP channels through which polyamines permeate (see above) might also contribute to polyamine transport into mammalian cells (56).

In contrast to mammalian cells, different polyamine transporters have been identified on microbial cells and their structures elucidated (61).

Transport in plasma For the relatively low plasma levels of polyamines [around 0.1 μM in healthy humans, up to 1 μM in pathophysiological states such as cancer (62)], binding studies to plasma proteins have not been a major issue until now. A recent study reveals significant binding of spermidine and spermine to serum albumin (K_d values around 6 and 3 μM, respectively), accompanied by conformational changes pointing to a partial unfolding of the protein (63); however, structural details on this binding are lacking. Albumin, the most abundant serum protein (around 600 μM) is the principal depot and transport vehicle in the circulation for a vast variety of endogenous and exogenous compounds. Within clefts of its subdomains, albumin comprises several distinct binding sites that allow for hydrophobic contacts as well as for hydrogen bonding and electrostatic interactions [for review see (64, 65)]. The affinity of polyamines for albumin matches or exceeds that of many drugs that are effective at comparably low plasma levels (≤ 1 μM) and show 'high' plasma protein binding (<90%) [listed in (66)]. Whether and which role albumin binding might have on the pharmacokinetic behavior of polyamines under physiological conditions – in the presence of numerous other ligands and an excess of inorganic cations – is still unexplored.

Interactions at protein surfaces

Polyamines are capable of interacting with protein surfaces in a reversible and irreversible manner (67, 68).

Inspection of published crystal structures reveals an irregular distribution of charged sites on protein surfaces, forming patches with several charged groups at shallow parts, at protruding domes or loops, and particularly accumulated at the tail of diverse proteins (69). The quantity, size, and distribution of negatively charged sites on protein surfaces reflect their capability of binding polyamines in a reversible way. In addition to multiple electrostatic interactions, H-bonding and hydrophobic contacts can contribute to specificity of binding, thus distinguishing polyamines from monovalent and divalent cationic competitors within cells and outside. For its extended size and flexibility, particularly spermine will allow a maximum of contacts on protein surfaces resulting in an efficient masking of negatively charged sites as well as a bridging between them – either on the same protein or between different proteins – and triggering conformational changes (Figure 2). Consequently, polyamines bound at protein surfaces might specifically affect the recognition of potential partners, thereby modulating important protein aggregation processes (e.g., in the formation of structural scaffolds) as well as transient protein-protein interactions in signaling pathways and in transferring metabolites, ions, and electrons.

Some representative examples of these reversible actions of polyamines will be given in the following sections.

Covalent binding to protein surfaces

Transglutaminases are a large family of enzymes that crosslink proteins by transferring ε-amino groups of lysines on one protein to the γ-carboxamide group of glutamine residues on a different protein [for review see (11, 70)] (Figure 3). Whereas transglutaminases show strict specificity for glutamines exposed on protein surfaces, they can also exploit small endogenous amines as substitutes for lysyl residues. Polyamines as substrates add positive charge to the (uncharged glutamine of) modified proteins and can thereby alter their function, illustrated, e.g., by the increased activity of polyaminated phospholipases A_2 (70) or the improved stability of polyaminated substance P against proteolysis (71). The remaining free amino group of the polyamine can then again undergo a further transglutaminase-catalyzed attach-

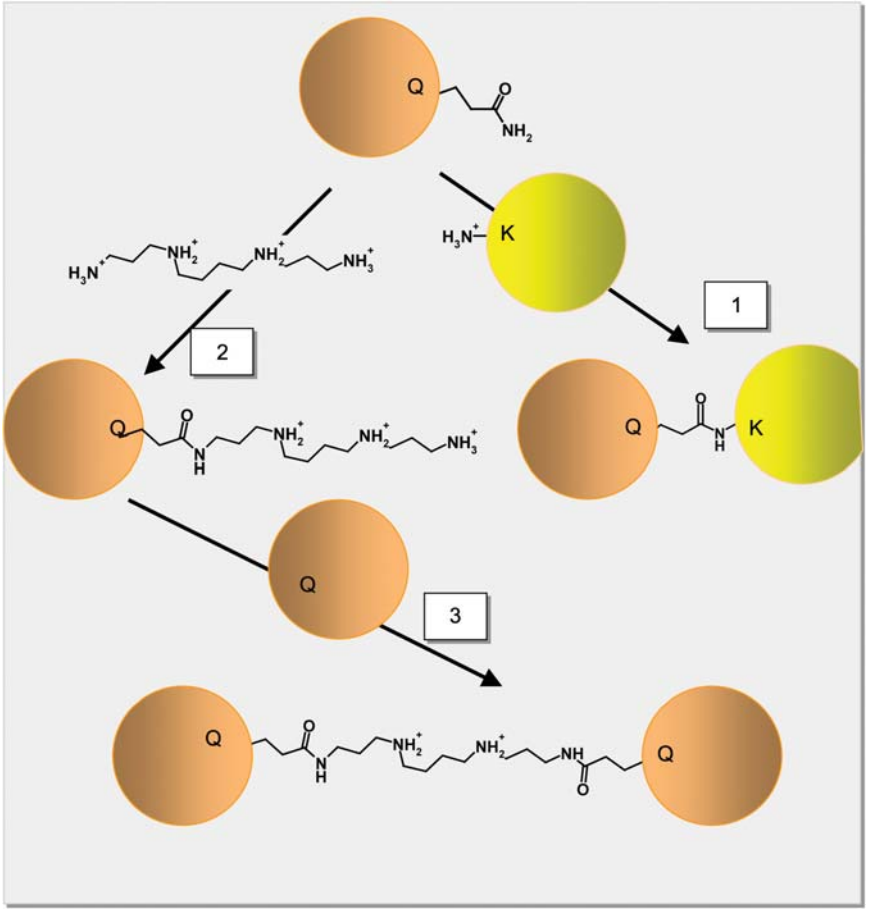

Figure 3 Covalent binding of polyamines on protein surfaces catalyzed by transglutaminases.
Transglutaminases crosslink proteins by transamidating the γ-carboxamide group of glutamine residues (Q) on one protein with ε-amino groups of lysines (K) on a different protein (1). Polyamines (here spermine) can substitute for lysines, first resulting in a covalently modified protein with increased positive charge (2); in a next step transamidation of a γ-carboxamide on a different protein leads to an aggregate with a flexible, charged linker (3).

ment to glutamines on other proteins, leading to different types of crosslinked proteins that are connected by a flexible bis-(γ-glutamyl)-polyamine bridge (68, 72).

Transglutaminases recognize a wide variety of proteins as substrates. So far, more than 150 substrates have been identified by functional proteomics, including proteins of the cellular and extracellular matrix, contractile proteins, proteins involved in signaling cascades, in energy metabolism, and many more (73, 74). These substrates reflect the involvement of transglutaminases in essential life functions, ranging from the architecture of the extracellular matrix and the extensive crosslinking of cytoskeletal proteins in the late stages of apoptosis to receptor mediated endocytosis, blood clotting, formation of cornified envelopes in epidermal differentiation, and a host of pathological consequences arising from dysfunctioning transglutaminases (74). Numerous polyaminated proteins have been identified in intact cells, making it plausible that transglutaminases can play a key role in distinct polyamine-associated functions. Owing to the limited space of the present review, we have to refrain from a broader description of these important post-transcriptional processes and their physiological and pathological consequences.

Polyamines and protein aggregation

Essential structures of the cytoskeleton such as actin filaments and microtubules result from the aggregation of distinct protein building blocks. Many normally soluble molecules can also undergo aggregation that – triggered by a conversion to misfolded β-sheet rich structures – eventually leads to insoluble 'toxic' aggregates. Prominent examples include amyloid β, α-synuclein, polyglutamine proteins, and prions that are associated with neurodegenerative diseases (e.g., Huntington's, Alzheimer's, Parkinson's and prion diseases). Detailed information on the 3D structure of some of these subunits and aggregates derived from crystal structure analyses, atomic force spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, etc., has given some insight into the association process and the role of modulators therein [3D structures of tubulin, actin, and α-synuclein (75–77)]. Polyamine binding to acidic patches of several such proteins has been found to accelerate the aggregation process as illustrated below by representative examples.

Spectrin and the cytoskeleton Spectrins are long flexible rods that are present in many different cell types as key components of the membrane skeleton, an intracellular polygonal network that plays a crucial role in cell membrane stability, flexibility, and function. In this network, spectrins are linked to short actin filaments and interact via adaptor proteins (ankyrin, protein 4.1) specifically with integral membrane proteins (ion channels, ion exchanger) (78, 79).

Starting from the observation that spermine prevented the lateral diffusion of transmembrane proteins in erythrocyte ghosts (80), subsequent electron paramagnetic resonance studies demonstrated a pronounced decrease in the segmental motion of spin-labeled cytoskeletal proteins (mainly spectrin and the cytoplasmic pole of the anion transporter band 3) indicating a strengthening of cytoskeletal protein-protein interactions by spermine (81–83). As spectrin is rich in electronegative charges, it was hypothesized that spermine might link spectrin to the also negatively charged band 3 protein by bridging. Crosslinking was supported by Triton X-100 extracted ghosts, which, in the presence of spermine retained abnormally high levels of band 3 and other proteins (82).

Crystal structure analyses of spectrin and of its complex with the adaptor protein ankyrin gave insights into the architecture of protein-protein recognition (84, 85) and the role of complementary charged residues therein that allows for speculation of the sites of spermine interference. Spectrin from erythroid membranes is a tetramer consisting of two large subunits α and β, each of them composed of multiple trihelical tandem repeats, which first form a heterodimer with high affinity followed by a head-to-head assembly of two antiparallel heterodimers. The crystal structure of the tetramerization domain complex regions near the N-terminus of α-spectrin (repeats 0–1) and C-terminus of β-spectrin (repeats 16–17) revealed an extended interface (1050 $Å^2$ per molecule) with a large number of mostly hydrophobic contacts (84). However, opposing surfaces showed complementarity of charged residues, acidic residues on the β-chain matched basic residues on the α-chain, suggesting an important role of these residues in correct positioning of the chains (84).

Structural analysis of a spectrin-ankyrin complex (comprising relevant fragments of β-spectrin and ankyrin) showed an interface of 630 $Å^2$ and again charge and shape complementarity (acidic charges on β-chain matching basic residues on the ankyrin fragment) resulting in a very tight complex (85).

Tubulin aggregation Tubulin, the structural subunit of microtubules, is an acidic heterodimer (α-tubulin/β-tubulin) with negative charges concentrated at both of its C-terminal ends (Figure 4). Neutralization of these negative charges, e.g., by chemical amidation of the carboxyl residues resulted in enhanced polymerization of tubulin (86). Likewise, masking the negative charges by polyamines, in particular by spermine and to a lesser extent spermidine (but not putrescine), was shown to greatly enhance the rate of microtubule formation in cell-free systems (independent of the presence of monovalent salts at levels comparable to intracellular ones) and in intact HeLa and epithelial NRK cells (87). Depletion of cellular polyamines reduced and disrupted the microtubular network (87); cleavage of the C-terminal end abrogated the accelerating effects of polyamines (88). Examining the role of polyamines in the assembly process, Mechulam et al. (88) proposed a model based on a sharing (bridging) of polyamines between the highly negatively charged C-terminal ends of tubulin dimers. The resulting attraction would increase the lifetime of encounter between an incoming tubulin and the growing nucleus and enable adopting the correct orientation by facilitated diffusion.

α-Synuclein aggregation α-Synuclein, a small 140 aa protein, is abundantly expressed in the central nervous system and can have various functions including regulation of

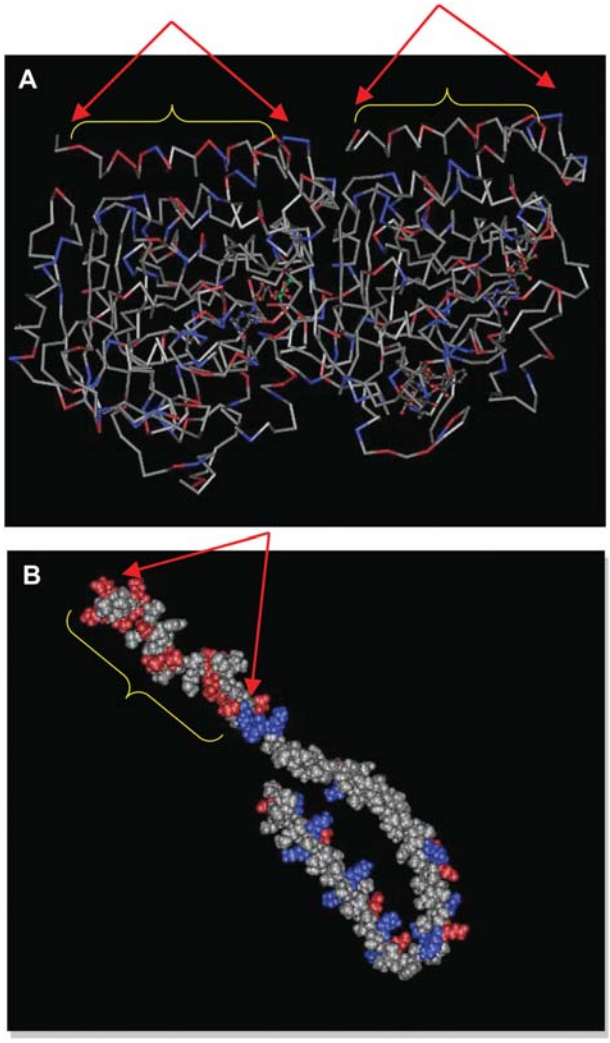

Figure 4 Crystal structures of tubulin and α-synuclein.
3D structures of the α/β-tubulin dimer [code 1TUB, (75)] (A) and the unfolded α-synuclein [code 1XQ8, (76)] (B). Acidic residues are colored in red, basic residues in blue; red arrows show the highly acidic C-terminal ends to which polyamines associate. The structures are derived from the Modeling DataBase (MMDB), also known as 'Entrez Structure', a freely accessible database of experimentally determined structures obtained from the Protein Data Bank (69).

lipid metabolism and dopamine synthesis and transport [for review see (89)]. Whereas native α-synuclein is soluble and unfolded, fibrillar aggregates are a hallmark in various neuropathies, in particular in Parkinson's disease (PD). In addition to other endogenous factors, polyamines are capable of massively accelerating the rate of α-synuclein aggregation and fibrillation (90, 91). Using NMR methods, moderate, however, specific binding of polyamines to a motif at the highly acidic C-terminal region (including 5 Asp and 10 Glu) was detected (K_d: spermine, 0.62 mM; spermidine, 3.3 mM; putrescine, 10 mM) (91). In parallel, perturbations were observed at a distant region indicative of initial steps to a β-sheet conformation that is characteristic for the fibrillar structure of α-synuclein. Binding of spermine reduced the net charge of the protein (from -10 to -6) and the concomitant conformational change reduced the size of the protein by a factor 2 leading to a highly compact structure (92).

A recent study underlines the role of polyamine induced α-synuclein aggregation in the pathogenesis of PD (93). Gene expression profiles of affected brainstem regions of patients revealed a disease-related reduction in the polyamine catabolic SSAT leading to imbalanced high polyamine levels. A causal link between decreased SSAT and neuropathology was shown *in vivo* in a transgenic mouse model that expressed human α-synuclein and developed PD inclusions: application of a SSAT activator (N^1,N^{11}-diethylnorspermine) reduced histopathology, application of a SSAT inhibitor (Berenil) worsened it.

Figure 4 displays the 3D structures of the α/β-tubulin dimer (75) and the unfolded α-synuclein (76), showing the highly acid C-terminal ends to which polyamines associate.

Polyamines and proteins in signaling pathways

Of the many pathways for which important involvement of polyamines has been reported or appears predictable, this review will focus on two facets only, namely the interaction with protein kinases, particularly with CK2, and blood coagulation.

Protein kinases Phosphorylation of proteins by a multitude of protein kinases (several hundred protein kinases are encoded in the human genome) is an essential mechanism in the broad signaling networks that regulate cell function and fate and overall physiology (94). Comprehensive tissue phosphoproteomics has identified thousands of non-redundant phosphorylation sites in a single tissue, suggesting that around one-third of eukaryotic proteins are phosphorylated (95). Polyamines interact with many of these kinases, resulting either in their activation (e.g., of CK2 or mitogen-activated kinases ERK1, ERK2) and stimulation of cell growth and malignant transformation or in counteracting activation [e.g., in calmodulin phosphorylation (9)]. As protein kinases are frequently dysregulated in proliferative diseases, they are gaining increasing importance as druggable therapeutic targets, particularly after the great success shown with the antitumor drugs Gleevec, Irressa, and Tarceva (96, 97).

The protein kinase CK2, an ubiquitous serine/threonine protein kinase, differs from other kinases by its relative unspecificity towards substrates, which are mainly characterized by an acidic cluster downstream to the phosphorylation site (98). A list of 308 substrates reported in 2003, including transcription factors, signaling proteins, effectors of DNA/RNA structure and function, structural proteins, metabolic enzymes, etc., certainly underestimates the real number (98). Many new CK2 substrates have been added, e.g., components of the steroid hormone cascade, capable of modulating the biosynthesis of steroid hormones (99, 100).

Various studies have demonstrated a strong activation of CK2 by polyamines at low micromolar concentrations (101–103) and crystal structures of CK2 revealed the sites of polyamine interactions (104, 105). The general architec-

ture of CK2 shows a tetramer consisting of two catalytic subunits CK2α – comprising the ATP-binding site and a substrate site – and two regulatory subunits CK2β, that are arranged to an α-β-β-α complex. Dimer formation between the regulatory CK2β subunits is mediated by a zinc finger motif, association of CK2β to CK2α by an activation domain at the C-terminus. The polyamine binding site was identified as a protruding, highly acidic stretch covering residues 55–64 on CK2β that is involved in the negative regulation of CK2. Neutralization of these negative charges by a polyamine (or another polybasic compound) as well as by mutation raised the basal activity of CK2 but abolished (further) effects of polyamines (94, 104).

Blood coagulation Reactions at protein surfaces play a pivotal role in autocatalytic zymogen activation and blood coagulation. The serine protease plasma hyaluronan binding protein (PHBP, also called factor VII activating protease: FSAP) is involved in coagulation as well as in fibrinolysis. PHBP circulates as a single chain proenzyme form (pro-PHBP – 'zymogen') and undergoes autoproteolytic cleavage to the active two chain form. This form consists of multiple domains including a highly acidic N-terminal region (NTR), three epidermal growth factor-like domains (E3), a kringle domain, and a protease domain (106). Polyamines (in the order spermine>spermidine>>putrescine) promote the formation of the autoactivation complex by modulating the intramolecular interactions between the NTR and E3 on one proPHBP thereby enabling intermolecular binding of the NTR to E3 on another proPHBP molecule without affecting the activity of the protease domain (107). The presence of the highly acidic NTR is indispensable for the autoactivation complex because a mutant lacking residues 1–52 (containing 14 negatively charged residues) was not able to enhance intermolecular binding of proPHBP (107). It seems conceivable to us that the polyamines transiently neutralize the negative charge of the NTR thereby allowing two molecules to dimerize. Interestingly, the negatively charged carminic acid inhibited spermidine promoted proPHBP autoactivation, but not the spermidine-independent one.

In vivo, activation of proPHBP is observed under inflammatory conditions. Inflammatory tissues (as well as tumor cells) have high levels of polyamines (108). Significant cell death in such tissues together with increased vascular permeability could result in elevated local polyamines. Whether these levels would be sufficiently high to achieve proPHBP activation is ambiguous.

Polyamines and protein-protein interactions in redox chains

Redox chains play a crucial role in cell metabolism and include processes such as photosynthesis in plants and light-harvesting bacteria, cell respiration, and oxidation of endogenous and exogenous compounds (xenobiotics). In many of these redox chains, salt bridges govern the recognition between proteins (109–113). Supposing a role of polyamines as endogenous modulators of the underlying protein-protein interactions, the mitochondrial CYP11A1 electron transfer system was chosen as an appropriate model to test this hypothesis. CYP11A1, a member of the cytochrome P450 (CYP) gene family, catalyses the side chain cleavage of cholesterol to pregnenolone, the rate-limiting step in the biosynthesis of all steroid hormones (114). CYP11A1 requires six electrons to activate oxygen for the three-step process that are provided from NADPH via a three-component electron transfer chain: forming a transient protein-protein complex, the FAD-containing reductase, adrenodoxin reductase (AdR) transfers an electron to the iron-sulfur protein, adrenodoxin (Adx) and, sequentially, Adx associates with CYP11A1 and shuttles the electron to this enzyme. Electrostatic interactions are crucially involved in the recognition between AdR and Adx, as well as between Adx and CYP11A1 (109, 115, 116).

When testing the complete CYP11A1 system and its individual components, substantial effects of the polyamines spermine, spermidine, and putrescine on protein-protein recognition and electron transfer were observed (23). With regard to the association of AdR to Adx (tested by using surface plasmon resonance), all three polyamines strengthened the binding by enhancing the association rate (k_{on}) up to eight-fold, but affecting less the dissociation rate (k_{off}). In contrast to tightening the AdR-Adx complex, polyamines weakened Adx-CYP11A1 binding, again mainly affecting the association rate. The effect on electron transfer corresponded to that on protein assembly: polyamines accelerated the electron transfer from reduced AdR to Adx, but slowed down Adx reduction of CYP11A1. The weakened Adx-CYP11A1 recognition governed the entire process of substrate conversion to pregnenolone. Examined in a cellular system, spermidine and spermine showed strong concentration-dependent inhibition (putrescine had negligible putrescine) (23).

Exploiting the known crystal structures of Adx, AdR, and the Adx-AdR complex (PDB codes 1AYF, 1CJC, and 1E6E, respectively), docking experiments identified five favorable binding sites of polyamines, four of them at or adjacent to the AdR and Adx interfaces (23) (Figure 5A). In particular, two high affinity binding sites were recognized on Adx at its so-called interaction region with AdR (115, 117, 118) that contains the negatively charged amino acids Asp72, Glu73, and Asp76 at site 1 and Asp15, Asp39, Asp41 at site 2 (118). Partial masking of charges at site 2 by polyamines (as well as by mutation of residues) accelerated recognition (23). Polyamine binding to site 1 might further reinforce protein-protein interactions by bridging between Glu73 on Adx and the close (7 Å distant) Glu212 on AdR.

Concerning Adx-CYP11A1 association, a crystal structure of the complex of CYP11A1 with (a binding fragment of) Adx is available (PDB ID 3NAO). It indicates a deep positively charged funnel around the key recognition residue Lys382 (including Lys378 and Arg386) to which Adx binds with its site 1. Masking of site 1 due to high-affinity interacting polyamines might impede recognition at the interface. Moreover, effects on protein-protein association might be caused by polyamine binding to negatively charged patches on the CYP11A1 surface that could result in a conformational change (possibly accounting for a reversed type I spec-

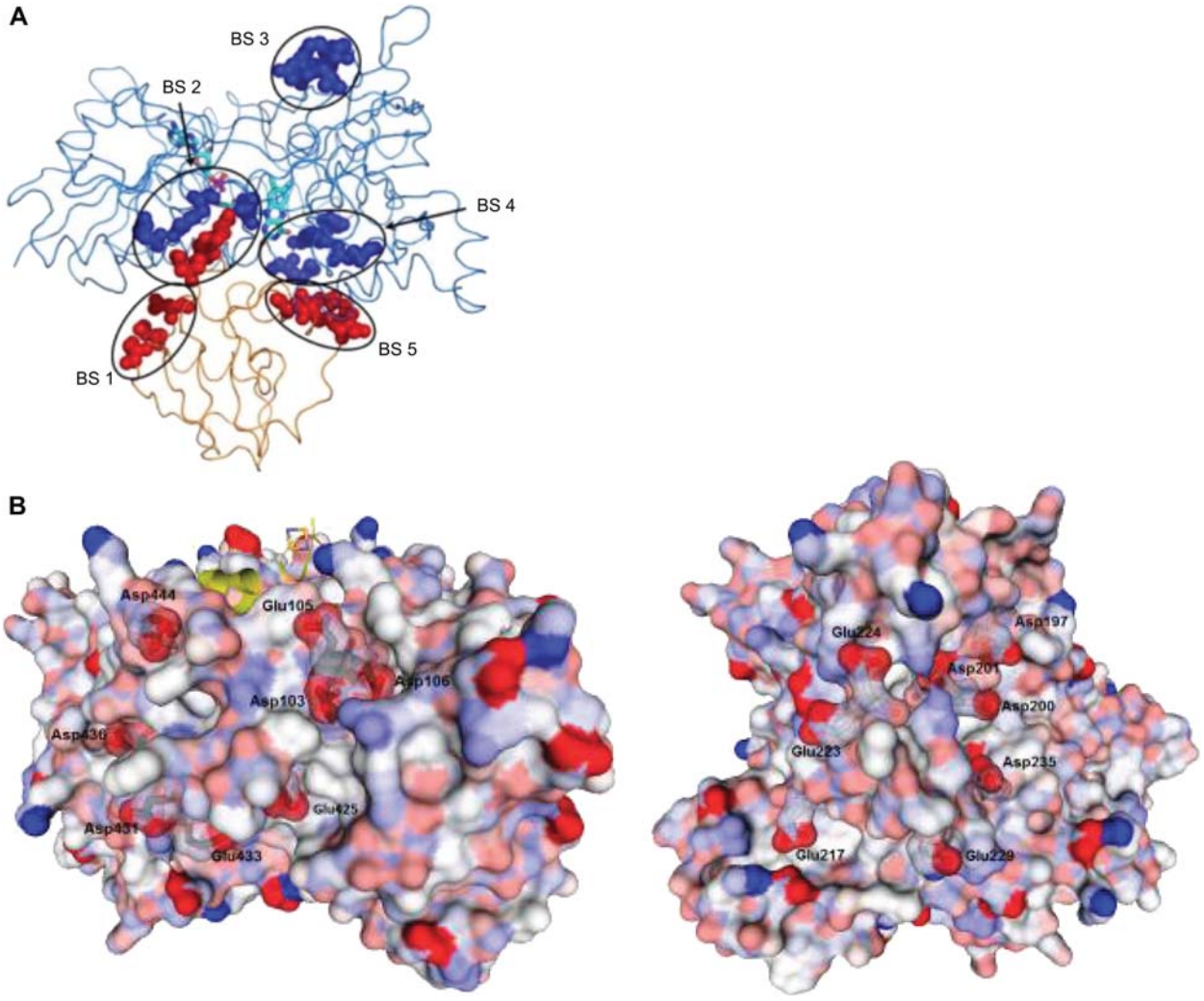

Figure 5 Structures of the Adx-AdR complex and the CYP11A1 surface.
(A) Location of the identified binding sites (BS) on the Adx-AdR complex. Adx is shown in orange, AdR in light blue. For both proteins, a ribbon representation is used except for the residues lining the binding sites (blue: AdR residues, red: Adx residues) that are shown as ball-and-stick models and FAD (colorized representation, colorized by element). Reprinted from Berwanger et al. (23), with permission from Elsevier. (B) Surface view of CYP11A1. View on the negatively charged surface adjacent to the adrenodoxin binding site (residues Asp103, Glu105, Asp106, Glu425, Asp431, Glu433, Asp436 and Asp444) (on the left hand site) and apart from the adrenodoxin binding site (residues Asp197, Asp200, Asp201, Glu208, Glu217, Glu223, Glu224, Glu229, and Asp235) (on the right hand site) of CYP11A1 (coordinates taken from PDB ID 3NAO). Negatively charged amino acid residues are depicted in red, positively charged ones in blue. Amino acid residues forming a negative channel are labeled. The iron-sulfur cluster and the interaction domain of adrenodoxin are depicted in yellow. The visualization of the structures was performed using the program Discovery Studio Visualizer 2.5.5 from Accelrys (http://accelrys.com/).

tral change observed with all polyamines). Two examples of negatively charged patches are depicted in Figure 5B,C.

In addition to CYP11A1, the AdR-Adx transfer chain also serves other mitochondrial CYPs such as CYP27B1 and CYP24A1 that are specifically involved in vitamin D activation and metabolism, CYP27A1 that catalyzes the first step in vitamin D activation as well as cholesterol oxidation to oxysterols and bile acids, and CYPs11B1/B2 that catalyze the synthesis of corticoid hormones [reviewed in (119, 120)]. It might be speculated that differing interactions of CYPs with polyamines could help distinguish in which direction the promiscuous Adx would transfer its electrons. Electrostatic interactions also play an important role in microsomal CYP systems involved in drug metabolism (121, 122, 123) and thus might be prone to modulation by polyamines.

Expert opinion: why nature chose polyamines

In a landmarking paper, that has been cited several hundred times since 1987, Westheimer reasoned about ‘Why nature chose phosphates’ (124). Listing the pleiotropic functions of

phosphates as intermediary metabolites, as principal reservoirs of biochemical energy, and in building and maintaining large biological structures, that were all related to their ionized character, Westheimer stated: 'no other residue appears to fulfil the multiple roles of phosphates in biochemistry'. Trying to answer 'Why nature chose polyamines', we are confronted with a comparably complex repertoire of capabilities of polyamines, however, still without a stringent confirmation of many of these supposed functions under (patho)-physiological conditions.

In fact, polyamines appear as positively charged counterparts of phosphates, capable of reversibly interacting with negatively charged sites, screening acidic charges, and/or bridging between them. An electrostatic partnership between phosphates and polyamines is obvious not only concerning a regulation of structure and function of nucleic acids, but also in controlling signaling pathways, e.g., by protein kinases. Nevertheless, polyamines add a new quality that is based on the high flexibility of their linear hydrophobic alkyl chains in which the charged residues are interspersed. This uniqueness enables polyamines to modulate an immense variety of biomolecules by interacting in or on each of them with multiple subsites – not only by electrostatic forces – in a more or less specific way. Achieving sufficiently high affinity could render polyamines successful competitors of other intracellular and extracellular cations. Focusing on proteins, polyamines have been demonstrated to serve as substrates or inhibitors in binding pockets, as regulators of ion flow owing to their capability to selectively plug or stimulate channels and, importantly, as modulators of protein-protein interactions. In the latter case, binding to acidic patches on protein surfaces has been shown to facilitate or impede protein-protein recognition and leads to – or prevents – formation of transient complexes, larger assemblies, and structural networks. As polyamine concentrations fluctuate during the cell cycle, we hypothesize that the extent of their interference with transient protein-protein complexes might change likewise, thereby contributing to switching metabolic pathways and signaling cascades.

Covalent binding of polyamines to proteins – catalyzed by transglutaminase – has been confirmed to take place in various tissues of mammalian species *in vivo*. This modification adds positive charge to the surface of proteins and could substantially alter their functions (possibly corresponding to the modified roles of protein kinase-created negatively charged proteins?). Further crosslinking by transglutaminase leads to a new class of aggregates, in which individual members are linked by flexible, positively charged chains. In addition to changed protein-protein recognition, polyamine binding/modification might affect the interactions of proteins with charged sites on other biomolecules, e.g., on nucleic acids or on phospholipids. However, the respective discussion of these issues is well beyond the scope of our article.

There is still little information on which of the afore-mentioned pleiotropic actions can/will play decisive roles under *in vivo* conditions. Polyamines are by no means selective agents: validating a target supposed from studying *in vitro* models appears to be problematic as the underlying reductionistic approach had focused just on a few aspects but ignored so many other important issues such as the multitude of concurrent different potential targets and the functional networks between them or the abundance/composition of other inorganic and organic cations that might efficiently compete. Although still in its infancy, systems biology will be the appropriate strategy to describe and understand the complexity of polyamine interactions in a physiological context. As a first step to this holistic approach, we propose to extract the widely dispersed published information and build a data bank containing comprehensive data on polyamines and their targets (i.e., chemical, biochemical, biological, pharmacological, and pharmaceutical data) and links to other databases.

Outlook

Research on polyamine-protein interactions appears a highly challenging and promising task as many facets of this topic are unexplored or far from being understood. This holds true for polyamine interactions with single partners such as the still unknown transporter systems, or distinct ion channels, and even more for complex systems in which polyamines are involved such as cellular signaling cascades or assembly of proteins to cytoskeletal networks or toxic aggregates. Information on any of these systems might indicate valuable new targets for drug discovery in a host of indications. Because polyamines are capable of binding to distinct regions on protein surfaces in a specific manner, they indicate the druggability of these regions and their potential to serve as targets for modulating protein-protein interactions, a new important therapeutic strategy.

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