Melting Down Protein Stability: PAPS Synthase 2 in Patients and in a Cellular Environment

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Within the crowded and complex environment of the cell, a protein experiences stabilizing excluded-volume effects and destabilizing quinary interactions with other proteins. Which of these prevail, needs to be determined on a case-by-case basis. PAPS synthases are dimeric and bifunctional enzymes, providing activated sulfate in the form of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) for sulfation reactions. The human PAPS synthases PAPSS1 and PAPSS2 differ significantly in their protein stability as PAPSS2 is a naturally fragile protein. PAPS synthases bind a series of nucleotide ligands and some of them markedly stabilize these proteins. PAPS synthases are of biomedical relevance as destabilizing point mutations give rise to several pathologies. Genetic defects in PAPSS2 have been linked to bone and cartilage malformations as well as a steroid sulfation defect. All this makes PAPS synthases ideal to study protein unfolding, ligand binding, and the stabilizing and destabilizing factors in their cellular environment. This review provides an overview on current concepts of protein folding and stability and links this with our current understanding of the different disease mechanisms of PAPSS2-related pathologies with perspectives for future research and application.

Keywords: sulfation pathways, PAPS synthase, quinary interaction, excluded volume effect, ligand stabilization, enzyme storage complex

INTRODUCTION

Sulfation pathways are centered around enzymatic conversion of sulfate to the activated sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and the transfer of the sulfuryl moiety to biological acceptor molecules (Foster and Mueller, 2018). The enzymes in charge are PAPS synthases (Mueller and Shafqat, 2013) and PAPS-dependent sulfotransferases (Coughtrie, 2016; Hirschmann et al., 2017), respectively. Sulfation pathways also include the removal of sulfate by sulfatases (Mueller et al., 2015) and the deactivation of the sulfation-byproduct 3′-phosphoadenosine-5′-phosphate (PAP) by dedicated PAP phosphatases (Chan et al., 2016). Sulfation impacts on many different acceptor molecules, such as carbohydrates, proteins, lipids, xenobiotics, and steroids as well as other hormones (Mueller et al., 2015). Several proteins from this pathway have been studied with regard to their stability; some of them giving rise to clinically observed pathologies (Oostdijk et al., 2015).

An experimental categorization into thermostable and thermolabile sulfotransferases (Reiter and Weinshilboum, 1982) was an early apprehension of the multiplicity of sulfation pathways...
we know of today. Unfolding was then monitored as loss-of-
activity measurements upon thermal increase. Midpoints of
unfolding of sulfation activity towards dopamine were 39.5°C,
buts 44°C for a phenol-targeting sulfotransferase (Reiter and
Weinshilboum, 1982). Hence, thermostability was understood
as being folded and active at about physiological temperature.
This system was in use more and more sulfotransferase
genes were cloned (Dubin et al., 2001). Later, sulfotransferase
proteins were purported to form dimers via an unusually small
protein interface (Petrotenko et al., 2001; Weitzner et al.,
2009). Dimerization is thought to increase structural stability
of human sulfotransferase SULT1A1, both with regards to thermal
inactivation and chemically induced unfolding (Lu et al., 2009).

Only in 2012, the first biophysical study of sulfate-activating
PAPS synthase enzymes (van den Boom et al., 2012) identified
one of them, human PAPSS2, as a fragile protein specifically
stabilized by ligand binding (van den Boom et al., 2012;
Mueller and Shafqat, 2013). PAPS synthases are bifunctional
enzymes comprising a C-terminal ATP sulfurylase and an N-
terminal APS kinase domain (Mueller and Shafqat, 2013). Their
physiological substrates and products are sulfate, ATP, ADP,
pyrophosphate, PAPS, and the reaction intermediate adenosine-
5′-phosphosulfate (APS) (Strott, 2002). Out of these, ADP
and PAPS had moderately stabilizing effects, but APS shifted
unfolding transitions by more than 16°C (van den Boom
et al., 2012). The relevance of this finding for intracellular
sulfation pathways is not yet clear as concentrations of the
intermediate APS are expected to change considerably within
the cell (Landsdon et al., 2004).

Various point mutations have been described for the PAPSS2
gene that lead to bone and cartilage malformations (Oostdijk
et al., 2013) as well as a steroid sulfation defect (Noordam et al.,
2009; Oostdijk et al., 2015). A subset of these mutations seems to
destabilize the PAPSS2 protein severely, inducing its intracellular
aggregation and triggering its ubiquitination and degradation via
the proteasome (Oostdijk et al., 2015). Interestingly, PAPSS2 is
involved in transient protein interactions with other sulfation
pathway proteins (Mueller et al., 2018) and these interactions
may stabilize or activate the PAPSS2 protein. This review will look
at sulfation pathways, central to healthy human physiology from
a protein-stability/protein-folding perspective.

PAPS SYNTHASE PROTEINS DISPLAY
SUBSTRATE-SPECIFIC FOLDING
PROPERTIES

Understanding structure, function, and stability of proteins
as the cellular workforce to generate vital biomolecules has
been of great interest ever since (Bryngelson et al., 1995).
Protein folding becomes especially important due to the many
examples of malfunctioning proteins causally linked to severe
diseases, such as Huntington’s (McColgan and Tabrizi, 2018)
and Parkinson’s disease (Poewe et al., 2017). Many proteins
are functional on their own, but intermolecular interactions
such as dimer or multimer formation are common features of
proteins (Marsh and Teichmann, 2015). These quaternary
structures result from highly specific interactions encouraged
by complementary surface properties of the proteins involved.
They may represent the functional form of many proteins
(Dobson et al., 2004), regulate activity (Grum et al., 2010) or
be included in signal pathways (Heldin, 1995) and trafficking
between compartments (Knauer et al., 2005; Schröder et al.,
2012; Eggert et al., 2015). Compared to smooth interactions
such as multimer formation, intermolecular protein interactions
may also have a fuzzy nature. Interactions resulting in
these fuzzy complexes usually involve intrinsically disordered
regions that interact with each other to form for example
guish complexes or phase-separating ribonucleoprotein granules
(Wu and Fuxreiter, 2016; Alberti et al., 2019).

Within the complex environment of the living cell, transient
interactions with other biomolecules may occur and these have been named quinary interactions (McConkey,
1982) as a continuation of primary, secondary, tertiary and
quaternary structure (Cohen and Pielak, 2017). However,
commonly biomolecules are probed in dilute buffer solutions
or in crystals by techniques such as NMR spectroscopy or
crystallography. Thus, protein functions relying on transient
protein interactions mostly remain unnoticed or are very hard to
study (Matena et al., 2013).

The different structural and interaction levels of proteins
create a multidimensional rugged energy landscape with
several small energetic minima representing different possible
conformations of the polypeptide chain (Bryngelson et al.,
1995). Even though the energy landscape in theory allows many
marginally stable conformations, protein folding and unfolding
of small single-domain proteins can often be described by a
two-state model (Figure 1A). The unfolded protein needs to
cross a transition state comprised of an ensemble of partially
folded structures in order to fold downhill into its native
conformation, by forming intramolecular interactions in a
cooperative manner (Bryngelson et al., 1995; Oliveberg and
Wolynes, 2005). This one-dimensional energy landscape model
allows to precisely determine rates of protein folding as a
folding “speed limit” (Kubelka et al., 2004). The influence of
intermolecular and intramolecular interactions as well as pH or
viscosity on folding and the ruggedness of the landscape are still
investigated extensively (Chung et al., 2015; Chung and Eaton,
2018). However, the simple model needs adjustments when
describing folding of large multi-domain proteins. Multi-domain
proteins may form stable and biologically relevant intermediates
representing distinct energy minima on the protein folding
energy landscape. This applies to proteins with independently
folding domains (Ferreiro et al., 2005) or those requiring specific
interactions with substrates or cofactors (Klein and Schwarz,
2012; van den Boom et al., 2012). The actual positioning on the
reaction coordinate and their stability compared to the unfolded
and folded state may differ according to the number of contacts
already formed in this structure. PAPS synthases with their two
independently folding domains are a good example for multi-
state folder.

The 140 kDa dimeric PAPS synthase proteins unfold
irreversibly thermally and chemically, when studied as
recombinant proteins in vitro (van den Boom et al., 2012).
Their reaction intermediate, the nucleotide adenosine-5′-phosphosulfate, has two effects on the unfolding thermographs (Figure 1B). At low APS concentrations, equal to or slightly higher than the protein, unfolding transitions shift remarkably by about 5°C. At higher APS concentrations, even the form of the unfolding transition changed and a clear unfolding intermediate is seen (van den Boom et al., 2012). The high-affinity effect can be explained by forming a stable dead-end enzyme-ADP-APS complex in the APS kinase domain (Mueller and Shafqat, 2013), further discussed below. The ADP for this complex most likely is carried over through the purification process as it is tightly enzyme-bound (Mueller and Shafqat, 2013). The low-affinity effect is most likely due to stabilization of the ATP sulfurylase domain (van den Boom et al., 2012). In summary, this shows that PAPS synthase proteins display complex and substrate-specific folding properties that could play a important role in the regulation of sulfation pathways. It will be crucial
to study PAPS synthase stability in a cellular environment with tightly regulated substrate concentrations and the crowded environment described below.

**PUSH AND PULL—CROWDED CELLS VS. BUFFER SOLUTIONS**

In comparison to dilute solution, the cellular environment is crowded with up to 300 g/L of biopolymers (Zimmerman and Trach, 1991; Ellis and Minton, 2003). Biomolecules in such environments are influenced by a many of specific and non-specific interactions. Metaphorically speaking, comparing a biomolecule in dilute solution to the situation in the cell is like comparing an elegant ballet dancer in an empty dancing hall with a person in an overcrowded night-club. Random interactions and collisions are rare in the first case, but unavoidable in the second. Scientifically speaking, the consequences of the random interactions and the collisions in the cellular “night-club” are referred to as excluded-volume effects (Minton, 1983) and quinary interactions (McConkey, 1982), respectively.

Within the concept of excluded-volume, macromolecules primarily repel each other. High macromolecule concentrations increase the occupied volume and decrease the volume accessible for other macromolecules, hence, reducing the configurational entropy of less compact conformations. With regards to proteins, this favors compact protein conformations that normally are the functional protein folds (Minton and Wilf, 1981). Indeed, *in vitro* experiments show that an artificially crowded environment using large polymers such as PEG or Ficoll-70 stabilizes native conformations (Alfano et al., 2017) and favors protein association (Batra et al., 2009) and catalytic activity (Dhar et al., 2010; Paudel et al., 2018). However, a physicochemical force driving proteins into their most compact conformation will unavoidably also affect aggregation-prone proteins and increase aggregation rates in a crowded environment (Hatters et al., 2002; Gao et al., 2015).

The concept of quinary interactions describes the organization of large biomolecules via weak and transient interactions (McConkey, 1982). In contrast to repulsive interactions, transient interactions depend on the chemical composition of the biomolecule and the crowding agent (Minton, 2013). Since crowding agents, used to mimic the cellular environment, differ in their chemical properties, their effects on a target protein studied *in vitro* may hence differ as well (Senske et al., 2014; Feig et al., 2017; Majumdar et al., 2018). While some crowding agents such as PEG directly interact with a protein, others such as dextran rather affect the water network and the solvation of the protein (Senske et al., 2014). This mechanism is comparable to the thermodynamic stabilization of proteins by osmolytes (Yancey et al., 1982; Senske et al., 2014, 2016). Recently, the effects of quinary interactions on protein stability have been correlated with protein surface properties – the increase in surface charge is a key factor for the formation of quinary interactions (Gnutt et al., 2019). These well-studied concepts show that in addition to the intrinsic stability of the protein fold, weak and repulsive interactions in a cellular environment need to be considered.
as well for a complete view on protein folding and stability. Detailed effects of the cellular environment and how intracellular crowding is mimicked in vitro were discussed here (Politou and Temussi, 2015; Gnutt and Ebbinghaus, 2016).

Understanding how a protein folds and how it is stabilized, when surrounded by hundreds of other macromolecules with a myriad of unspecific interactions is a complex task. Several approaches have been applied to disentangle the physicochemical effects exerted on biomolecules inside the cell, ranging from fluorescent crowding sensors (Boersma et al., 2015; Gnutt et al., 2015) to case-studies in cell lysates (Martin and Hartl, 1997) or whole cell simulations (Feig et al., 2017). Within living cells, similar studies have been performed using an RNA hairpin (Gao et al., 2016), the glycolytic PGK enzyme (Dhar et al., 2011; Wirth et al., 2013), superoxide dismutase 1 (Gnutt et al., 2019), cell volume changes (Wang et al., 2018), and intracellular osmolytes (Sukenik et al., 2018). Very recently, the repertoire of in-cell protein analysis has been expanded to live zebrafish (Feng et al., 2019). It appears that the net-effect on thermal stability of a specific protein or sensor inside cells cannot be generalized and highly depends on the surface exposed. This complexity makes it inevitable to use in-cell techniques to study proteins within their native environment complemented with in vitro experiments.

An orthogonal method to study in-cell protein stability represents mass-spectrometry of isotopically labeled cells. A pioneering study in this regard reported thermal profiling of the cellular proteome (Savitski et al., 2014). From this dataset, we derived midpoints of unfolding $T_{50}$ of 54.6 and 46.2°C for PAPSS1 and PAPSS2, respectively, and compared these to the $T_{50}$ values of 45.8 and 39.8°C for PAPSS1 and PAPSS2, measured before (van den Boom et al., 2012), revealing a difference in $T_{50}$ of 8.3 and 5.8°C for the two PAPS syntheses. Even though these numbers are from two studies using different methods, the observed differences in $T_{50}$ suggest that PAPS synthase protein stability within cells may differ compared to previous in vitro studies (van den Boom et al., 2012). Whether this is related to intrinsic protein stability or protein interactions is a question which remains to be answered.

**HOLDING ON TO SOMETHING—HOW LIGANDS, COFACTORS AND SUBSTRATES AFFECT PROTEIN STABILITY**

Functional proteins have not evolved to preserve a certain protein fold, but rather to fulfill a certain biological function. Enzymes have specific binding sites for strong and specific interactions with their small-molecule substrates and products (Martinez Cuesta et al., 2015). This specific binding relies on many cooperatively acting interactions and may stabilize the protein, making it more rigid. Indeed, many experiments studying thermal or mechanical stability show strong stabilization of proteins by binding their substrates or cofactors. This includes, among others, adenylate kinases binding substrate and inhibitory nucleotides (Mazal et al., 2018), PAPS syntheses binding their substrates or products ADP, APS, and PAPS (van den Boom et al., 2012) as well as cohesins binding calcium (Verdorfer and Gaub, 2018). But how do these newly formed interactions contribute to the actual stability of the protein?

The Gibbs energy $\Delta G$ is genuinely used to compare the energetic differences between two states and in protein-ligand-binding this refers to the bound and the unbound state of the ligand. The Gibbs energy can further be split into contributions of the enthalpy $\Delta H$ describing the total energy of the system observed and the temperature-dependent entropy $\Delta S$ describing the degrees of freedom or conformational states of similar energy. The thermodynamic descriptions of protein-ligand binding focus either on the ligand, especially in drug design (Claveria-Gimeno et al., 2017), or how the energy landscape of protein folding and stability is affected (Kabir et al., 2016; Hingorani et al., 2017). One factor regarding ligand binding is the process of breaking existing water networks in the unbound state in order for the ligand to bind (Fox et al., 2017; Verteramo et al., 2019). Breakage and reorganization of a strong water network displays an energetic cost which ultimately reduces the affinity of drugs to their targets (Fox et al., 2017). Thermodynamic analysis focusing mainly on the binding effects on the protein itself include changes in protein structure (Reyes et al., 2017) or stability (Kabir et al., 2016). In some cases, binding of ligands may actually stabilize non-native or intermediate conformations and therefore not always lead to a stabilization of the protein target (Kabir et al., 2016). However, stabilizing non-native interactions may be desirable in drug design to reduce formation of, for example, toxic aggregates (Vöpel et al., 2017; Perni et al., 2018).

PAPS syntheses show consecutive binding and release of sulfate, ATP, ADP, APS, and PAPS (Sekulic et al., 2007; Figure 2). As all nucleotides involved are generated from ATP, it becomes apparent that ATP availability fuels the cycle. Looking into crystal structures and experimental data, ADP and APS appear to be tightly bound to the protein (Harjes et al., 2005; Mueller and Shafqat, 2013) and the structure of the protein is known to be stabilized by these nucleotides (van den Boom et al., 2012). The inhibitory or “dead-end” complex forms after the release of the product PAPS, by re-binding of an APS nucleotide to the ADP-kinase complex. This is a highly stabilized form of the APS kinase (Sekulic et al., 2007; Mueller and Shafqat, 2013). Considering thermal unfolding experiments in presence and absence of APS described above (van den Boom et al., 2012), one of the observed thermal unfolding transitions must be caused by the formation of this inhibitory complex with APS (Figure 1B). Due to the marked increase in stability by binding ADP and especially APS, the formed complex may not only be of inhibitory nature, but serves as a storage form in times of ATP depletion.

Even though ATP is often thought to be a ubiquitously available source of energy inside cells at up to two-digit millimolar concentration, the levels of ATP are regulated by different factors and may fluctuate significantly during the lifetime of a cell (Beis and Newsholme, 1975; Ataullakhanov and Vitvitsky, 2002; Bonora et al., 2012). However, in response to ATP depletion resulting from stresses, kinases such as S′-AMP protein kinase become more active in order to rebalance the ATP/AMP-ratio by phosphorylating substrates, regulating downstream gene expression and ultimately the activity of ATP-generating systems.
DNA damage from irradiation and oxidative stress caused by endogenous or environmental factors are stresses that affect ATP levels temporarily (Budanov and Karin, 2008). However, hyperosmotic shock (Dmitrieva et al., 2011), resulting in an imperfect crowding adaptation (Gnutt et al., 2017), as well as cellular starvation (Maddocks et al., 2013) are known to cause DNA lesions as well. Further, as ATP has been suggested to act as a hydrotrope helping to solubilize proteins in the cellular environment, proteins may even become prone to aggregation, if ATP is depleted for a longer period of time (Patel et al., 2017). Considering changing ATP levels and PAPS synthase stabilization via ligand binding, we propose that the PAPSS2-ADP-APS complex within the APS kinase domain represents a stable storage form not only of the enzyme, but also for the APS nucleotide, of relevance in times in which ATP levels are depleted.

### INTRACELLULAR RECYCLING—STABILITY AND DEGRADATION OF PAPS SYNTHASES IN CELLS

Proteins within the cell are not only exposed to the physicochemical forces described above, but also to enzymatic post-translational modifications and some of these modifications influence protein stability again. In Alzheimer’s disease, as an example, the phosphorylated tau protein forms neurofibrillary tangles and glycosylation defects have been observed in the amyloid precursor protein (APP) and other proteins (Schedin-Weiss et al., 2014). Ubiquitination is noteworthy here as it serves, among other functions, to target proteins for active degradation (Chaugule and Walden, 2016). A tetra-ubiquitin chain attached to a lysine side chain of a target protein is sufficient for recognition and degradation by the proteasome (Singh et al., 2016). The attachment of ubiquitin is catalyzed by hundreds of ubiquitin ligase-complexes (Chaugule and Walden, 2016). Interestingly, this attachment depends on physicochemical stability of the target protein as ubiquitination requires the target peptide to be unfolded or intrinsically disordered (Prakash et al., 2009); structured domains need to unfold partially before ubiquitination can occur (Hagai et al., 2011) and conversely ubiquitination destabilizes the protein fold (Hagai and Levy, 2010). Preliminary data for PAPS synthases indeed show enhanced ubiquitination for PAPSS2 than for PAPSS1 upon 6 h treatment with the proteasome inhibitor MG-132 (data not shown). Taken together, biophysical instability may translate to biological instability involving active degradation by the ubiquitin/proteasome machinery.

### ONE PROTEIN, DIFFERENT MUTANTS AND TWO MECHANISMS—UNDERSTANDING PAPS SYNTHASE-RELATED DISEASES AND APPLYING THIS KNOWLEDGE TO DRUG DESIGN

Several neurodegenerative diseases present with aggregates of certain proteins such as Aβ peptide in Alzheimer’s disease (Masters et al., 2015), poly-Q repeats in Huntington’s disease (McColgan and Tabrizi, 2018), and α-synuclein in Parkinson’s disease (Poewe et al., 2017). Once disease progression was linked to the cytotoxicity of the observed protein aggregates, aggregation had been studied in detail and the actual toxic oligomeric species identified (Knowles et al., 2014). Most
recently, small molecules have been developed to alter aggregation properties of the affected protein to treat the according disease (Vöpel et al., 2017; Perni et al., 2018; Limbocker et al., 2019).

While neurodegenerative diseases are well-studied, diseases related to sulfation pathways remained underexplored with the exception of heparin as an anti-coagulant (Tsai et al., 2017). Furthermore, genetic defects in the gene for PAPS synthase 2 have been described with phenotypes of various forms of bone and cartilage formation as well as androgen regulation and polycystic ovary syndrome (Oostdijk et al., 2015). These PAPSS2 missense mutations include G78R (Kurima et al., 1998), T48R (Noordam et al., 2009), C43Y, L76Q, V540D (Iida et al., 2013), and G270D (Oostdijk et al., 2015). They are mapped on a schematic representation of the PAPS synthase protein structure (Figure 3), which shows that the APS kinase mutants are in close spatial proximity.

Some of the mutations in PAPSS2 occurred homozygous such as V540D (Iida et al., 2013; Oostdijk et al., 2015). However, two studies describe clinical cases of compound heterozygous mutations, where a missense mutation is observed in the genetic background of a non-sense mutation (Noordam et al., 2009; Oostdijk et al., 2015). Messenger RNAs of non-sense mutations with their premature stop codon are substrates for non-sense-mediated mRNA decay (Lloyd, 2018) and hence they are regarded as null alleles. For three of the missense mutations, G78R, T48R, and G270D, it is known that they are hypomorphic; they are alleles with residual activity. For the G78R variant, very little residual APS kinase activity was reported, but the ATP sulfurylase activity remained similar to wild type (Kurima et al., 1998), suggesting a folded and functioning ATP sulfurylase that may act as a solubility anchor and prevent immediate aggregation of the protein. For T48R and G270D, residual ability to support SULT2A1-dependent DHEA sulfation was reported, however, both mutated protein variants were also ubiquitinated and degraded (Oostdijk et al., 2015), suggesting severely destabilized protein folds.

These data suggest the existence of two different disease mechanisms for the same PAPS synthase proteins. Residue solvent accessibility extracted from DSSP values further illustrates that G78R is more solvent exposed compared to the buried amino acids (keeping in mind that glycine residues show small solvent accessible surfaces in general due to the lack of a side chain). For an understanding of PAPSS2-related pathologies, it will be essential to investigate whether biophysical observation can be correlated with disease progression.

Generally, once a protein’s molecular disease mechanisms is classified as misfolding or aggregation, designing small molecule-drugs is often the best way to recover that protein from its disease states (Arosio et al., 2014; Vöpel et al., 2017; Chia et al., 2018). This may be applicable to any other disease that compromises the protein-folding homeostasis (Wood et al., 2018). However, if the catalytic function of the protein is affected, this approach appears not promising and cure is probably best to achieve by supplementing patients with the lacking compounds that are no longer synthesized by the affected protein. For defects in the biosynthetic pathway of a cofactor, such supplementation can involve a precursor of that cofactor (Edwards et al., 2015); for PAPS synthase defects it may turn out to be a small subset of sulfated biomolecules.

CONCLUSION AND OUTLOOK

Activated sulfate in form of PAPS is essential for driving sulfation pathways inside cells. Even though a lack of PAPS synthase activity results in severe disease states, biophysical studies have only focused on measurements in buffered solutions so far. These findings implicate that PAPS synthases may be highly influenced by the cellular interior with regard to ligand binding and protein ubiquitination and degradation. At times, PAPS synthases form an inhibitory complex with ADP and APS bound. This complex may be reinterpreted as a storage form of the intrinsically unstable PAPSS2 protein and its substrate APS. In-cell studies are required to determine the intracellular stability of PAPS synthases. These findings shall provide further insight into proposed molecular disease mechanisms and help to design suitable drugs or treatment strategies of PAPS synthase-related diseases.

AUTHOR CONTRIBUTIONS

SE and JWM defined the scope for this review. OB and JWM collected material and wrote the paper. All authors read and approved the final version of this review.

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