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Sulfate activation and transport in mammals: system components and mechanisms

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Abstract

Extensive studies on the mammalian sulfate-activating enzymes and PAPS translocase have enhanced our understanding of the overall pathway of sulfate activation and utilization. Isolation of the PAPS-synthesizing activities from rat chondrosarcoma and preparation of stable non-hydrolyzable analogs of APS and PAPS have facilitated the kinetic characterization of mammalian ATP sulfurylase and APS kinase. These studies provided the basis for further experimental work showing that APS, the labile intermediate product, is channeled directly between the sulfurylase and kinase active sites. The defect in the brachymorphic mutant mouse lies in this channeling mechanism, thus interfering with efficient PAPS production. The rat chondrosarcoma ATP sulfurylase and APS kinase activities, in fact, reside in a single bifunctional cytoplasmic protein, which has now been cloned and expressed. The mechanism by which PAPS reaches its sites of utilization in the Golgi lumen has also been elucidated: The PAPS translocase is a 230-kDa integral Golgi membrane protein which functions as an antiport.

Keywords: ATP-sulfurylase; APS-kinase; PAPS-translocase; Bifunctional; Channeling; Antipor

Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate; PPi, inorganic pyrophosphate (P/O).

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1. Introduction

The sulfate activation pathway in mammals consists of two activities, ATP sulfurylase (EC 2.7.7.4) which catalyzes synthesis of APS from ATP and SO_4^{-2} and APS kinase (EC 2.7.1.25) which phosphorylates APS in the presence of another molecule of ATP (Fig. 1). The same two activities are involved in sulfate activation in simpler organisms where they appear to be relatively small, separate enzymes. We have previously studied the PAPS-synthesizing enzymes in the context of a defect in the production of PAPS in the brachymorphic mutant mouse, where a reduction in the activities of both enzymes was clearly demonstrated [1–3]. In order to understand this intriguing double enzyme defect, it was necessary to elucidate the relationship between the two activities by first purifying and attempting to determine whether they represented two separate polypeptides or a single bifunctional polypeptide. Toward this end, the two sulfate-activating enzymes, ATP sulfurylase and APS kinase, were each purified from rat chondrosarcoma and shown to have nearly identical molecular properties and fractionation behavior [4]. To further our evidence as to whether a single polypeptide with multiple active sites or two tightly complexed polypeptides pertain, characterization of both kinetic mechanisms, as well as further affinity purification and eventual cloning of the sulfate activation enzymes was accomplished. These studies identified the mammalian sulfurylase/kinase as a bifunctional enzyme that uses a channeling mechanism to transfer the intermediate APS efficiently from the sulfurylase to the kinase active site. The finding of multiple functions on a single polypeptide suggests that this complex enzyme is a critical locus for regulation and a vunerable site for mutations.

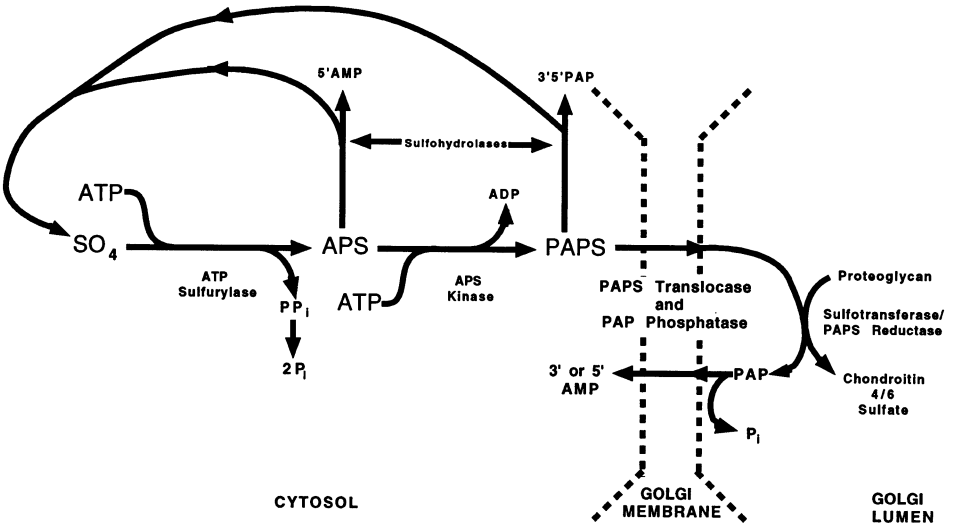

Fig. 1. Integrated pathway for sulfate activation, delivery to the Golgi and utilization by sulfotransferases.

In addition, components which are involved in processes distal and proximal to the sulfurylase/kinase complex, thereby constituting the entire sulfate uptake, activation and utilization pathway (Fig. 1), remained unknown with respect to the number and nature of participants, rate-limiting steps and modes of regulation. Maximum efficiency of this overall pathway requires transport of inorganic sulfate into the cell and then transport of the product of the activation pathway, PAPS, from the site of synthesis in the cytosol to the site(s) of utilization in the Golgi lumen. Clearly transport across inter- and intra-cellular membranes is a hallmark of this overall pathway. Thus, another potential site of regulation of the sulfation process relates to the mechanism by which PAPS generated in the cytosol is transferred (or translocated) across the Golgi membrane to intralumenal sites of PAPS utilization. It is hypothesized that this translocation process must be exquisitely coupled to biosynthesis of PAPS for maximum efficiency of the overall process. Thus identification and characterization of the PAPS translocase was necessary for gaining a better understanding of these individual steps as well as the process of sulfation in general.

2. Synthesis of APS and PAPS analogs

In concert with the purification and molecular characterization, kinetic analysis of the ATP sulfurylase and APS kinase activities individually and in coupled reactions, have contributed to understanding the nature of their relationship to each other. Mechanistic studies of the enzymes involved in synthesis and utilization of APS and PAPS were hampered by the extreme liability of APS and PAPS, the vulnerability of the compounds to enzymatic degradation, the unfavorable equilibrium constant for initial sulfate activation and the lack of suitable inhibitors. Therefore, proper analysis of these enzymes as well as further affinity purification necessitated the generation of stable substrate analogs.

Nucleotide analogs in which the bridging oxygen of the phosphoanhydride linkage is replaced by a methylene group which have been useful in elucidating the mechanisms of many enzymes that use nucleoside polyphosphates as substrates. These analogs are non-hydrolyzable and isosteric to the naturally occurring polyphosphates and, therefore, frequently bind to the active site of the enzyme as competitive inhibitors. In order to produce a stable APS analog we used an approach in which the bridging oxygen atom is replaced by a methylene group by synthesizing 2′3′-*O*-isopropylidene-adenosine-5′-*O*-chloromethylphosphonate, followed by nucleophilic substitution of the chlorine atom with sulfite ion. The product, β-methylene APS, is both acid and base stable and was obtained with an overall molar yield of 40–50%; the structure was confirmed by mass spectrometry and NMR [5].

Because PAPS is neither bound as a substrate by, nor acts as an inhibitor of the sulfurylase enzyme, it was reasoned that an analog of PAPS would prove efficacious in permitting separation of the individual sulfate activation enzymes, to facilitate a more complete kinetic investigation of the APS kinase and expedite the

purification and characterization of PAPS-utilizing activities such as sulfotransferases and PAPS translocases. Also, photoaffinity labeling of the individual activities was deemed possible following generation of such a compound. Our synthesis of a stable PAPS analog employed a cyclic intermediate, tri(tetramethylammonium) trimetaphosphate in the selective phosphorylation of the cis-2′,3′-diol of unprotected ribonucleotides and the product was separated from unreacted β-methylene-APS by ion-exchange chromatography [6]. A new method was then devised for separating the resulting mixed 2′(3′)-isomers since the charge density is identical in both molecules, obviating the use of conventional ion-exchange chromatography. The mixed isomers of β-methylene-2′(3′)-PAPS were separated by competitive ion-pairing reversed phase HPLC on an octadecyl silica column using triethylamine as the primary hetaeron and 1,3-diaminopropane as the competing hetaeron [7]. These unique analogs have been invaluable for the following studies; they should also be useful for investigating PAPS binding proteins in other systems.

3. Mechanistic studies of PAPS enzymes

Based on our early work showing that the brachymorphic mouse exhibits a dual defect affecting both PAPS enzymes [1–3] and then showing that they fractionate together through extensive purification and exhibit several common properties and behavior [4], additional strategies for exploring their relationship were sought. Since defects in consecutive activities of a metabolic sequence are uncommon in mammals, such observations suggest ways in which single abnormalities may alter both enzymes. Kinetic analysis of these activities individually and in coupled reactions, is one way of studying their interactions.

The kinetic mechanism of rat chondrosarcoma ATP sulfurylase was investigated by steady-state methods in the physiologically forward direction as well as the reverse direction. In the forward direction, the double reciprocal initial velocity plots for the sulfurylase reaction intersect to the left of the ordinate with the $K_m(ATP) = 200\ \mu M = K_m(SO_4)$ and 97 μM. Chlorate, a competitive inhibitor with respect to sulfate showed uncompetitive inhibition with respect to ATP with an apparent K_i of 1.97 μM. In the physiologically reverse direction, the double reciprocal initial velocity patterns intersect to the left of the ordinate axis with $K_m(APS) = 39\ \mu M$ and $K_m(pyrophosphate) = 18\ \mu M$. The results of steady-state experiments using magnesium indicated that the true substrate is the magnesium pyrophosphate complex. The APS analog, 5′-β-methylene phosphosulfate, was an inhibitor competitive with APS and non-competitive with respect to magnesium pyrophosphate. The simplest formal mechanism in accord with all the data is an ordered, steady-state single displacement with MgATP as the leading substrate in the forward direction and APS as the leading substrate in the reverse direction [8].

A complete kinetic characterization of APS kinase has also been carried out. Initial velocity patterns for rat chondrosarcoma APS kinase indicate a single displacement formal mechanism with $K_m(APS) = 76$ nM and $K_m(ATP) = 24\ \mu M$. Inhibition studies with analogs of substrates and products were performed in order

to determine the complete formal mechanism. The analog of PAPS, 5′-β-methylene phosphosulfate, exhibited inhibition competitive with APS and non-competitive with respect to ATP. The analog of APS, was also competitive with APS and non-competitive with respect to ATP. Imido-ATP showed inhibition competitive with ATP and produced mixed-type inhibition, with a pronounced intercept effect and a small slope effect, with respect to APS. These results are in accord with the formulation of the predominant pathway as a steady-state, ordered mechanism with APS as the leading substrate and PAPS as the final product released [9].

The results of these kinetic investigations provided the first indication for a functional interaction between these two activities. Since APS is the last product to be released from the individual sulfurylase reaction and APS is the first substrate to bind to the kinase activity, it is possible that the enzymes transfer the APS intermediate between the active sites, or that the APS remains bound to the same site for action by the phosphorylation step. This specific order of substrate addition and product release results in a more efficient pathway and helps to overcome the many obstacles to the synthesis of PAPS through this pathway. These results have led to the subsequent studies to elucidate the physical and functional relationships between sulfurylase and kinase.

4. Intermediate channeling between ATP sulfurylase and APS kinase

Three types of experiments were used to examine the relationship of these two sequential activities from rat chondrosarcoma. In the first, an assay system that permits measuring the accumulation of both APS and PAPS in the presence of both enzyme activities yielded a PAPS/APS ratio corresponding to a channeling efficiency of 96%. Second, the velocity of the APS kinase reaction measured in the overall system, utilizing endogenously synthesized APS, was 8-fold greater than that of the isolated kinase reaction using exogenous APS. In the third type of experiment, isotope dilution showed that the APS intermediate was not released into the bulk medium but remained bound in the overall system. Addition of a 10^4-fold higher concentration of exogenous unlabeled APS to a system initiated with ATP and labeled sulfate produced only a 26% decrease in the production of the labeled product. Conversely, when labeled APS (at a concentration of about 2-fold higher than the steady-state level) was added to the overall reaction initiated with unlabeled substrates, less than 10% of the PAPS product was labeled. In contrast, control experiments with a system containing both the sulfurylase and kinase from *P. chrysogenum* (generously supplied by I. Segel, UC Davis) gave the results expected for a non-channeled pathway. These data indicate that APS is channeled between the active sites of ATP sulfurylase and APS kinase during the production of PAPS in rat chondrosarcoma [10]. Furthermore, using these same approaches we have recently demonstrated that the defect in the brachymorphic mouse primarily affects the function of this unique coupling mechanism between these two activities causing a decrease in the ability to channel APS and produce PAPS efficiently [11] Taken together with the kinetic analyses described above, the interaction of ATP

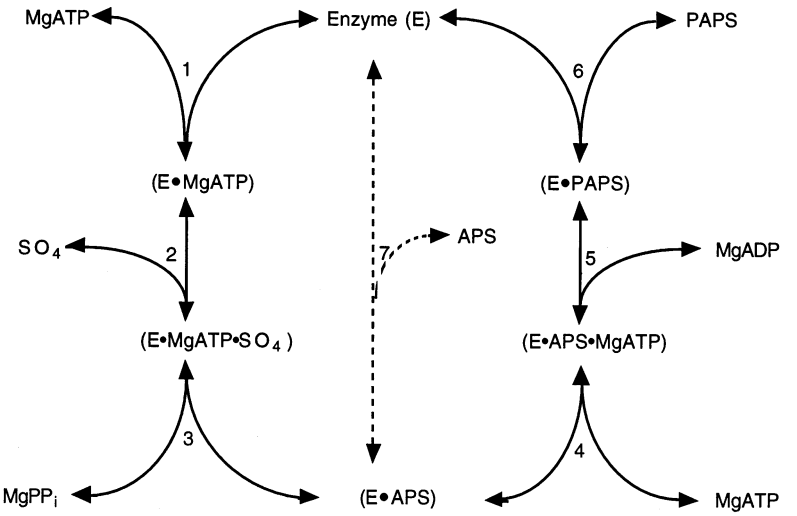

Fig. 2. ATP sulfurylase and APS kinase as a 3-substrate, 3-product enzyme.

sulfurylase and APS kinase can be described in the functional context of the overall sulfate activation pathway. In this proposed mechanism, the ATP sulfurylase and APS kinase act essentially as a 3-substrate, 3-product enzyme, as shown in Fig. 2.

5. ATP sulfurylase and APS kinase reside on a single bifunctional protein

As mentioned, both sulfurylase and kinase from rat chondrosarcoma co-purified over 2000-fold through S300 gel filtration, hydroxylapatite and ATP affinity chromatography, suggesting the activities are inseparable [4]. In subsequent studies both activities co-purified through substrate affinity chromatography using stable analogs of APS and PAPS. Approximately 56-kDa protein, containing both activities, was purified to apparent homogeneity through reversed-phase chromatography and observed on denaturing polyacrylamide gels. The molecular mass of the native active unit containing both activities in the purified preparation corresponded to approximately 60 kDa by analytical gel filtration. Coincident binding and elution of ATP sulfurylase and APS kinase by immunoaffinity chromatography, using polyclonal serum generated against the 56-kDa protein, also demonstrated that the enzyme contains both activities. Lastly, a single N-terminal amino acid sequence was obtained from the 56-kDa band isolated by gel electrophoresis. These biochemical results all suggest that ATP sulfurylase and APS kinase from rat chondrosarcoma reside on a single bifunctional protein [18].

A fused mammalian sulfurylase/kinase exists and was recently confirmed by cDNA cloning, sequencing and expression of a bifunctional enzyme [12]. Comparison against sequence databases suggested that the composite sequence was a fused sulfurylase/kinase product, since the deduced amino acid sequence showed exten-

sive homology to known separate sequences of both ATP sulfurylase and APS kinase from several sources. The first 199 amino acids corresponded to APS kinase sequence, followed by a tract of 37 amino acids which did not match any known sequence, followed in turn by 389 amino acids which are highly homologous to known ATP sulfurylase sequences. Finally, recombinant enzyme expressed in COS-1 cells exhibited both ATP sulfurylase and APS kinase activity. These results verify our biochemical studies, indicating that ATP sulfurylase and APS kinase constitute a bifunctional enzyme and demonstrate the feasibility of the expression system for future mutagenesis studies and expression of recombinant protein.

6. Identification and characterization of PAPS translocase

Our interest in overall synthesis and utilization of the universal high energy sulfate donor, PAPS, also extends to the process by which the PAPS generated in the cytosol is transferred across the Golgi membrane to intralumenal sites of PAPS utilitization (Fig. 1). Toward this end, we found that the protein responsible for the PAPS translocating activity could be solubilized from vesicles enriched in enzyme markers for the Golgi apparatus and reconstituted into liposomes [13]. In order to identify, characterize and isolate the entities involved, we have studied PAPS transport in Golgi vesicles, a reconstituted system and in an intact cell system in which we can access the cytosol, in both rat liver and rat chondrosarcoma [14,15]. The following evidence suggests that the PAPS translocase is a membrane-spanning protein of approximately 230 kDa: isolation by affinity chromatography on β-methylene PAPS matrices of a 230-kDa Golgi membrane protein concomitant with PAPS translocase activity; demonstration that the 230-kDa protein possesses the only PAPS binding site accessible to the cytoplasmic face of intact Golgi membranes, while several other PAPS binding proteins are labeled in solubilized membrane preparations; reduction in size of the 230-kDa membrane protein and loss of PAPS translocase activity following protease treatment; estimation via hydrodynamic analysis of the molecular size of the membrane protein associated with PAPS translocase activity; and correlation of β-methylene PAPS binding and labeling of the 230-kDa Golgi protein with PAPS translocase activity in artificial liposomes [16]. These data have permitted the identification of the first of a potentially large class of Golgi membrane nucleotide-metabolite transporters.

In order to gain a better understanding of the mechanisms involved in the transfer of PAPS from the cytosol, where it is synthesized to the Golgi lumen, where it serves as the universal sulfate donor for sulfate ester formation in higher organisms, we have also undertaken a kinetic characterization of the PAPS translocase from rat liver Golgi. Strong competitive inhibition in zero-*trans* uptake experiments only with β-methylene PAPS and adenosine 3′,5′-biphosphate (PAP) suggest the transporter is highly specific for the 3′-phosphate. The demonstration of *trans* acceleration as observed by stimulation of transport activity under exchange conditions suggests that the translocase is a carrier with distinct binding sites accessible from both faces of the membrane. The behavior of the PAPS translocase

in the presence of equilibrium concentrations of PAP supports the function of an antiport mechanism. Thus the translocase is characterized by its kinetic properties as a specific transporter of PAPS which acts through an antiport mechanism with PAP as the returning ligand [17]. This analysis, coupled with the earlier mentioned identification and characterization, represents a key step toward the purification and utilization of the PAPS translocase in the dissection and reconstitution of the overall pathway.

In sum, our studies represent a comprehensive approach to identifying the major components at several stages of the sulfation process and to elucidating the mechanisms which regulate the integrative process. Using now a selective in vitro reconstitution and studying naturally occurring mutants or creating new ones after cloning and expressing relevant components, a more thorough understanding of the integrated pathway for sulfate activation-transport-utilization should be forthcoming.

Acknowledgements

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