Critical Reviews in Biochemistry and Molecular Biology, 28(6):515–542 (1993)

The Physical Biochemistry and Molecular Genetics of Sulfate Activation

Thomas S. Leyh

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, New York 10461

Referee: Dr. Nicholas Kreditch, Dept. of Medicine, Duke University Medical Ctr., Box 3100, Durham, NC 27710.

ABSTRACT: This article is an overview of current research in the area of sulfate activation. Emphasis is placed on presenting unresolved issues in an appropriate context for critical evaluation by the reader. The energetics of sulfate activation is reevaluated in light of recent findings that demonstrate that the synthesis of activated sulfate is thermodynamically driven by GTP hydrolysis. The structural and functional bases of this GTPase activation are discussed in detail. The bonding and hydrolysis of the high-energy, phosphoric-sulfuric acid anhydride bond of activated sulfate are presented along with an analysis of the importance of the divalent cation and pyrophosphate protonation in the equilibria governing activated sulfate formation. The molecular genetics of sulfate assimilation in prokaryotes is reviewed with an emphasis on the regulation of the pathway. Recent discoveries connecting sulfate activation to plant/microbe symbiogenesis are presented, as are several examples of the importance of activated sulfate in human metabolism and disease.

KEY WORDS: sulfate activation, GTpase.

I. INTRODUCTION

Sulfate, by most chemical norms, is unreactive under moderate conditions of pH, temperature, and ionic strength; yet, it is readily assimilated by most, if not all, living things. The biological solution to this problem is a familiar one: sulfate is chemically activated via adenylylation. Once activated, sulfate is energetically poised for facile entry into its metabolic fates of reduction and group transfer. This article discusses the chemistry, enzymology, molecular genetics, and mammalian relevance of sulfate activation in the context of recent literature. Other excellent reviews provide stepping stones to earlier literature and topics not discussed here.[1–5]

II. IN THE BEGINNING

The concept that a chemically activated form of sulfate might be a metabolic intermediate in the transfer of sulfate to various acceptors appeared in the literature for the first time in 1952. Three articles published that year in the *Journal of Biological Chemistry* demonstrated that the sulfation of phenolic compounds required adenosine triphosphate (ATP) and Mg^{2+} to potentiate sulfate, rather than the phenolic-acceptor, for the sulfuryl group transfer.[6–8] This work intrigued Fritz Lipmann,[9] who had been working in the area of biochemical activation for some time: "The problem of sulfate activation is somewhat unusual in so far as it represents an ATP-linked activation of an inorganic compound other than phosphate. . . . In view of certain similarities to the carboxyl group activation, we have recently turned our attention to this unusual reaction . . . " (Fritz Lippmann[10]). In 1955, using partially purified enzyme preparations, he and Helmuth Hiltz showed that the activating reaction(s) produced one molar equivalent of PP_i and adenine per mole of activated sulfate from ATP and sulfate.[10] Based on this evidence and formidable chemical intuition, they tentatively proposed that adenosine-5′-phosphosulfate (APS) was the structure of activated sulfate. This landmark article was the first to describe the chemical structure of activated sulfate. In a subsequent article, published in 1956, Lipmann and Robbins[11] purified enough activated

1040-9238/93/$.50

sulfate to perform more detailed structural analyses and found that the sulfuryl group donor was, in fact, adenosine-3′-phosphate-5′-phosphosulfate (PAPS) rather than the previously suspected APS.

The aforementioned studies were performed with protein extracts obtained from rat or lamb liver that were sensitive and difficult to work with. In 1956, Lloyd Wilson and Robert Bandurski[12] published their work using more stable extracts prepared from yeast. Although yeast extract catalyzed the formation of activated sulfate, its activity was "lost" on fractionation; however, the activity could be reconstituted by combining two of the fractions, each of which was heat labile. This was the first suggestion that two proteins were involved in the activation. In back-to-back publications later that same year, Wilson and Bandurski[13] and Robbins and Lipmann[14] independently published their findings that the yeast fractions separately contained either ATP sulfurylase or APS kinase and that these enzymes catalyzed consecutive steps in the activation of sulfate. The chemical synthesis of APS, published in 1957,[15] provided access to large quantities of APS, which considerably aided these studies. By the end of 1958, both ATP sulfurylase and APS kinase had been purified from yeast, the extremely unfavorable equilibrium constant for the ATP sulfurylase reaction had been measured, and its metabolic implications discussed.[16,17]

III. CHEMICAL POTENTIAL, BONDING, AND REACTIVITY OF ACTIVATED SULFATE

A. Thermodynamics

The equilibrium constant for the ATP sulfurylase reaction can be written

$$K'_{eq} = \sum (APS) \cdot \sum (PP_i) / \sum (ATP) \cdot \sum (SO_4) = 1.1 \times 10^{-8} \quad (1)$$

where the total concentration of a given reactant is represented by the sum of the concentrations of the protonated and/or metal ion complexed forms of that species. Because this expression does not explicitly include either the proton or Mg^{2+} concentration, the equilibrium constant is apparent — indicated by the prime — and is a function of both pH and Mg^{2+} concentration. The value of 1.1×10^{-8} was determined under the following conditions and initial reactant concentrations: 10 m*M* ATP, 20 m*M* SO_4, 5.0 m*M* $MgCl_2$, 0.1 *M* Tris, pH = 8.0, T = 37°C.[17]

This equilibrium constant presents a considerable energetic barrier to the accumulation of activated sulfate. It is often suggested that the chemical potential of the APS kinase and inorganic pyrophosphatase reactions might energetically compensate the unfavorable ATP sulfurylase reaction. This scheme is outlined in Reactions 2–4. The ΔGs associated with these reactions are taken from published literature.[17–19] The data were determined at or extrapolated to pH = 8.0 and 5 m*M* Mg^{2+}, temperatures varied slightly.

	ΔG (Kcal/mol)	
$ATP + SO_4 \longleftrightarrow APS + PP_i$	10.8	(2)
$APS + ATP \longleftrightarrow ADP + PAPS$	–4.5	(3)
$PPi \longleftrightarrow 2Pi$	–7.8	(4)
Net (2 – 4): $2ATP + SO_4 \longleftrightarrow PAPS + ADP + 2P_i$	–1.5	(5)

Although the chemical potential of Reactions 3 and 4 is adequate to shift the thermodynamic balance toward formation of activated sulfate, the extent to which this potential is harnessed to drive PAPS formation is determined by how near equilibrium these reactions are. In exponentially growing cultures of *Escherichia coli*, the PP_i concentration is estimated at 0.5 m*M*.[20] Thus, Reaction 4, the major thermodynamic compensating reaction, appears to lie far from equilibrium *in vivo* and may not significantly drive APS formation. Assuming steady-state intracellular concentrations of PP_i, ATP, and SO_4 of 0.5 m*M*, 4 m*M*, and 10 m*M*, respectively, one calculates an intracellular APS concentration of 8.8×10^{-10} *M*. Although this demonstrates just how low the APS concentration might be, it assumes that the reactants behave ideally *in vivo* (i.e., their activity coeffi-

cients approach unity) and does not treat the possibility of subcellular compartmentalization or substrate channeling.

1. The Role of the Proton

Over the physiological pH range 7–9, the displacement of PP_i from ATP results in the donation of a proton from solvent to PP_i. This is because the most basic pK of the ATP tripolyphosphate chain is 7.0, whereas that of pyrophosphate is 9.0.[19] Thus, after or during its departure from ATP, PP_i will pick up a proton. The resulting pH dependence of the apparent equilibrium is shown in Figure 1.

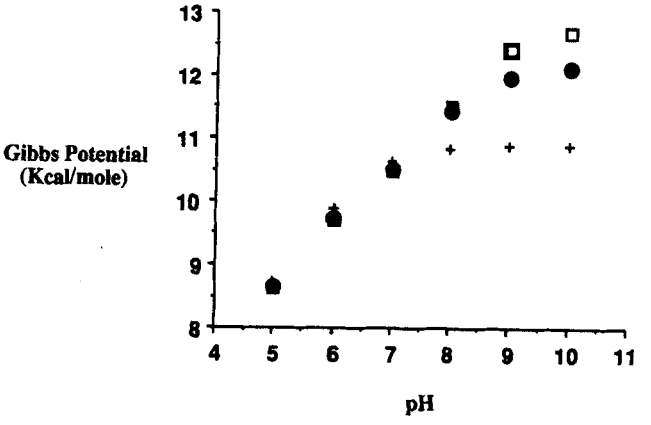


FIGURE 1. pH dependence of the Gibbs potential of the ATP sulfurylase reaction. The simulated conditions correspond to those used for the experimental determination of the equilibrium constant (i.e., 10 m*M* ATP, 20 m*M* SO_4). The chemical potential was simulated at 10 m*M* (+), 1.0 m*M* (•), 0.1 m*M* (□) Mg^{2+}. The equilibria used in the simulations are shown in Table 1. The simulations were performed using the Kinsim program.[21]

The ΔG for protonation of PP_i^{4-} is quite large, ~12 kcal/mol at 37°C,[22] and contributes substantially to the overall equilibrium. Most of this chemical potential is entropic and associated with the hydration of pyrophosphate; solvent is more ordered around the tetra- than the tri-anionic species. Ultimately then, solvent organization provides the lion's share of the thermodynamic potential used to drive PP_i protonation and APS formation. This discussion holds for any nucleotidyl transfer reaction involving displacement of PP_i from a tripolyphosphate chain, not withstanding other compensatory changes in reactant pKs.

Reaction 6 provides a measure of the relative transfer potential of PP_i and SO_4; clearly, SO_4 is a much better leaving group than PP_i. At pH 8.0, the pH at which the ΔG was determined, Reaction 6 is a composite of both the adenylyl transfer and pyrophosphate protonation reactions. It is instructive to separate these to assess the proton independent transfer potential of SO_4 vs. PP_i. This can be estimated by adding Reactions 6 and 7 as follows:

	ΔG (Kcal/mol)	
$H^+ + ATP^{4-} + SO_4^{2-} \longleftrightarrow APS^{2-} + HP_2O_7^{3-}$	10.8	(6)
$HP_2O_7^{3-} \longleftrightarrow P_2O_7^{4-} + H^+$	12	(7)
$ATP^{4-} + SO_4^{2-} \longleftrightarrow APS^{2-} + HP_2O_7^{3-}$	22.8	(8)

Equation 8, the proton independent reaction, shows that the group transfer potential of sulfate is

extraordinarily high compared with that of pyrophosphate and demonstrates just how well suited APS is in providing a chemically activated sulfuryl moiety.

2. Simulated Equilibria

To assess the influence of pH and Mg^{2+} concentration on the apparent equilibrium, it was simulated as a function of both of these variables — see Figures 1 and 2 and Table 1. For comparative purposes, the concentrations of ATP and SO_4 used in the simulations are the same as those used for the experimental determination of the equilibrium constant, 10 m*M* and 20 m*M*, respectively.[11] The equilibrium constants used for the simulations are presented in Table 1.[11,19] Simulations were performed using the KINSIM program.[21]

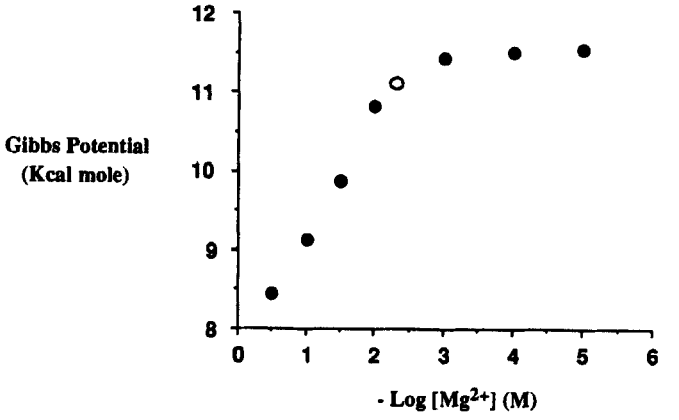


FIGURE 2. Mg^{2+} dependence of the Gibbs potential of the ATP sulfurylase reaction. The simulated conditions correspond to those used for the experimental determination of the equilibrium constant (i.e., 10 m*M* ATP, 20 m*M* SO_4). The open circle was simulated using the experimental Mg^{2+} concentration, 5 m*M*. The equilibria used in the simulations are shown in Table 1. The simulations were performed using the Kinsim program.[21]

Table 1
Reactions and Constants Used in the Equilibrium Simulations[a]

Reaction	Equilibrium constant
$MgP_2O_7P^{2-} \longleftrightarrow Mg^{2+} + P_2O_7^{4-}$	$3.89\ e^{-6}$
$MgHP_2O_7^{1-} \longleftrightarrow Mg^{2+} + HP_2O_7^{3-}$	$8.71\ e^{-4}$
$Mg_2P_2O_7^{0} \longleftrightarrow 2Mg^{2+} + P_2O_7^{4-}$	$4.57\ e^{-3}$
$H_2P_2O_7^{2-} \longleftrightarrow H^{+} + HP_2O_7^{3-}$	$7.59\ e^{-7}$
$HP_2O_7^{3-} \longleftrightarrow H^{+} + P_2O_7^{4-}$	$1.12\ e^{-9}$
$H_2ATP^{3-} \longleftrightarrow H^{+} + ATP^{4-}$	$1.12\ e^{-7}$
$HATP^{3-} \longleftrightarrow H^{+} + ATP^{4-}$	$8.71\ e^{-5}$
$MgATP^{2-} \longleftrightarrow Mg^{2+} + ATP^{4-}$	$1.00\ e^{-4}$
$MgHATP^{1-} \longleftrightarrow Mg^{2+} + HATP^{3-}$	$3.24\ e^{-2}$
$ATP^{4-} + SO_4^{2-} \longleftrightarrow APS^{2-} + P_2O_7^{4-}$	$1.10\ e^{-8}$

[a] Constants were determined at or adjusted to 25°C and 0.2 ionic strength. The equilibrium constants were obtained from References 17 and 19.

a. pH Effects

The apparent equilibrium constant for APS formation increases with increasing pH over the pH range 6–10 (see Figure 1 and previous discussion). This is caused primarily by the mass action associated with deprotonation of $HP_2O_7^{3-}$. Because the coordination of Mg^{2+} can result in deprotonation of $HP_2O_7^{3-}$, the pH and Mg^{2+} effects are coupled. At $Mg^{2+}/ATP << 1$, Mg^{2+} complex formation contributes little to the apparent equilibrium. At $Mg^{2+}/ATP \geq 1$, the Mg^{2+}-associated deprotonation of $HP_2O_4^{3-}$ causes the equilibrium to become pH independent between 7 and 10. At lower pH (5 to 6), the equilibria are Mg^{2+} independent because the major pyrophosphate species, $H_2P_2O_4^{2-}$, does not appreciably complex Mg^{2+}.

b. Mg^{2+} Effects

The influence of Mg^{2+} concentration on the apparent equilibrium at pH = 8.0 is shown in Figure 2. The curve is biphasic. The plateau is due to the negligible thermodynamic contribution of Mg^{2+} at low Mg^{2+}/ATP. The linear decrease in ΔG at $Mg^{2+}/ATP \geq 1$ is caused by the greater affinity of PP_i than ATP for Mg^{2+} and the accumulation of the Mg_2PP_i at the higher Mg^{2+} concentrations.

C. Bonding

Sulfur, a group VIB element, contains six electrons in its valence shell, which has the electronic configuration $(3s)^2(3P_x)^2(3P_y)(3P_z)$. Its atomic number, 16, is sufficiently large that the d- and p-orbital energies are close enough to allow the d-orbitals to participate extensively in bonding.[23] The higher symmetry of the d- compared with the s- and p-orbitals allows a greater diversity of bonding geometries and coordination number — sulfur forms covalent bonds to as many as six atoms. Qualitatively, sulfur-oxygen bonding in sulfate and, presumably, the $-SO_4$ group of activated sulfate involves both σ- and π- bonds. The σ-bonds are formed between the p-orbitals of oxygen and sp^3 hybridized orbitals of sulfur and provide the tetrahedral framework of the molecule. The π-bonds between the sulfur, d- , and oxygen, p- , orbitals form the basis of the familiar sulfate resonance bonding scheme. The π-bonds are believed to involve overlap of each of the d_Z^2 and $d_{x^2-y^2}$ orbitals of sulfur with four p-orbitals, one from each oxygen. Each of these molecular orbitals contributes 1/4 bond order to the S-O bond. The resultant π-bond order is 1/2 and the total bond order for each S-O bond is 1.5.

An interesting feature of the π-bonds is that they are believed to involve d-orbital contraction.[24–26] Various self-consistent field radial-wave functions for the d-orbitals of sulfur suggest that these orbitals are too large and diffuse to allow significant overlap and bonding with the much smaller p-orbitals of oxygen. However, oxygen is considerably more electronegative than sulfur, 3.5 and 2.5, respectively, on the Pauling electronegativity scale.[27] This difference in electronegativity is believed to cause an increased positive charge at the sulfur nucleus that, in turn, acts to draw down or contract the d-orbitals to a point where they form significant bonds with the oxygen p-orbitals, thereby reducing the positive charge at sulfur in accord with Pauling's principle of electroneutrality.[28]

D. Reactivity

The mechanism of sulfate ester hydrolysis is predominantly dissociative with slight associative character (see Figure 3).[29] The rate-limiting step is believed to be the scission of the S-O bond with the concomitant release of sulfur trioxide. This mechanism is based, in large measure, on the fact that the rate of hydrolysis is quite sensitive to the pK of the leaving group ($\beta = -1.2$ for hydrolysis of monanions of nitro- and dinitrophenylsulfates[30]) and insensitive to the pK of the incoming nucleophile ($\beta = 0.20$ for the nucleophilic reactions of *p*-nitrophenylsulfate with 3′, 2′, and 1′-amines[31]). These β values are similar to those for hydrolysis of the corresponding phosphate monoesters, which are well-characterized, dissociative reactions believed to involve the release of the metaphosphate monanion.

Studies with phenylphosphosulfate show that the unprotonated dianion slowly hydrolyzes in water; the protonated monanion hydrolyzes three

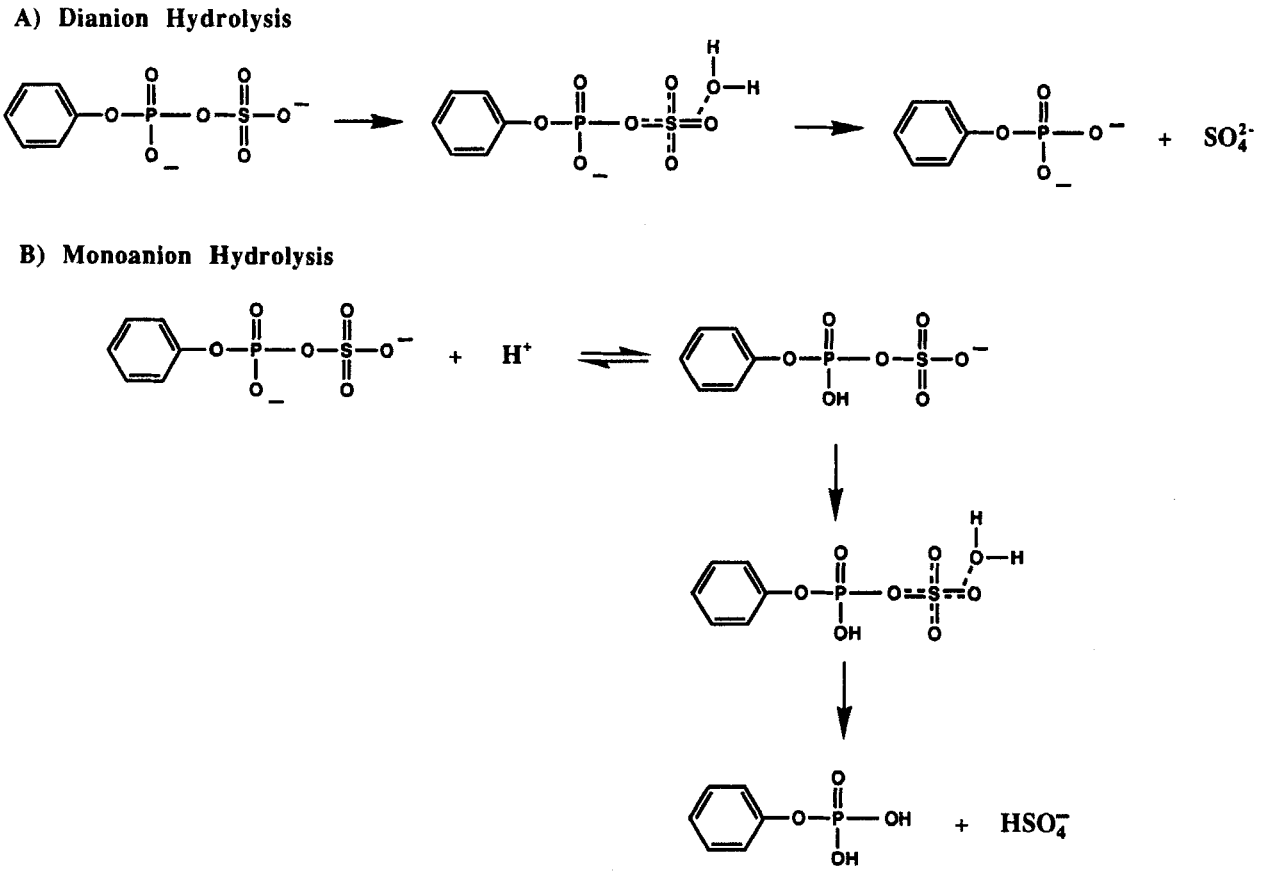


FIGURE 3. The chemical mechanism of phenylphosphosulfate hydrolysis in aqueous solution.

orders of magnitude faster.[29] This acid activation is due to the protonation of the phosphoryl oxygen in a step that is fast, compared with release of the sulfur trioxide. Approximately 5% of phosphosulfate cleavage occurs by a different mechanism: displacement of sulfate, presumably by nucleophilic attack, at the phosphoryl group in a reaction that mimics those known to occur for phosphate diesters.[29] Activation of sulfuryl group transfer by protonation of the phosphoryl group provides a means for the biological substrate, APS, to remain stable *in vivo* yet be catalytically activated for transfer by enzymatic protonation at the α-phosphoryl group.

Although there are many similarities between sulfate and phosphate hydrolysis, an important difference lies in the molecularity of the transition states.[29–31] The ΔS† for hydrolysis of phosphate mono- and dianions is typically slightly positive; in contrast, sulfate ester hydrolysis shows a negative ΔS†. Positive values are consistent with a dissociative mechanism in which the entropy of the system increases in going to the transition state because of the dissociation of the leaving group.[32] Reciprocally, a negative ΔS† is indicative of an associative mechanism in which the entropy of the transition state is decreased because of reactant association in the transition state.[32] Negative values for dissociative mechanisms have been observed and suggest a small degree of bond formation in the transition state. Rather than actual bond formation, this modest, negative ΔS† could reflect a reorganization of solvent in the transition state in response to redistribution of charge caused by the dissociating leaving group. Whatever the mechanism, sulfate ester hydrolysis appears to involve significantly more solvent participation in the transition state than phosphate ester hydrolysis.

IV. GENETICS AND BIOCHEMISTRY OF SULFATE ACTIVATION IN *E. COLI*

The metabolic pathways of sulfate utilization in *E. coli* and *Salmonella typhimurium* are well studied and extremely similar — analogous structural genes often showing 70–80% identity. The chemical steps and associated genes in the *E. coli* pathway are presented in Figure 4. Cysteine, the end product of the pathway, is synthesized in four phases: transport, activation, and reduction of sulfate, followed by incorporation into cysteine. Genetic lesions in this pathway often result in a nutritional requirement for cysteine; hence, the genetic acronym, *cys*. Currently, 18 genes are known to be involved in cysteine biosynthesis (see Table 2). Reviews describing the metabolic use of sulfate by other organisms can be found elsewhere.[2,3,33]

A. Regulation of *cys* Genes: Transcriptional Activation by the CysB Protein

The cysteine (*cys*) genes are found alone or in clusters that are well isolated from one another on the chromosome. As is often the case, many of these functionally related genes are organized into polycistronic transcriptional units. The activities of the proteins involved in the cysteine biosynthesis, are coincidentally regulated in response to specific sulfur nutrients. (They are inhibited by end products of the pathway, cystine, and cys-

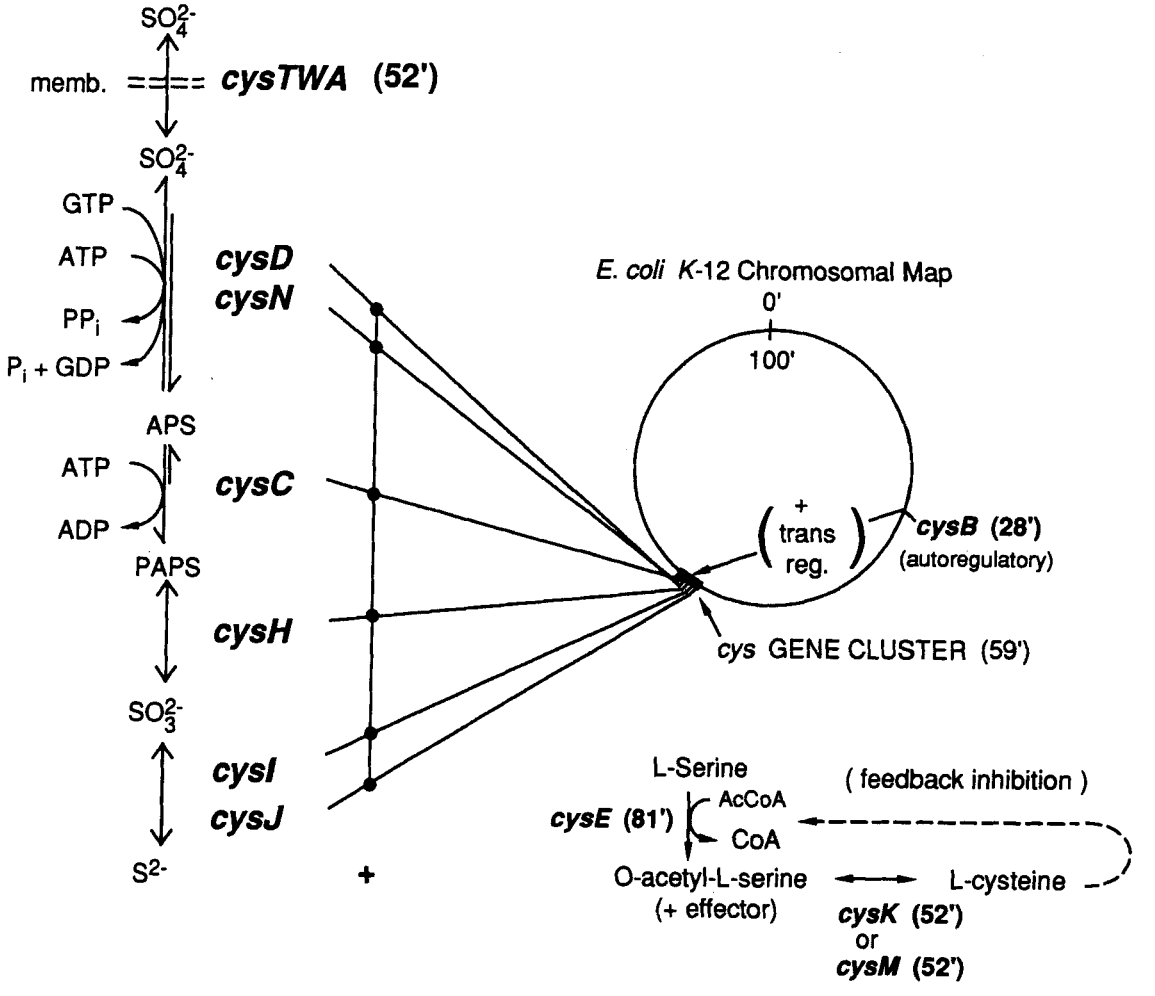


FIGURE 4. The genetics and biochemistry of sulfate utilization in *E. coli*.

Table 2
Genes and Proteins Involved in Cysteine Biosynthesis in *E. coli* and *S. typhimurium*

Gene	Protein/function	Cysteine regulon
cysA	ATP binding subunit of the membrane-associated thiosulfate/sulfate transport complex[a]	Yes
cysB	Transcriptional activator of the cysteine regulon	Yes
cysC	APS kinase	Yes
cysD	ATP sulfurylase, smaller subunit	Yes
cysN	ATP sulfurylase, larger subunit, putative GTP binding subunit	Yes
cysE	Serine transacetylase	Yes
cysG	Sulfite reductase, siroheme	?
cysH	PAPS sulfotransferase	Yes
cysI	Sulfite reductase, heme protein	Yes
cysJ	Sulfite reductase, flavoprotein	Yes
cysL	Selenate resistance	?
cysM	*O*-Acetyl-l-serine sulfhydrylase-B	Yes
cysP	Thiosulfate binding protein	?
cysQ	PAPS homeostatic protein[a]	?
cysS	Cysteinly tRNA ligase	?
cysT	Subunit of the membrane-associated thiosulfate/sulfate transport complex[a]	Yes
cysW	Subunit of the membrane-associated thiosulfate/sulfate transport complex[a]	Yes
cysZ	Sulfate transport (possibly sulfate binding protein[a])	Yes
sbp	Sulfate binding protein (possibly *cysZ* [a])	Yes
?	Cystine transport system	Yes

[a] Function inferred from sequence.

teine, and stimulated by metabolic precursors of cysteine: sulfate and *O*-acetyl-L-serine.) The known exceptions are serine transacetylase and the CysB protein that regulate the pathway. The cysteine regulon includes all of the operons in the cysteine biosynthetic pathway. It is now well established that most, if not all, of the operons in the regulon are transcriptionally activated by the CysB protein and the inducer *O*-acetyl-L-serine, the immediate precursor of cysteine.[34,35]

cysB has been cloned and sequenced, the encoded protein has been overexpressed and purified to apparent homogeneity.[36,37] It is a 144-kDa homotetramer that plays a dual regulatory role: it stimulates transcription from promoters in the regulon while inhibiting transcription from its own promoter. CysB is a member of the LysR family of bacterial, transcriptional activators.[38] The DNA sequence of each of the nine family members predicts a helix-turn-helix motif beginning roughly 23 residues from its amino-terminus. The six of the family members that have well-defined function are transcriptional activators whose activities are regulated by small molecule inducers. Four, possibly five, of these regulate amino acid biosynthetic operons and at least four are autoregulatory.

The mechanism of transcriptional activation by CysB and its inducers has been studied by DNA footprinting, gel shift, and transcription runoff experiments using purified reagents at several *cys* operon promoters (i.e., *cysB*, *cysJIH*, *cysK*, and *cysPTWA*).[39–42] CysB binds with reasonably high affinity (1–10 n*M*) at each of these promoters; however, transcriptional activation requires *O*- or *N*-acetyl-L-serine, which also increases the affinity and specificity of the CysB promoter interactions. The influences of sulfide,[41] cysteine,[41]

thiosulfate,[42] and *O*-acetyl-L-serine[40] on the ability of CysB to activate transcription *in vitro* have been tested. Thiosulfate and sulfide inhibit both transcriptional activation and binding. Interestingly, these binding effects occur only in the presence of *N*-acetyl-L-serine. Cysteine has no effect on binding or activation at concentrations as high as 5 m*M*.

In contrast to the positively regulated operons, the *in vivo* concentration of CysB protein and its mRNA are insensitive to growth on different sulfur nutrients.[39,43] The CysB binding site at its own operon overlaps the canonical –10 and –35 RNA polymerase binding site. Thus, CysB appears to downregulate its own expression by competing with RNA polymerase for the *cysB* promoter. Its expression is self-limiting; the higher its concentration, the slower its expression at saturation, transcription stops. *In vitro*, *N*-acetyl-L-serine causes release of CysB from its promoter, allowing transcription to proceed.

B. The DNA Sequence of the Sulfate Activation Operon from *E. coli* K-12

The DNA sequence of the sulfate activation operon has been determined recently.[44] In addition to the expected findings (i.e., the organization of structural genes and promoter elements, the nature of intergenic regions, the amino acid sequence of the encoded polypeptides, and the likely transcription termination signals), the sequence analysis has predicted novel catalytic functions for ATP sulfurylase with far-reaching metabolic implications. It also provided a likely structural framework for one of the ATP sulfurylase subunits and offered important insight into the symbiogenesis of the nitrogen fixing, soil bacterium *Rhizobium meliloti* and its host, alfalfa.

1. The Operon Anatomy

a. The Promoter

A physical map of the sulfate activation operon is shown in Figure 5.[44] The expression of ATP sulfurylase and APS kinase is regulated by the transcriptional activator, CysB, and the inducer, *O*-acetyl-L-serine. A comparison of the known CysB operator sequences with the *cysDNC* promoter sequence yielded the putative operator shown in Figure 6. Typical of positive operator sequences, the putative *cysDNC* operator is AT rich.[45] The –35 and –10 RNA polymerase consensus binding sequences,[46] Shine-Dalgarno sequence,[47] and the operon transcription start site,

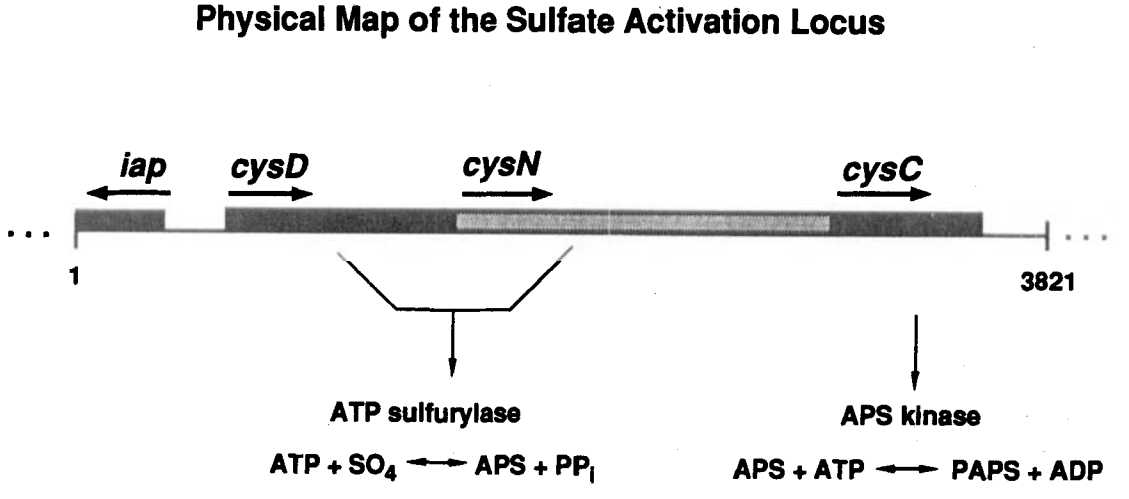


FIGURE 5. The physical map of the sulfate activation locus. *cysN* and *cysD* encode ATP sulfurylase; *cysC*, APS kinase. Arrows beneath gene symbols indicate the 3′-5 orientation of that gene. The catalytic activities associated with these enzymes are shown.

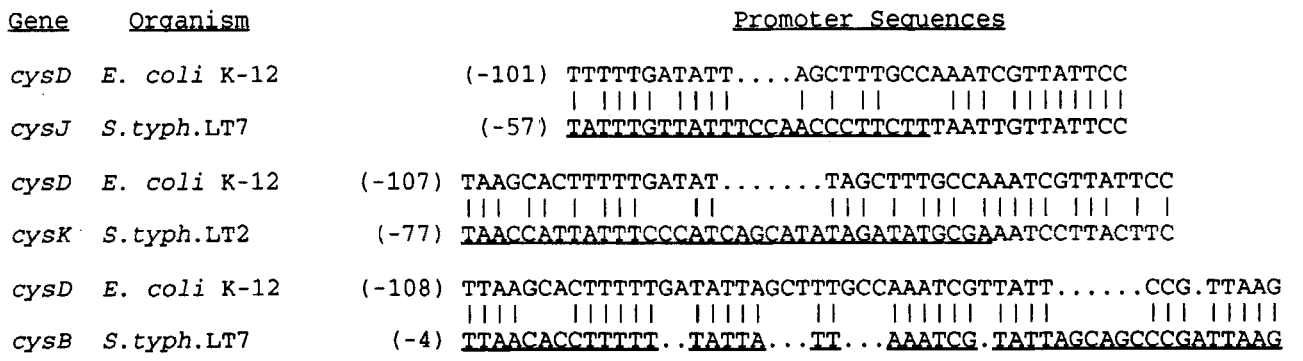


FIGURE 6. Sequence comparison of *cys* promoters that are regulated by the CysB protein. The underlined bases are protected by CysB in footprinting studies. The numbers in parenthesis indicate the position of the 5′-most base in the sequence relative to the major transcriptional start site for that gene.

mapped by primer extension,[48] have been determined.[44]

b. Translational Coupling

The translational termination and initiation sequences of the *cysD-cysN* and *cysN-cysC* gene pairs overlap; the termination and initiation codons are separated by 1 base at the *cysD-cysN* junction, the *cysN-cysC* pair overlap by 1 base (see Figure 7). This imbricated motif, known as *translational coupling*, is believed to enhance translational efficiency and allow stoichiometrically well-defined — often 1:1 — expression of contiguous genes.[49,50] The *cysN* and *cysC* Shine-Dalgarno sequences, believed to be important for proper alignment during translational reinitiation, are indicated in Figure 7.

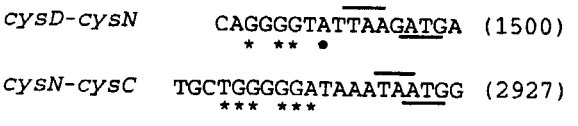


FIGURE 7. Translational coupling of the *cysDN* and *cysNC* gene pairs. Translational stop and start codons are overlined and underlined, respectively. Asterisks indicate possible sites for translational reinitiation.

c. Transcription Terminator

Transcription terminators are classified as rho-dependent or rho-independent, according to whether termination is facilitated by the rho protein.[51] Most rho-independent terminators share well-defined sequence traits, including a palindromic G/C rich sequence capable of forming a stable RNA hairpin, immediately preceding 4–8 contiguous Us that are closely followed by UCUG-like sequences.[52] Hairpin formation during transcription is important for the pausing and subsequent dissociation of RNA polymerase.[51] A sequence that conforms well to the features of the rho-independent terminator can be found ~90 bp downstream from the translational stop codon of *cysC*. Termination at this site would result in a polycistronic message containing all the genes known to reside within the sulfate activation operon. The sequence of this putative terminator is shown in Figure 8.

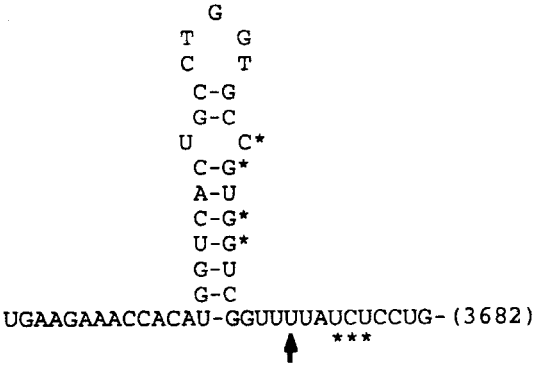


FIGURE 8. A putative transcriptional terminator for the sulfate activation operon.

V. THE ROLE OF GTP IN SULFATE ACTIVATION

A. GTPase-Mediated Activation of ATP Sulfurylase

GTP-binding proteins, or GTPases, comprise a superfamily of metabolic regulatory proteins.[53,54] Members of this diverse family are involved in many cellular processes, including signal transduction,[55–58] vesicular trafficking,[59] protein synthesis,[60] and cellular proliferation and differentiation.[61] GTPases often act at an early stage in a metabolic cascade by binding to and stimulating the activity of a target enzyme. The majority of the known GTPases have been identified solely on the basis of sequence similarity with other known GTP-binding proteins. To date, few of the roughly 100 known, or presumed, GTPases have well-defined targets. In cases where the target is known, purification and reconstitution of the activation system often present major technical obstacles.

It is frequently, if not exclusively, the GTP-bound form of the GTPase that is active in stimulating a target's activity; the GDP-bound form is inactive.[53,54] The cell typically uses two classes of proteins to regulate the activity of a given GTPase: guanine nucleotide release proteins (GNRPs) and GTPase-activating proteins (GAPs). GNRPs, as their name implies, facilitate the release of guanine nucleotide (GDP) from the GTPase. GDP release is often the rate-limiting step in the regeneration of the active GTPase. GAPs increase the k_{cat} for GTP hydrolysis — binding to a GAP inactivates the GTPase. The k_{cat} for GTP hydrolysis temporally regulates target activation by defining the time interval during which activation can occur. GNRPs and GAPs determine when the GTPase is activated and how long it will remain active, respectively.

The primary sequences of GTP-binding proteins show regions of high similarity separated by intervening sequences of well-defined length. These findings were codified into a consensus GTP-binding sequence that has proven extremely useful in the identification of new members of most, but not all, classes of GTP-binding proteins. The original consensus sequence contained three similarity elements with constraints on the interelement spacing.[62,63] These three elements reside in regions of the protein that contact GTP. The consensus sequence has been expanded and refined recently.[64]

A comparison of the GTP-binding consensus sequence with the primary sequence of CysN and several other known GTP-binding proteins is shown in Table 3. When the functional implications of the GTP-binding site were investigated, it was found that the initial rate of APS formation was stimulated 116-fold at a saturating concentration of GTP (see Figure 9).[68] The stimulation of APS formation is concomitant with the hydrolysis of GTP. The GTPase activity is stimulated by the presence of ATP and/or SO_4. This is the first demonstration that the sulfate activation pathway is a target for GTPase activation in any species and establishes a meta-

Table 3
Sequence Comparison of the Putative GTP-Binding Region of ATP Sulfurylase with Other Known GTP-Binding Proteins and a Consensus GTP-Binding Site.

Gene	Protein	Organism	Element I	IES[a]	Element II	IES	Element III	Ref.
cysN	ATP sulfurylase	*E. coli*	GSVDDGK	73	DTPG	51	NKMD	44
tufA	Ef-Tu	*E. coli*	GHVDHGK	56	DCPG	51	NKCD	65
tufB	Ef-G	*E. coli*	AHIDHGK	65	DTPG	50	NKMD	66
	EF1-α	*H. sapien*	GHVDSGK	71	DAPG	58	NKMD	67
	Consensus sequence		GXXXXGK	40–80	DXXG	40–80	NKXD	62

[a] Interelement spacing (IES) is the number of amino acids between consecutive elements.

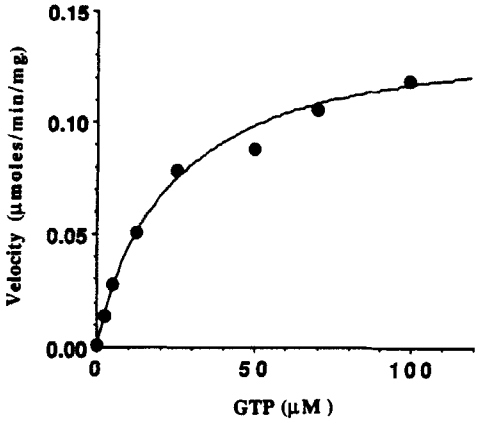


FIGURE 9. GTP stimulation of ATP sulfurylase activity. The assay mixtures contained GTP, at the concentration indicated, and ATP (5.0 m*M*), $^{35}SO_4$ (2.5 m*M*, SA = 120 Ci/mol), KCl (39 m*M*), PEP (0.54 m*M*), $MgCl_2$ (1 m*M* + [total nucleotide]), HEPES-K^+ (50 m*M*), pH 8.0, ATP sulfurylase (20 μg/ml), pyruvate kinase (27 μm/μl), and inorganic pyrophosphatase (0.12 μm/μl).

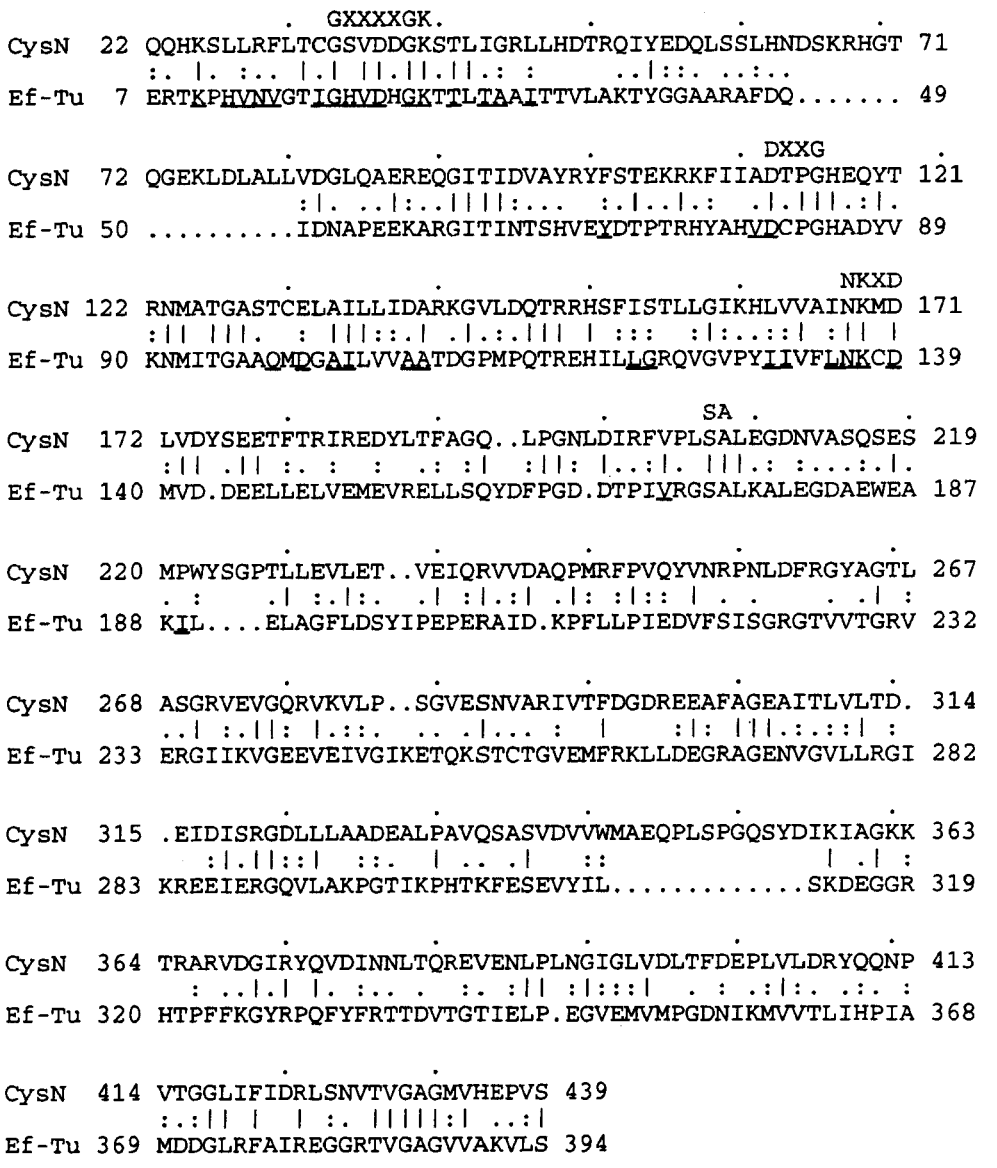
bolic connection between GTP and sulfate activation that could function *in vivo* to regulate the assimilation of sulfur.

In several GTP-hydrolyzing systems, the energy released by GTP hydrolysis is used to thermodynamically drive another chemical process. This coupling occurs by one of two different mechanisms: direct chemical coupling in which chemical groups are exchanged between GTP and the other reactants (e.g., succinyl-CoA synthetase[69,70] and PEP carboxykinase[71]), or conformational coupling, which requires an ordered sequence of chemical events to wed GTP hydrolysis to the coupled process[72] (e.g., protein translation).[73–75] Either mechanism could contribute as much as ~–7 kcal/mol of chemical potential to drive APS formation (discussed in the thermodynamic section). Preliminary work in our laboratory clearly shows that GTP hydrolysis and APS formation are energetically linked. The mechanism of this coupling is currently under investigation.

There are several properties of the ATP sulfurylase-associated GTPase in addition to its metabolic context that appear unique among the members of the GTPase superfamily. The well-characterized GTPases are different from their target protein(s), and their inherent GTP-hydrolyzing activity is stimulated by interaction with GAPs and GNRPs. In contrast, the hydrolytic activity of the ATP sulfurylase-GTPase is stimulated by the small molecule substrates for the reaction that is regulated, and the GTPase and target activities are tightly associated in a single enzyme that is composed of two types of subunits. Current models of GTPase/target interactions suggest that the CysN subunit is the GTPase and CysD is the catalytic subunit of ATP sulfurylase.

B. Implications from Known GTPase Structures

The three-dimensional structures of the nucleotide-bound complexes of two known GTPases, ras and the GTP-binding domain of elongation factor Tu (EF-Tu),[76,77] have been determined. Although the structures of these molecules are remarkably alike, their amino acid sequences are not. Their GTP-binding domains are ~17% identical.[78,79] It appears that although nature has allowed the amino acid sequences of these proteins to diverge considerably, it has preserved the three-dimensional architecture that underlies their functional relatedness. Optimal alignment of the RAS and EF-Tu structures results in a positional deviation of 1.4 Å (rms) at a given α-carbon; however, the structural elements involved in nucleotide binding are virtually identical. After careful analysis of the sequences and structures of RAS and EF-Tu, Valencia et al.[79] proposed a set of core residues that are structurally important for the proper functioning of these and probably other GTPases. The criteria used for the selection of core residues were that they lie in the conserved primary sequences of the GTPase superfamily, that they have a large number of intraresidue contacts, and that they are located in regions that are structurally conserved between EF-Tu and RAS. A comparison of the primary sequences of CysN and EF-Tu is shown in Figure 10. The comparison metrics for this overall alignment were 27% identity and 49% similarity; when the core residues were



Comparison Metrics		
Comparison	% Identity	% Similarity
CysN with Ef-Tu	27	49
CysN with Ef-Tu Structural Core	43	71

FIGURE 10. A comparison of the primary sequences of CysN and Ef-Tu. The GTP binding consensus elements are placed over their corresponding regions in CysN and EF-Tu. The structural core residues of Ef-Tu are underlined, and the alignment metrics are given.

used as the query sequence, the metrics increased to 43% identity and 71% similarity. These comparisons suggest that a common GTP-binding structural motif provides the basis for the functional and primary sequence similarities of ATP sulfurylase with other known GTPases.

VI. SULFATE ACTIVATION/GTPASES IN NITROGEN FIXATION

Rhizobium melilotti, a soil bacterium, shares a symbiotic relationship with legumes, a large family of angiosperms.[80,81] The bacterium converts dinitrogen to ammonia for the plant, which, in turn, supplies it with photosynthetically derived metabolites. The interactions between the plant and bacterium that led to symbiosis are complex and fascinating. Plants communicate with soil bacteria by releasing chemical signals from their roots. Among these signals are chemoattractants, which draw the bacterium toward the root, and bacterial gene inducers. Once the bacterium is sufficiently close to the root, the inducers activate specific sets of bacterial genes. One such set of genes, the *nod* genes, is essential for the formation of plant root nodules. This growth response is one of a number of physiological changes undergone by both the plant and bacterium that ultimately result in the transport of the bacterium into the plant's cytosol, its dedifferentiation, and the initiation of its nitrogen-fixing activity.

The *nodP* and *nodQ* genes from *R. melilotti* are essential for nodulation.[82] They belong to the subset of *nod* genes that are responsible for determining the bacterium's host specificity. Based on the similarity of NodPQ and CysDNC amino acid sequences (see Figure 11), it appeared likely that the *nod* genes encoded ATP sulfurylase and APS kinase. Subsequently, this was verified experimentally.[83,84] The GTP-binding elements of NodQ and CysN are identical, suggesting that the nod system would also be activated by GTP. Experiments using partially purified NodPQ showed that this was indeed the case. The initial velocity of activated sulfate formation was stimulated minimally 50-fold by the presence of GTP. Furthermore, no detect-

A

```
CysD   1 MDQIRLTHLRQLEAESIHIIREVAAEFSNPVMLYSIGKDSSVMLHLARKA 50
NodP   1    MSLPHLRRLEAEAIHVIREVVATFSNPVVLYSIGKDSSVLLHLAMKA 47
CysD  51 FYPGTLPFPLLHVDTGWKFREMYEFRDRTAKAYGCELLVHKNPEGVAMGI 100
NodP  48 FYPAKPPFPFLHVDTKWKFREMIEFRDRMARELGFDLLVHVNQDGVEQGI 97
CysD 101 NPFVHGSAKHTDIMKTEGLKQALNKYGFDAAFGGARRDEEKSRAKERIYS 150
NodP  98 GPFTHGSNVHTHVMKTMGLRQALEKYGFDAALAGARRDEEKSRAKERIFS 147
CysD 151 FRDRFHRWDPKNQRPELWHNYNGQINKGETIRVFPLSNWTEQDIWQYIWL 200
NodP 148 IRSAQHGWDPQRQRPEMWKTYNTRVGQGETMRVFPLSNWTEFDIWQYILR 197
CysD 201 ENIDIVPLYLAAERPVLERDGMLMMIDDNRIDLQPGEVIKKRMVRFRTLG 250
NodP 198 EEIPIVPLYFAARRPVVKREGMLIMVDDDRMPIQPEEEVTEQLVRFRTLG 247
CysD 251 CWPLTGAVESNAQTLPEIIEEMLVSTTSERQGRVIDRDQAGSMELKKRQG 300
NodP 248 CYPLTGAVESDAVTVPEILREMLTVRTSERQSRLIDTDEVGAMEKKKREG 297
CysD 301 YF 303
NodP 298 YF 299
```

A

FIGURE 11. Comparison of *cysDNC* and *nodPQ*. (**Panel A**) Comparison of *cysD* with *nodP* — alignment metrics and schematic peptide alignments are shown. (**Panel B**) Comparison of *cysN* and *cysC* with *nodQ*.

B

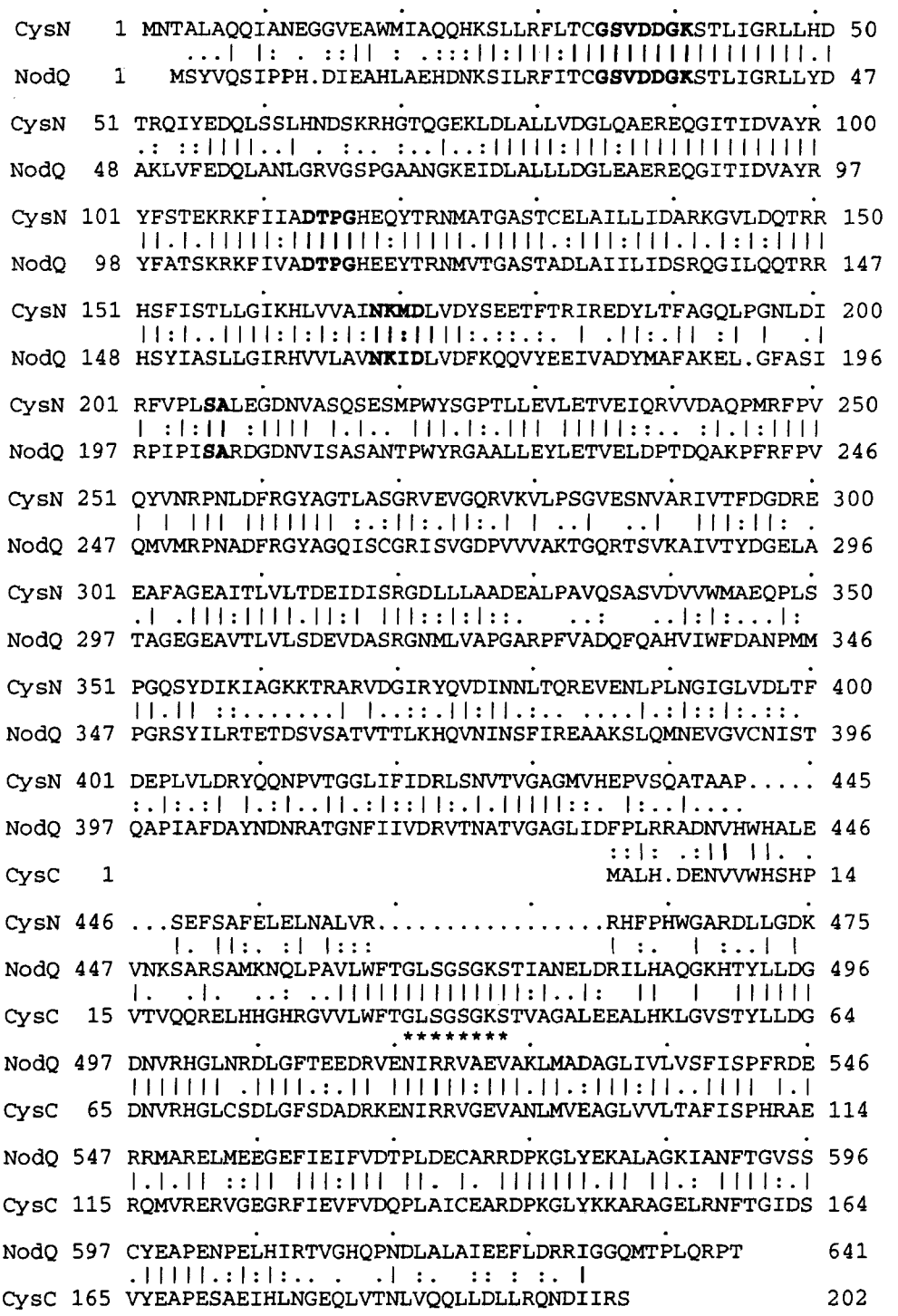


FIGURE 11B

able activated sulfate was formed in the absence of GTP, suggesting that GTP is essential for root nodule formation and symbiogenesis.[84]

NodP appears to be the smaller subunit of ATP sulfurylase; it is 61% identical and 81% similar to CysD and their lengths differ by only

three amino acids.[44] NodQ contains homologues of CysN and CysC, suggesting that it encodes both the larger subunit of ATP sulfurylase and APS kinase.[44] Western blots using antibodies raised against CysC, also active against NodQ, show that the NodQ is not proteolyzed into separate APS kinase and ATP sulfurylase fragments. The ATP sulfurylase and APS kinase activities of the NodPQ system comigrate during size exclusion and anion exchange chromatography, and the partially purified complex converts ATP and SO_4 into PAPS and ADP with little or no APS formation. It appears that the ATP sulfurylase and APS kinase activities in *R. meliloti* are associated in a multifunctional protein complex, the so-called *sulfate activating complex* (SAS).[84]

The fusion of ATP sulfurylase and APS kinase into a multifunctional complex is of particular interest because these enzymes are considered likely candidates to channel APS (i.e., to transfer APS directly between the enzymes without releasing it into solution).[85] This view is fostered by the extremely unfavorable equilibrium constant for APS formation and the potent inhibition of APS kinase and ATP sulfurylase by APS.[86] These obstacles to the formation of activated sulfate could be overcome by channeling APS; this might circumvent the equilibrium problem by using the protein surface as the APS "solvent" and avoid the inhibition by appropriate steric constraints in the transfer step. It appears that the *Penicillium chrysogenum* enzymes do not channel APS; however, these enzymes have not been reported to form a complex.[85] It will be interesting to test the channeling hypothesis in the multifunctional systems.

A molecular link between host specificity and sulfate activation in *R. melilotti* has been established recently.[87] A sulfated phytohormone, released by the bacterium in response to the *nod* gene inducers, has been purified. It is an *N*-acetylated glucose tetramer containing a single sulfuryl group at carbon 6 of the reducing glucosyl moiety. The pure compound causes distortion and branching of alfalfa root hair, a symbiogenic plant activity. The unsulfated morphogen, no longer active on alfalfa roots, becomes active on a different host, vetch. Hence, the sulfuryl group modification determines host specificity.

The *nodPQ* genes of another nitrogen-fixing soil bacterium, *Azospirillum brasilense*, are also extremely similar to *cysDNC*[84] and *nodPQ*.[88] The sequence similarities strongly suggest that the *Azospirillum* genes also encode a multifunctional ATP sulfurylase/APS kinase protein complex that will be activated by GTP. The striking similarities between these Gram-negative organisms suggest that GTP-dependent stimulation of sulfate activation may be widespread among these species.

VII. ENZYMOLOGY OF THE SULFATE-ACTIVATING ENZYMES

A. ATP Sulfurylase

Although ATP sulfurylase (EC 2.7.7.4. ATP:sulfate adenylyltransferase) has been purified, or partially purified, from a wide variety of sources,[17,89–100] only the *P. chrysogenum* and rat liver enzymes have been mechanistically characterized in detail. The *P. chrysogenum* enzyme is a hexamer of identical ~69 kDa subunits. Initial velocity kinetic work, including product and dead-end inhibition studies, and ligand-binding measurements suggest that ATP and SO_4 add randomly to the enzyme; APS and PP_i add in a preferred order with APS binding first (see Figure 12).[101,102] The rat liver enzyme appears to be a dimer of 62-kDa subunits. Its mechanism is the

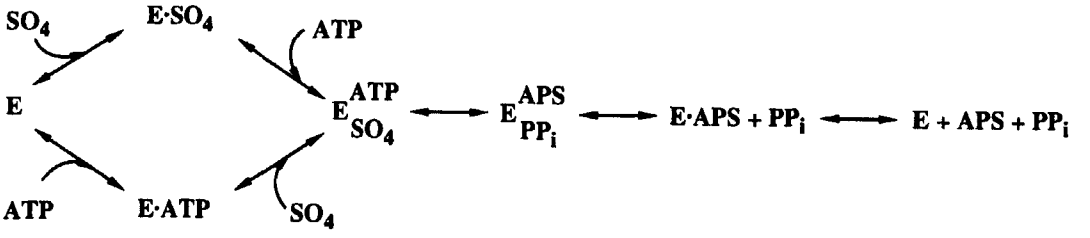


FIGURE 12. The chemical mechanism of ATP sulfurylase purified from *P. chrysogenum*.

same as that of the *P. chrysogenum* enzyme.[100] The stereochemical course of the reaction catalyzed by the ATP sulfurylase purified from yeast has been determined. It proceeds with inversion of stereochemistry at the α-phosphoryl moiety, suggesting that the reaction follows an in-line nucleophilic attack by SO_4 at the α-phosphoryl group of ATP.[103]

When the *Penicillium* enzyme is treated with any of several sulfhydryl reactive reagents, one cysteine/subunit is modified. The modified enzyme behaves as though a K-type allosteric effector is bound to it (K-type effectors modify K_m, not V_{max}).[104] ATP and sulfate bind to the modified enzyme in a positive cooperative fashion resulting in sigmoidal plots of velocity vs. substrate concentration. Segel and co-workers,[105] responsible for all of the work done with the *Penicillium* enzyme, propose a minimal two-site model to explain this allostery in which the hexameric enzyme behaves as a trimer of noninteracting dimers — substrate binding to one subunit of a given dimer only influences binding to the other subunit of that same dimer. Alternative models are discussed elsewhere.

The allosteric behavior prompted a search for possible *in vivo* allosteric regulators. PAPS was found to mimic the effects of the sulfhydryl modification. The apparent K_i for PAPS ranges from ~35–200 μ*M* over a presumed physiological range of ATP and sulfate concentrations.[106] A comparison of PAPS inhibition among enzymes purified from fungal and nonfungal sources suggests that the allosteric regulation may be unique to filamentous fungi.[106]

B. APS Kinase

APS kinase (MgATP:APS 3′-phosphotransferase, EC 2.7.1.25) catalyzes the transfer of the γ-phosphoryl group of ATP to the 3′ position of the APS ribose ring, yielding PAPS (phosphorylated APS) and ADP (see Reaction 9).

$$\text{APS} + \text{ATP} \longleftrightarrow \text{PAPS} + \text{ADP} \qquad (9)$$

This reaction requires divalent, not monovalent, cations. Because APS kinase does not catalyze formation of the high-energy phosphoric-sulfuric acid anhydride bond, which is the chemical basis of sulfate activation, it does not formally activate sulfate; yet, it is ubiquitous in organisms that carry out assimilatory sulfate reduction. Although the metabolic use of this kinase is not yet clear, the enzyme does use chemical potential of the β-γ-bond of ATP to drive PAPS, or activated sulfate, synthesis. Although at the outset this seems useful, PAPS is believed to be toxic; in this case, too much of a good thing could prove ruinous.[107,108]

APS kinase has been purified to apparent homogeneity from *P. cyrysogenum*,[109] *E. coli*,[18] rat liver,[110] and rat chondrosarcoma.[90] The *E. coli* enzyme is a dimer of identical 21-kDa subunits that forms a tetramer when it is phosphorylated. The native *P. chrysogenum* enzyme, 60-kDa, is composed of identical 30-kDa subunits. The molecular masses reported for the rat liver and chondrosarcoma enzymes are complicated because of protein aggregation.

The kinetic mechanisms of the *E. coli* and *P. chrysogenum* APS kinases are well characterized (see Figure 13 and Table 4). The *E. coli* enzyme is well adapted to handling the low *in vivo* concentrations of APS. It is an extremely efficient catalyst, PAPS formation is rate limited by reactant diffusion and the K_m for APS is quite low, <250 n*M*.[18] Both mechanisms are ordered in substrate addition and product release; ATP binds first, ADP releases last. The mechanisms differ sharply on two principle counts: (1) the mode of APS inhibition and (2) the nature of the chemical steps.[18,111] Although potent substrate inhibition of APS kinase has often been noted, its mechanistic basis has rarely been pursued. This is due, in part, to the technical obstacles associated with developing assays that operate at the low, subinhibitory, concentrations of APS. Kinetic and ligand binding studies suggest that a dead-end E·APS·ADP complex causes the inhibition of the *P. chrysogenum* enzyme. The *E. coli* enzyme, however, is inhibited by formation of a binary, E·APS, complex. Perhaps a more fundamental difference between these mechanisms is that a covalent E~P intermediate, believed to be essential for PAPS synthesis, is detected in the *E. coli* enzyme but not in the *P. chrysogenum* APS kinase. This modification, which occurs at serine-109, is stable. It can be isolated in a 1:1 complex with the enzyme and subsequently reacted with APS to form

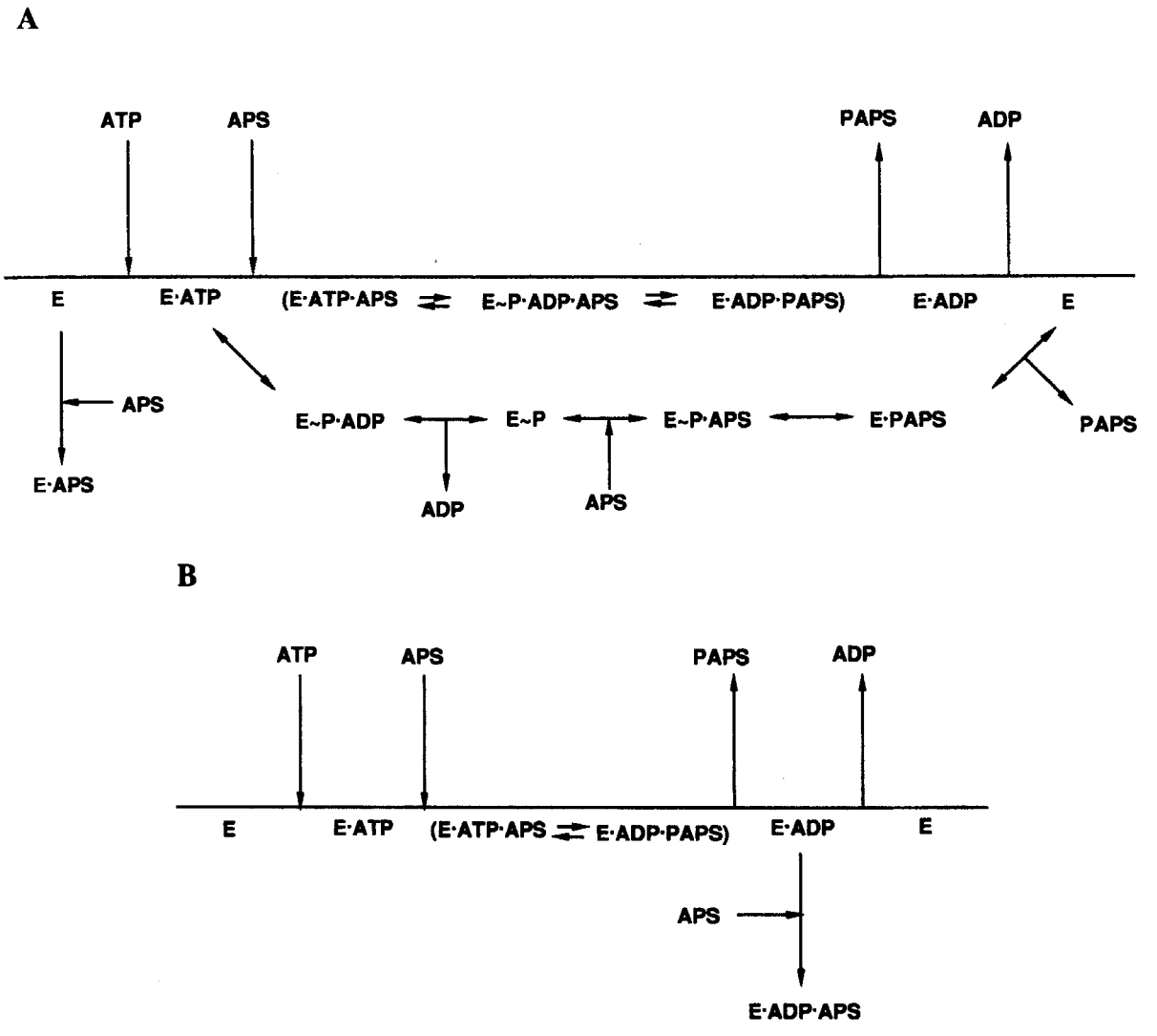


FIGURE 13. The chemical mechanism of APS kinase from (**Panel A**) *E. coli* and (**Panel B**) *P. chrysogenum.*

Table 4
Kinetic Constants for APS Kinase Isolated from *E. coli* and *P. chrysogenum*

	Source of APS kinase	
	E. coli	*P. chrysogenum*
Kinetic constant	**Value**	
Forward reaction		
K_m APS	~0.25 (μ*M*)	1.4 (μ*M*)
K_i APS	0.4 (μ*M*)	23 (μ*M*)
K_m ATP	10 (μ*M*)	1500 (μ*M*)
V_{max} (forward)	153 (μmol/min/mg)	39 (μmol/min/mg)
k_{cat}	54 s^{-1}	19 s^{-1}
k_{cat}/K_m APS	2.2 M^{-1} s^{-1} (× 10^8)	0.14 M^{-1} s^{-1} (× 10^8)
Reverse reaction		
K_m ADP	130 (μ*M*)	<<560 (μ*M*)
K_m PAPS	370 (μ*M*)	8 (μ*M*)
V_{max} (reverse)	0.63 (μmol/min/mg)	0.16 (μmol/min/mg)
k_{cat}	0.22 s^{-1}	0.078 s^{-1}

PAPS.[112] Interestingly, the aggregation of the enzyme depends on its phosphorylation. The E~P enzyme is dimeric; the dephosphorylated species is a tetramer. The preferred kinetic mechanism of the *E. coli* enzyme is determined by the relative rates of APS addition and ADP release. The *E. coli* enzyme displays sequential or ping-pong behavior, depending on whether the concentration of APS is high enough to bind and react with E~P·ADP quickly compared with ADP release. If ADP release is slow, sequential profiles are observed; fast release results in ping-pong kinetics.

VIII. MAMMALIAN SIGNIFICANCE

3′-Adenosine 5′-phosphosulfate (PAPS) is the only known sulfuryl group donor in mammalian metabolism. In the transfer reactions, the $-SO_3$ moiety is covalently attached to carbohydrate hydroxyls or protein tyrosine residues. Sulfation is an extremely widespread modification. The transfer is catalyzed by the class of enzymes known as the PAPS sulfotransferases. Table 5 presents a list of metabolites for which the functional consequences of sulfation are known. The sulfation of small molecules often acts as a switch to toggle "on" or "off", or otherwise change, the function of the molecule. Sulfation of proteins is known to promote protein-protein association; in which case, the modification occurs at a tyrosine(s) at the interface of the associating proteins. In the majority of cases, the function of sulfation has not been investigated. There are many recent articles and reviews dealing with the importance of sulfation in mammalian metabolism.[5,125–132] Here we have selected a few detailed examples that demonstrate the ways in which sulfation is used to regulate metabolism.

A. Sulfation Occurs in the *Trans*-Golgi

The posttranslational maturation of proteins synthesized at the rough endoplasmic reticulum begins there and continues as the proteins are

Table 5
Compounds with Functionally Significant $-SO_3^-$ Modifications

Compound(s)	Activation (+) inactivation (–)	Ref.
Catecholamine neurotransmitters		
Epinephrine	(–)	113
Norepinephrine	(–)	113
Opiate hormone		
Leu-enkephalin	(–)	114
Peptide hormones		
Gastrin	(+)	115
Cholecystokinin	(+)	116
Steroid hormone		
Estrogen	(–)	117
Hemostasis		
Factor VIII (antihemophilic factor)	(+)	118
Hirudin (anticoagulant)	(+)	119
Heparin	(+)	120
Dermatin sulfate	(+)	121
Immune system		
Complement C4	(+)	
Connective tissue		
Heparin sulfate	(+)	122
Therapeutic drugs		
Minoxidil	(+)	123
CRE 10904 (antihypertensive)	(+)	124

vesicularly transported to and through the *cis*-, medial-, and *trans*-cisternae of the Golgi apparatus.[133] These modifications are compartmentalized within the Golgi cisternae. Sulfation of tyrosine residues is among the last modifications to occur, prior to passage from the *trans*-Golgi.[134] This reaction, represented by Reaction 10, is catalyzed by the enzyme tyrosine protein sulfotransferase (TPST).

$$\text{protein - tyr} + \text{PAPS} \longleftrightarrow \text{PAP} + \text{protein - tyr - } SO_3 \quad (10)$$

TPST has been purified to apparent homogeneity from Golgi-enriched membranes of the bovine adrenal medulla.[135] It is a sialylated, integral membrane protein of subunit molecular weight of 50–54 kDa. The activity of the enzyme requires Mg^{2+} and is optimal at a slightly acidic pH (pH = 6), the pH of the *trans*-Golgi cytoplasm. The apparent K_m for PAPS is 1.4 μM; for native protein substrates, it is in the vicinity of 20 μM. The active site of TSPS is luminally oriented in the *trans*-cisternae. Amino acid sequence comparisons of the tyrosine sulfate regions of a number of proteins has resulted in a tyrosine sulfation consensus sequence.[136] The fact that acidic residues often flank tyrosine sulfate suggests that this may be an important requirement for substrate recognition and catalysis. Studies using purified TPST and synthetic peptides confirm that these residues are indeed functionally important.[137]

B. The Metabolic Importance of Sulfation In Hemostasis

1. Heparin Cofactor II and Antithrombin III

Heparin cofactor II (HCII) and antithrombin III are members of the serpine (serine protease inhibitor) superfamily.[138] They are both suicide inhibitors of thrombin, the protease that initiates blood clot formation by converting fibrinogen to fibrin.[139] HCII is an extremely potent and specific inhibitor of thrombin ($K_i = 20 \times 10^{-15}$).[119] Antithrombin III inhibits thrombin as well as many other proteases in the clotting cascade. The rate of inactivation of thrombin by HCII is stimulated ~1000-fold by the sulfated glycosaminoglycans heparin and dermatan sulfate.[140] HCII is a glycoprotein with an acidic amino terminus that contains tyrosine sulfate residue.[141] The sequence of this amino terminus is similar to that of hirudin, a leech anticoagulant, the most potent thrombin inhibitor known.[142] Sulfation of hirudin at tyrosine 63 results in a 10-fold increase in its inhibitory potency.[119] The structure of the thrombin-hirudin complex has provided a basis for understanding the mechanism of inhibition as well as for the functional role of the tyrosine sulfate. Hirudin is a 65 AA protein. Its structure in a cocrystal with thrombin displays a disulfide-linked, globular N-terminus positioned at the thrombin-active site and a highly acidic, extended C-terminus.[143,144] This C-terminus lies in a 40–50 Å structural groove of thrombin that is lined with basic amino acid residues, the so-called exosite I binding site of thrombin. Because of the difficulties associated with obtaining the native, sulfated hirudin, these structures were of the recombinant, unsulfated hirudin/thrombin complex. Recently, the structure of thrombin complexed to a tyrosine sulfate containing peptide that corresponds to the acidic C-terminus of hirudin has been determined. The structure of the sulfated tail shows that the sulfuryl group interacts with the protein predominantly through a hydrogen-bonding network; there are no counterion salt linkages directly to the sulfate.

2. Heparin

Heparin is a sulfated glycosaminoglycan found almost exclusively in the intracellular granules of the mast cells in the lining of arterial walls.[131] It is released through injury and is an important regulatory agent in the prevention of blood clotting.[139] Heparin binding to antithrombin III (ATIII) stimulates the rate of ATIII inactivation of thrombin and factor Xa. Factor Xa, or Stuart factor, proteolytically converts prothrombin, the inactive precursor of thrombin, to thrombin.[139] The importance of the heparin sulfuryl moieties in acceler-

ating this inhibition has been studied in detail. A specific heparin-derived tetramer appears to be the minimum oligosaccharide competent to bind ATIII and stimulate inhibition of factor Xa.[145] Inhibition of thrombin requires a larger ~16-mer fragment, which contains the tetramer. The longer fragment appears necessary to tether thrombin to ATIII, thereby stimulating inhibition by promoting the association of the two.[146] Regiospecific incorporation of sulfuryl groups into synthetic tetramers allows assessment of the contribution of these individual groups to the tetramers ability to bind to and activate ATIII.[147] The results of this study are summarized in Figure 14. The sulfuryl moieties, enclosed in boxes, are the major binding determinants of the tetrasaccharide. Molecular modeling studies based on the crystal structure of dermatin sulfate predict that these sulfuryl groups will be aligned in the same face of the heparin helix.[148,149] Interestingly, the Gibbs potential of these groups alone is significantly greater than that for the entire tetramer, suggesting that they may interact in binding to ATIII and that some fraction of their combined potential is used to stabilize an ATIII conformational change.[120] The conformationally induced fluorescence change that occurs on binding of the tetramer to ATIII is strongly suppressed by the loss of either of the two sulfuryl moieties.[147]

3. Factor VIII (Antihemophilic Factor)

One in 5000 humans suffers from hemophilia A, a bleeding disorder that often arises from point mutations or deletions that inactivate factor VIII — they can be treated by intravenous injections of normal factor VIII.[150] Factor VIII, or antihemophilic factor, stimulates the proteolytic activation of factor X by factor IXa by three orders of magnitude.[151] Factor VIII is itself activated by specific proteolytic cleavages by thrombin and/or factor Xa; further digestion results in its inactivation.[152,153] Thus, only certain intermediates in the proteolytic pathway are the active cofactors. In serum, factor VIII is tightly, noncovalently bound to von Willebrand factor, a large glycoprotein involved in the adhesion of platelets to injured cell walls. The half-life of factor VIII in plasma is reduced 10- to 20-fold if it is not complexed to the von Willebrand factor and shows little ability to stop bleeding in dogs that are von Willebrand deficient.[154]

Factor VIII contains approximately 5 tyrosine sulfate/mol.[118] One of these, at position 1680, maps to an acidic region of the factor that is important for its interaction with the von Willebrand protein. If the sulfuryl group is removed from tyrosine 1680, or if the tyrosine is replaced by a phenylalanine, binding to von Willebrand factor appears to be completely abol-

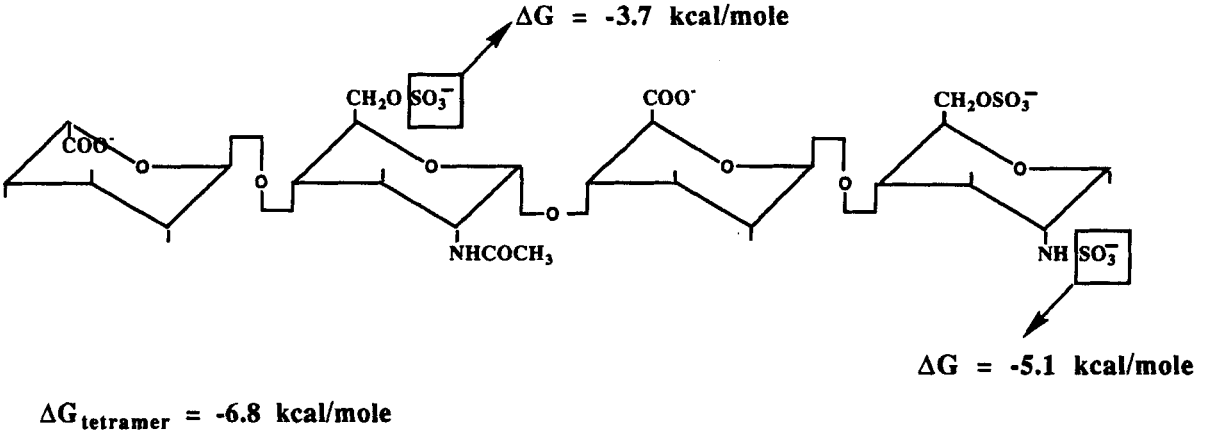


FIGURE 14. The minimum antithrombin III-binding heparin fragment (iduronic acid — *N*-acetylglucosamine 6-*O*-sulfate — glucuronic acid — glucosamine 3-*N*, 6-*O* disulfate). The Gibbs potential is given for the association of the tetramer with antithrombin III and for the binding contribution of each of the important sulfuryl groups.

ished.[118] Point mutations that substitute amino acids for tyrosine 1680 are known to result in a severe form of hemophilia A. Thus, the sulfuryl group appears to be a chemical linchpin in the factor VIII/von Willebrand interaction. Its absence likely results in accelerated proteolytic degradation of factor VIII, the consequence of which is hemophilia. In this case, tyrosine sulfation acts both to promote protein/protein interactions and to kinetically regulate protein degradation.

C. Sulfuryl Modification in Neuronal Protein Transport

Neuronal protein synthesis occurs primarily in the cell body or perikaryon.[155,156] The axon of the cell, thousands of times the length of the perikaryon and hundreds of times its volume, is devoid of ribosomes and relies virtually entirely on the perikaryonic activity for its protein complement. The transport of proteins, organelles, and other metabolites from the cell body toward the axon and presynaptic termini, so-called *anterograde axonal transport*, is catagorized as either fast or slow, according to the velocity at which the materials are transported. Fast transport occurs at 250–400 mm/day, whereas the slowly transported material progresses along the axon at 1–5 mm/d. Each category has been subclassified on the basis of differences in the rate of progression of different materials within a given transport.

The composition of the fast and slow transport differs markedly.[156] The synthesis and transport of these proteins is dramatically increased during axonal regeneration following trauma.[157,158] The fast transport consists primarily of membranous organelles, including the smooth endoplasmic reticulum, vesicles (including synaptic vesicles), acetylcholine-containing structures, and mitochondria. In contrast, the slow transport contains largely cytoplasmic proteins, neurofilaments, and tubulin.[156] The sulfation of the fast transport components has been studied.[159–161] The majority of fast transport proteins are sulfated; the modification is found either on a glycosyl moiety or tyrosine residue. The sulfotyrosine proteins comprise a group of acidic, low-molecular-weight (15–25 kDa) species, many of which appear to be secretable proteins destined for the presynaptic termini; others are more likely integral membrane-bound proteins. These observations may provide a mechanistic clue as to the targeting of proteins for the fast transport and/or the synaptic terminus.

IX. SUMMARY

Although much is known about the metabolic importance of activated sulfate, there are far more questions than answers in this field. The area of tyrosine sulfation continues to catalogue previously unidentified proteins that are sulfated at tyrosine. In the majority of these cases, we do not yet understand the function of the sulfation. When it has been investigated, tyrosine-sulfation has proven a novel and critical regulatory modification. Today, it is an easy task to scan the sequence of a protein of interest for a tyrosine-sulfation consensus sequence. This seems a prudent exercise for any protein but particularly for those processed in the Golgi, where the modification occurs. Similarly, the carbohydrate sulfation field is rife with opportunity. There are many important sulfate carbohydrates for which the functional consequences of the sulfation are not yet understood.

In the area of energetics there is much to do. The recent findings that GTP hydrolysis is thermodynamically and kinetically coupled to APS synthesis dramatically alters our understanding of the thermodynamics and regulation of this pathway. The first step in sulfate activation, a step that must occur for an organism to assimilate sulfate, has gone from extremely energetically unfavorable to very reasonable. This connection must be pursued; to understand it, the mechanism of the enzyme must be worked out. This will reveal the chemical events that couple these reactions and may well provide an important paradigm for the general problem of energetic coupling and, more specifically, for the GTPase activation of target enzymes. It is also important to address the universality of this phenomenon. To date, we know

that it occurs in several Gram-negative bacteria. As more sequences become available, we can hope to trace the evolutionary lineage of what seems likely to be an acquired functional adaptation.

REFERENCES

1. **De Meio, R. M.,** Sulfate activation and transfer, in *Metabolic Pathways,* Vol. VII, 3rd ed., Greenberg, D. M., Ed., Academic Press, New York, 1975.
2. **Siegel, L. M.,** Biochemistry of the sulfur cycle, in *Metabolic Pathways,* Vol. VII, 3rd ed., Greenberg, D. M., Ed., Academic Press, New York, 1975.
3. **Fauque, G. D., Barton, L. L., and Le Gall, J.,** Oxidative phosphorylation linked to the dissimilatory reduction of elemental sulfur by *Desolfovibrio,* in *Sulphur in Biochemistry,* Excerpta Medica, New York, 1980, 49.
4. **Postgate,** The sulfur cycle, in *Inorganic Sulfur Chemistry,* Nickless, G., Ed., Elsevier, Amsterdam, 1968, 7.
5. **Mudd, H. S.,** Diseases of sulphur metabolism: implications for the methionine-homocysteine cycle, and vitamin responsiveness, in *Sulphur in Biochemistry,* Excerpta Medica, New York, 1980, 239.
6. **Bernstein, S. and McGilvery, R. W.,** Substrate activation in the synthesis of phenyl sulfate, *J. Biol. Chem.,* 199, 745, 1952.
7. **Bernstein, S. and McGilvery, R. W.,** The enzymatic conjugation of m-aminophenol, *J. Biol. Chem.,* 198, 195, 1952.
8. **De Meio, R. H. and Tkacz, L.,** Conjugation of phenol by rat liver slices and homogenates, *J. Biol. Chem.,* 195, 175, 1952.
9. **Lipmann, F.,** Metabolic generation and utilization of phosphate bond energy, *Adv. Enzymol.,* 1, 99, 1949.
10. **Hiltz, H. and Lipmann, F.,** The enzymatic activation of sulfate, *Proc. Natl. Acad. Sci. U.S.A.,* 41, 880, 1955.
11. **Robbins, P. and Lipmann, F.,** Identification of the enzymatically active sulfate as adenosine-3′-phosphate-5′-phosphosulfate, *JACS,* 78, 2652, 1956.
12. **Wilson, L. and Bandurski, R. S.,** The mechanism of "active sulfate" formation, *JACS,* 78, 6408, 1956.
13. **Wilson, L. and Bandurski, R. S.,** An enzymatic reaction involving adenosine triphosphate and selenate, *Arch. Biochem. Biophys.,* 62, 503, 1956.
14. **Robbins, P. and Lipmann, F.,** The enzymatic sequence in the biosynthesis of active sulfate, *JACS,* 78, 6409, 1956.
15. **Baddiley, J., G., B. J., and Letters, R.,** Synthesis of adenosine-5′ sulphatophosphate. A degradation product of an intermediate in the enzymic synthesis of sulphuric esters, *J. Chem. Soc.,* 1067, 1957.
16. **Robbins, P. W. and Lipman, F.,** Separation of the two enzymatic phases in active sulfate synthesis, *J. Biol. Chem.,* 233, 681, 1958.
17. **Robbins, P. W. and Lipmann, F.,** Enzymatic synthesis of adenosine-5′-phosphosulfate, *J. Biol. Chem.,* 233, 686, 1958.
18. **Satischandran, S. and Markham, G. D.,** Adenosine 5′-phosphosulfate kinase from *Escherichia coli* K-12, *J. Biol. Chem.,* 264, 15012, 1989.
19. **Alberty, R. A.,** Standard Gibbs free energy, enthalpy, and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates, *J. Biol. Chem.,* 244, 3290, 1969.
20. **Kukko-Kalske, E., Lintunen, M., Inen, M. K., Lahti, R., and Heinonen, J.,** Intracellular PP_i concentration is not directly dependent on amount of inorganic pyrophosphatase in *Escherichia coli K*-12 cells, *J. Bacteriol.,* 171, 4498, 1989.
21. **Barshop, B. A., Wrenn, R. F., and Frieden, C.,** Analysis of numerical methods for computer simulation of kinetic processes: development of KINSIM — a flexible, portable system, *Anal. Biochem.,* 130, 134, 1983.
22. **Izatt, R. M. and Christensen, J. J.,** Heats of protonation and ionization, pK, and related thermodynamic quantities, in *CRC Handbook of Biochemistry and Molecular Biology,* Vol. I, 3rd ed., Fasman, G. D., Ed., CRC Press, Boca Raton, 1976, 151.
23. **Cruickshank, D. W. J. and Webster, B. C.,** Orbitals in sulphur and its compounds, in *Inorganic Sulfur Chemistry,* Nickless, G., Ed., Elsevier, Amsterdam, 1968, 7.
24. **Craig, D. P., Maccoll, A., Nyholm, R. S., Orgel, L. E., and Sutton, L. E.,** Chemical bonds involving d-orbitals. I, *J. Chem. Soc.,* 332, 1954.
25. **Craig, D. P. and Magnusson, E. A.,** d-Orbital contraction in chemical bonding, *J. Chem. Soc.,* 4895, 1956.
26. **Cilento, G.,** The expansion of the sulfur outer shell, *Chem. Rev.,* 60, 147, 1960.
27. **Pauling, L. C.,** *The Chemical Bond,* Cornell University Press, Ithaca, 1956, 64.
28. **Pauling, L. C.,** Interatomic distances and bond character in the oxygen acids and related substances, *J. Phys. Chem.,* 56, 361, 1952.
29. **Benkovic, S. J. and Hevey, R. C.,** Studies in sulfate esters: the mechanism of hydrolysis of phenyl phosphosulfate, a model system for 3′-phosphoadenosine 5′-phosphosulfate, *JACS,* 92, 4971, 1970.
30. **Fendler, E. J. and Fendler, J. H.,** Hydrolysis of nitrophenyl and dinitrophenyl sulfate esters, *J. Org. Chem.,* 33, 3852, 1968.
31. **Benkovic, S. J. and Benkovic, P. A.,** Studies on sulfate esters. I. Nucleophilic reactions of amines with *p*-nitrophenyl sulfate, *JACS,* 88, 5504, 1966.
32. **Schaleger, L. L. and Long, F. A.,** Entropies of activation and mechanisms of reactions in solution, *Adv. Phys. Org. Chem.,* 1, 1, 1963.

33. **Schiff, J. A.,** Pathways of assimilatory sulphate reduction in plants and microorganisms, in *Sulphur in Biochemistry,* Excerpta Medica, New York, 1980, 49.
34. **Jones-Mortimer, M. C.,** Positive control of sulphate reduction in *Escherichia coli*, *Biochem. J.*, 110, 589, 1968.
35. **Kreditch, N. M.,** Regulation of L-cysteine biosynthesis in *Salmonella typhimurium, J. Biol. Chem.*, 246, 3474, 1971.
36. **Ostrowski, J., Jagura-Burdzy, G., and Kredich, N. M.,** DNA sequences of the *cysB* regions of *Salmonella typhimurium* and *Escherichia coli, J. Biol. Chem.*, 262, 5999, 1987.
37. **Miller, B. E. and Kredich, N. M.,** Purification of the cysB protein from *Salmonella typhimurium, J. Biol. Chem.*, 262, 6006, 1987.
38. **Heinkoff, S., Haughn, G. W., Calvo, J. M., and Wallace, J. C.,** A large family of bacterial activator proteins, *Proc. Natl. Acad. Sci. U.S.A.*, 85, 6602, 1988.
39. **Ostrowski, J. and Kreditch, N. M.,** Negative autoregulation of *cysB* in *Salmonella typhimurium:* in vitro interactions of CysB protein with the *cysB* promoter, *J. Bacteriol.*, 173, 2212, 1991.
40. **Ostrowski, J. and Kreditch, N. M.,** Molecular characterization of the *cysJIH* promoters of *Salmonella typhimurium* and *Escherichia coli:* regulation by cysB protein and *N*-acetyl-L-serine, *J. Bacteriol.*, 171, 130, 1989.
41. **Ostrowski, J. and Kreditch, N. M.,** In vitro interactions of CysB protein with the *cysJIH* promoter of *Salmonella typhimurium:* inhibitory effects of sulfide, *J. Bacteriol.*, 172, 779, 1990.
42. **Hryniewicz, M. M. and Kreditch, N. M.,** The *cysP* promoter of *Salmonella typhimurium:* characterization of two binding sites for CysB protein, studies of in vivo transcription initiation, and demonstration of the anti-inducer effects of thiosulfate, *J. Bacteriol.*, 173, 5876, 1991.
43. **Jagura-Burdzy, G. and Hulanicka, D.,** Use of gene fusions to study expression of *cysB,* the regulatory gene of the cysteine regulon, *J. Bacteriol.*, 147, 744, 1981.
44. **Leyh, T. S., Vogt, T. F., and Suo, Y.,** The DNA sequence of the sulfate activation locus from *Escherichia coli K*-12, *J. Biol. Chem.*, 267, 10405, 1992.
45. **Raibaud, O. and Schwartz, M.,** Positive control of transcription initiation in bacteria, *Annu. Rev. Genet.*, 18, 173, 1984.
46. **Hawley, D. K. and McClure, W. R.,** Compilation and analysis of *Escherichia coli* promoter DNA sequences, *Nucleic Acids Res.*, 11, 2237, 1983.
47. **Shine, J. and Dalgarno, L.,** The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 1341, 1974.
48. **Malo, M. S. and Loughlin, R. E.,** Promoter elements and regulation of expression of the *cysD* gene of *Escherichia coli K*-12, *Gene,* 87, 127, 1990.
49. **Oppenheim, D. S. and Yanofsky, C.,** Translational coupling during expression of the tryptophan operon of *Escherichia coli, Genetics,* 95, 785, 1980.
50. **Yates, J. L. and Nomura, M.,** Feedback regulation of ribosomal protein synthesis in *E. coli:* localization of the mRNA target sites for repressor action of ribosomal protein LI, *Cell,* 21, 243, 1981.
51. **Yager, T. D. and von Hippel, P. H.,** Transcript elongation and termination in *Escherichia coli,* in *Escherichia coli and Salmonella typhimurium,* Vol. 2, Neidhardt, F. C., Ed., Am. Soc. for Microbiol., Washington, DC, 1987, 1241.
52. **Brendel, V., Hamm, G. H., and Trifonov, E. N.,** Terminators of transcription with RNA polymerase from *Escherichia coli:* what they look like and how to find them, *J. Biomol. Struct. Dynamics,* 4, 705, 1986.
53. **Bourne, H. R., Sanders, D., and McCormick, F.,** The GTPase superfamily: a conserved switch for diverse cell functions, *Nature,* 348, 125, 1990.
54. **Bourne, H. R., Sanders, D., and McCormick, F.,** The GTPase superfamily: a conserved structure and molecular mechanism, *Nature,* 349, 117, 1991.
55. **Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Scherer, N., Toro, M., and Brown, A. M.,** Signal transduction by G proteins, *Kidney Int.*, 32, S-14, 1987.
56. **Birnbaumer, L.,** Transduction of receptor signal into modulation of effector activity by G proteins: the first 20 years or so..., *FASEB J.*, 4, 3178, 1990.
57. **Gilman, A. G.,** G proteins: transducers of receptor-generated signals, *Annu. Rev. Biochem.*, 56, 615, 1987.
58. **Iyengar, R. and Birnbaumer, L.,** Roles of G proteins and G protein subunits in signal transduction, *Lymphokine Res.*, 9, 533, 1990.
59. **Balch, W. E.,** Small GTP-binding proteins in vesicular transport, *TIBS,* 15, 473, 1990.
60. **Riis, B., Rattan, S. I. S., Clark, B. F. C., and Merrick, W. C.,** Eukaryotic protein elongation factors, *TIBS,* 15, 420, 1990.
61. **Barbacid, M.,** *ras* Genes, *Annu. Rev. Biochem.*, 56, 779, 1987.
62. **Dever, T. E., Glynias, M. J., and Merrick, W. C.,** GTP-binding domain: three consensus sequence elements with distinct spacing, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 1814, 1987.
63. **Halliday, K. R.,** Regional homology in GTP-binding proto-oncogene products and elongation factors, *J. Cyclic Nucleotide Protein Phosphor. Res.*, 9, 435, 1984.
64. **Valencia, A., Chardin, P., Wittinghoffer, A., and Sander, C.,** The *ras* protein family: evolutionary tree and role of conserved amino acids, *Biochemistry,* 30, 4637, 1991.
65. **Arai, K., Clark, B. F. C., Duffy, L., Jones, M. D., Kaziro, Y., Laursen, R. A., L'Italien, J., Miller, D. L., Nagarkatti, S., Nakamura, S., Nielsen, K. M., Petersen, T. E., Takahasi, K., and Wade, M.,** Primary structure of elongation factor Tu from *Escherichia coli, Proc. Natl. Acad. Sci. U.S.A.*, 77, 1326, 1980.

66. **Ovchinnikov, Y. A., Alakhov, Y. B., Bundulis, Y. P., Bundule, M. A., Dovgas, N. V., Kozlov, V. P., Motuz, L. P., and Vinokurov, L. M.,** The primary structure of elongation factor G from *Escherichia coli, FEBS Lett.*, 139, 130, 1982.
67. **Brands, J. H. G. M., Maassen, J. A., Van Hemert, F. J., Amons, R., and Moller, W.,** The primary structure of the α subunit of human elongation factor 1, *Eur. J. Biochem.*, 155, 167, 1986.
68. **Leyh, T. S. and Suo, Y.,** GTPase-mediated activation of ATP sulfurylase, *J. Biol. Chem.*, 267, 542, 1992.
69. **Bridger, W. B.,** Succinyl-CoA synthetase, in *The Enzymes*, Vol. X, 3rd ed., Boyer, P. D., Ed., Academic Press, New York, 1974, 581.
70. **Frey, P. A.,** Nucleotidyl transferases and phosphotransferases: stereochemical and covalent adducts, in *The Enzymes*, Vol. XX, 3rd ed., Sugman, D. S., Ed., Academic Press, New York, 1992, 141.
71. **Arnell, D. R. and O'Leary, M. H.,** Binding of carbon dioxide to phosphoenolpyruvate, *Biochemistry*, 31, 4363, 1992.
72. **Jencks, W. P.,** The utilization of binding energy in coupled vectorial processes, *Adv. Enzymol.*, 51, 75, 1980.
73. **Kaziro, Y.,** The role of guanosine 5′-triphosphate in polypeptide chain elongation, *Biochem. Biophys. Acta*, 505, 95, 1978.
74. **Hopfield, J. J.,** Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4135, 1974.
75. **Thompson, R.,** EFTu provides an internal kinetic standard for translational accuracy, *TIBS*, 13, 91, 1988.
76. **Jurnak, F.,** Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene proteins, *Science*, 230, 32, 1985.
77. **la Cour, T. F. M., Nyborg, J., Thirup, S. and Clark, B. F. C.,** Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by X-ray crystallography, *EMBO J.*, 4, 2385, 1885.
78. **Pai, E. F. and Wittinghofer, A.,** The structure of Ras protein: a model for a universal switch, *TIBS*, 32, 382, 1992.
79. **Valencia, A., Kjeldgaard, M., Pai, E. F., and Sander, C. A.,** GTPase domains of ras p21 oncogene protein and elongation factor Tu: analysis of three-dimensional structures, sequence families, and functional sites, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 5443, 1991.
80. **Fisher, R. F. and Long, S. R.,** *Rhizobium*-plant signal exchange, *Nature*, 357, 655, 1992.
81. **Long, S. R.,** *Rhizobium*-legume nodulation: life together in the underground, *Cell*, 56, 203, 1989.
82. **Schwedock, J. and Long, S. R.,** Nucleotide sequence and protein products of two new nodulation genes of *Rhizobium meliloti, nodP* and *nodQ, Mol. Plant-Microbe Interact.*, 2, 181, 1989.
83. **Schwedock, J. and Long, S. R.,** ATP sulfurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti, Nature*, 348, 644, 1990.
84. **Schewedoc, J., Leyh, T. S., Liu, C., and Long, S. R.,** unpublished data, 1993.
85. **Renosto, F. R., Martin, R. L., and Segel, I. H.,** Sulfate-activating enzymes of *Penicillium chrysogenum, J. Biol. Chem.*, 264, 9433, 1989.
86. **Seubert, P. A., Renosto, F., Knudson, P., and Segel, I. H.,** APS kinase substrate inhibition, *Arch. Biochem. Biophys.*, 240, 509, 1985.
87. **Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Deénarié, J.,** Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal, *Nature*, 344, 781, 1990.
88. **Vieille, C. and Emerich, C.,** Characterization of two *Axzospirillum brasilinse* Sp7 plasmid genes homologous to *Rhizobium meliloti nodPQ, Mol. Plant-Microbe Interact.*, 3, 389, 1990.
89. **Burnell, J. N. and Roy, A. B.,** Purification and properties of the ATP sulfurylase of rat liver, *Biochem. Biophys. Acta*, 527, 239, 1978.
90. **Geller, D. H., Henry, J. G., Belch, J., and Schwartz, N. B.,** Co-purification and characterization of ATP-sulfurylase and adenosine-5′-phosphosulfate kinase from rat chondrosarcoma, *J. Biol. Chem.*, 262, 7374, 1987.
91. **Kanno, N., Sato, M., and Sato, Y.,** Properties of ATP-sulfurylase from marine alga *Porphyra yezoensis, Nippon Susian Gakkaishi*, 54, 1635, 1988.
92. **Leyh, T. S., Taylor, J. T., and Markham, G. H.,** The sulfate activation locus of *Escherichia coli K*-12: cloning, genetic and enzymatic characterization, *J. Biol. Chem.*, 263, 2409, 1987.
93. **Nozawa, A.,** Purification and some properties of ATP-sulfurylase from developing sea urchin embryos, *Biochim. Biophys. Acta*, 611, 309, 1980.
94. **Renosto, F., Martin, R. L., Borrell, J. L., Nelson, D. C., and Segel, I. H.,** ATP sulfurylase from trophosome tissue of *Riftia pachyptila* (hydrothermal vent tube worm), *Arch. Biochem. Biophys.*, 290, 66, 1991.
95. **Shaw, W. H. and Anderson, J. W.,** Purification, properties and substrate specificity of adenosinetriphosphate sulphurylase from spinach leaf tissue, *Biochem. J.*, 127, 137, 1972.
96. **Shaw, W. H. and Anderson, J. W.,** Comparative enzymology of the adenosine triphosphate sulphurylase from leaf tissue of selenium-accumulator and non-accumulator plants, *Biochem. J.*, 139, 37, 1974.
97. **Shoyab, M. and Marx, W.,** Purification and properties of ATP sulfurylase from furth mouse mastocytoma, *Biochim. Biophys. Acta*, 258, 113, 1971.
98. **Tweedie, J. W. and Segel, I. H.,** ATP sulfurylase from *Penicillium chrysogenum.* I. Purification and characterization, *Prep. Biochem.*, 1, 91, 1971.
99. **Varma, A. K. and Nicholas, D. J. D.,** Purification and properties of ATP-sulphurylase from *Nitrobacter agilis, Biochim. Biophys. Acta*, 227, 373, 1971.
100. **Yu, M., Martin, R. L., Jain, S., Chen, L. J., and Segel, I. H.,** Rat liver ATP-sulfurylase: purification,

kinetic characterization, and interaction with arsenate, selenate, phosphate and other inorganic oxyanions, *Arch. Biochem. Biophys.*, 269, 156, 1989.

101. **Seubert, P. A., Renosto, F., Knudson, P., and Segel, I. H.,** Adenosinetriphosphate sulfurylase from *Penicillium chrysogenum:* steady-state kinetics of the forward and reverse reactions, alternative substrate kinetics and equilibrium binding studies, *Arch. Biochem. Biophys.*, 240, 509, 1985.
102. **Seubert, P. A., Hoang, L., Renosto, F., and Segel, I. H.,** ATP sulfurylase from *Penicillium chrysogenum:* measurements of the true specific activity of an enzyme subject to potent product inhibition and a reassessment of the kinetic mechanism, *Arch. Biochem. Biophys.*, 225, 679, 1983.
103. **Bicknell, R., Cullis, P. M., Jarvest, R. L., and Lowe, G.,** The stereochemical course of nucleotidyl transfer catalyzed by ATP sulfurylase, *J. Biol. Chem.*, 257, 8922, 1982.
104. **Renosto, F., Martin, R. L., and Segel, I. H.,** ATP sulfurylase from *Penicillium chrysogenum, J. Biol. Chem.*, 262, 16279, 1987.
105. **Martin, R. L., Daley, L. A., Lovric, Z., Wailes, L. M., Renosto, F., and Segel, I. H.,** The "regulatory" sulfhydryl group of *Penicillium chrysogenum* ATP sulfurylase, *J. Biol. Chem.*, 264, 11768, 1989.
106. **Renosto, F., Martin, R. L., Wailes, L. M., Daley, L. A., and Segel, I.,** Regulation of inorganic sulfate activation in filamentous fungi, *J. Biol. Chem.*, 265, 10300, 1990.
107. **Gillespie, D. M., Demerec, M., and Itakawa, H.,** Appearance of double mutants in aged cultures of *Salmonella typhimurium* cysteine-requiring mutants, *Genetics*, 59, 433, 1968.
108. **Neuwald, A. F., Krishnan, B. R., Brikun, I., Kulakauskas, S., Suziedelis, K., Tomcsanyi, T., Leyh, T. S., and Berg, D. E.,** *cysQ*, a gene needed for cysteine biosynthesis in *Escherichia coli K*-12 only during aerobic growth, *J. Bact.*, 174, 415, 1992.
109. **Renosto, F., Seubert, P. A., and Segel, I. H.,** Adenosine 5′-phosphosulfate kinase from *Penicillium chrysogenum, J. Biol. Chem.*, 259, 2113, 1984.
110. **Hommes, F. A., L., M., and Touchton, J.,** Purification and some properties of liver adenylsulfate kinase, *Biochim. Biophys. Acta*, 924, 270, 1987.
111. **Renosto, F., Martin, R. L., and Segel, I. H.,** Adenosine-5′-phosphosulfate kinase from *Penicillium chrysogenum:* ligand binding properties and the mechanism of substrate inhibition, *Arch. Biochem. Biophys.*, 284, 30, 1991.
112. **Saticshandran, S., Hickman, Y. N., and Markham, G. D.,** Characterization of the phosphoryl enzyme intermediate formed in the adenosine 5′-phosphosulfate kinase reaction, *Biochemistry*, 31, 11684, 1992.
113. **Roth, J. R. and Rivett, A. J.,** Does sulfate conjugation contribute to the metabolic inactivation of catecholamines in humans?, *Biochem. Pharm.*, 31, 3017, 1982.
114. **Unsworth, C. D., Hughes, J., and Morley, J. S.,** O-Sulfated Leu-enkephalin in brain, *Nature*, 295, 519, 1982.
115. **Brand, S. J., Andersen, B. N., and Rehfeld, J. F.,** Complete Tyrosine-*O*-Sulphation of gastrin in neonatal rat pancreas, *Nature*, 309, 456, 1984.
116. **Jensen, R. T., Lemp, G. F., and Gardner, J. D.,** Interactions of COOH-terminal fragments of cholecystokinin with receptors on dispersed acini from guinea pig pancreas, *J. Biol. Chem.*, 257, 5554, 1982.
117. **Pasqualini, J. R., Schatz, B., Varin, C., and Nguyen, B. L.,** Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer, *J. Steroid Biochem. Mol. Biol.*, 41, 323, 1992.
118. **Leyete, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verbeet, M. P., Mertens, K., and van Mourik, J. A.,** Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor, *J. Biol. Chem.*, 266, 740, 1991.
119. **Stone, S. R. and Hofsteenge, J.,** Kinetics of inhibition of thrombin by hirudin, *Biochemistry*, 25, 4622, 1986.
120. **Atha, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D., and Choay, J.,** Contribution of monosaccharide residues in heparin binding to antithrombin III, *Biochemistry*, 24, 6723, 1985.
121. **Maarouei, R. M., Tapon-Bretaudiere, J., Mardiguian, J., Sternberg, C., Dartzenberg, M. D., and Fischer, A. M.,** Influence of the oversulfation method and the degree of sulfation on the anticoagulant properties of dermatan sulfate derivatives, *Thromb. Res.*, 59, 749, 1990.
122. **Brauer, P. R., Keller, K. A., and Keller, J. M.,** Concurrent reduction in the sulfation of heparan sulfate and basement membrane assembly in a cell model system, *Development*, 110, 805, 1990.
123. **Meisheri, K. D., Oleynek, J. J., and Puddington, L.,** Role of protein sulfation in vasodilation induced by minoxidil sulfate, a K+ channel opener, *J. Pharmacol. Exp. Ther.*, 258, 1091, 1991.
124. **Garay, R. P., Labaune, J. P., Mesangeau, D., Nazaret, C., Imbert, T., and Moinet, G.,** CRE 10904 [2-(p-fluorophenoxy), 1-(o-hydroxyphenyl)-ethane]: a new diuretic and antihypertensive drug acting by in vivo sulfation, *J. Pharmacol. Exp. Ther.*, 255, 415, 1990.
125. **Christensen, C., Rozhin, J., Pack, B. A., and Brooks, S. C.,** *In situ* control of estrogen activity by sulfurylation in uterus and other targets, in *Sulfate Metabolism and Sulfate Conjugation,* Mulder, G. J., Caldwell, J., Van Kempen, G. M. J., and Vonk, R. J., Eds., Taylor Francis, London, 1982, 145.
126. **Finkelstein, J. D.,** Enzyme defects in sulfur amino acid metabolism in man, in *Metabolic Pathways,* Vol. VII, 3rd ed., Greenberg, D. M., Ed., Academic Press, New York, 1975.

127. **Mulder, G. J. and Meermen, J. H. N.,** The role of sulfation in carcinogenesis by *N*-hydroxyy-2-acetylaminofluorine, in *Sulfate Metabolism and Sulfate Conjugation,* Mulder, G. J., Caldwell, J., Van Kempen, G. M. J., and Vonk, R. J., Eds., Taylor Francis, London, 1982, 145.
128. **Hille, A., Braulke, T., von Figure, K., and Huttner, W. B.,** Occurrence of tyrosine sulfate in proteins — a balance sheet. I. Secretory and lysosomal proteins, *Eur. J. Biochem.,* 188, 577, 1990.
129. **Hille, A. and Huttner, W. B.,** Occurrence of tyrosine sulfate in proteins — a balance sheet. 2. Membrane proteins, *Eur. J. Biochem.,* 188, 577, 1990.
130. **Jakoby, W. B. and Ziegler, D. M.,** The enzymes of detoxication, *J. Biol. Chem.,* 265, 20715, 1990.
131. **Lindahl, U. and Hook, M.,** Glycosaminoglycans and their binding to biological macromolecules, *Annu. Rev. Biochem.,* 47, 385, 1978.
132. **Huttner, W. B.,** Tyrosine sulfation and the secretory pathway, *Annu. Rev. Physiol.,* 50, 363, 1988.
133. **Vote, D. and Vote, J.,** Lipids and membranes, in *Biochemistry,* John Wiley and Sons, New York, 1990, 301.
134. **Baeuerle, P. A. and Huttner, W. B.,** Tyrosine sulfation is a trans-Golgi-specific protein modification, *J. Cell. Biol.,* 105, 2655, 1987.
135. **Niehrs, C. and Huttner, W. B.,** Purification and characterization of tyrosylprotein sulfotransferase, *EMBO J.,* 9, 35, 1990.
136. **Hortin, G., Folz, R., Gordon, J. I., and Strauss, A. W.,** Characterization of sites of tyrosine sulfation in proteins and criteria for predicting their occurrence, *Biochem. Biophys. Res. Commun.,* 141, 326, 1986.
137. **Niehrs, C., Kraft, M., Lee, R. W., and Huttner, W. B.,** Analysis of the substrate specificity of tyrosylprotein sulfotransferase using synthetic peptides, *J. Biol. Chem.,* 265, 8525, 1990.
138. **Griffith, M. J., Noyes, C. M., and Church, F. C.,** Reactive site structural similarity between heparin cofactor II and antithrombin III, *J. Biol. Chem.,* 260, 2218, 1985.
139. **Vote, D. and Vote, J.,** Molecular physiology, in *Biochemistry,* John Wiley and Sons, New York, 1990, 1087.
140. **Tollefesen, D. M., Majerus, D. W., and Blank, M. K.,** Heparin cofactor II, *J. Biol. Chem.,* 257, 2162, 1982.
141. **Van Deerlin, V. M. D. and Tollefsen, D. M.,** The N-terminal acidic domain of heparin cofactor II mediates the inhibition of anthrombin in the presence of glycosaminoglycans, *J. Biol. Chem.,* 266, 20223, 1991.
142. **Mao, S. J. T., Yates, M. T., Owen, T. J., and Krstenansky, J. L.,** Interaction of hirudin with thrombin: identification of a minimal binding domain of hirudin that inhibits clotting activity, *Biochemistry,* 23, 8170, 1988.
143. **Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J. W., II,** The structure of a complex of recombinant hirudin and human α-thrombin, *Science,* 249, 277, 1990.
144. **Grutter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., and Stone, S. R.,** Crystal structure of the thrombin-hirudin complex: a novel mode of serine protease inhibition, *EMBO J.,* 9, 2361, 1990.
145. **Atha, D. H., Stephens, A. W., Rimon, A., and Rosenberg, R. D.,** Sequence variation in heparin octasaccharides with high affinity for antithrombin III, *Biochemistry,* 23, 5801, 1984.
146. **Oosta, G. M., Gardner, W. T., Beeler, D. L., and Rosenberg, R. D.,** Multiple functional domains of the heparin molecule, *Proc. Natl. Acad. Sci. U.S.A.,* 78, 829, 1981.
147. **Atha, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D., and Choay, J.,** Contribution of 3-O- and 6-O-sulfated glucosamine residues on the heparin-induced conformational change in antithrombin III, *Biochemistry,* 26, 6454, 1987.
148. **Villanueva, G. B.,** Predictions of the secondary structure of antithrombin III and the location of the heparin-binding site, *J. Biol. Chem.,* 259, 2531, 1984.
149. **Atkins, E. D. T. and Isaac, D. H.,** X-ray diffraction studies on the connective tissue polysaccharides. Molecular conformation of dermatin sulfate, *J. Mol. Biol.,* 80, 773, 1973.
150. **Tuddenham, E. G. D., Cooper, D. N., Gitschier, J., Higuchi, M., Hoyer, L. W., Yoshioka, A., Peake, I. R., Schwaab, R., Olek, K., Kazazian, H. H., Lavergne, J. M., Giannelli, F., and Antonarakis, S. E.,** Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene, *Nucleic Acids Res.,* 19, 4821, 1991.
151. **Van Dieijen, G., Tans, G., Rosing, J., and Hemker, H. C.,** The role of phospholipid and factor VIIIa in the activation of bovine factor X, *J. Biol. Chem.,* 256, 3433, 1981.
152. **Eaton, D., Rodriguez, H., and Vehar, G. A.,** Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa and activated protein C with activation and inactivation of factor VIII coagulant activity, *Biochemistry,* 25, 505, 1986.
153. **Pittman, D. D. and Kaufman, R. J.,** Proteolytic requirements for thrombin activation of anti-hemophilic factor (factor VIII), *Proc. Natl. Acad. Sci. U.S.A.,* 85, 2429, 1988.
154. **Brinkhous, K. M., Sandberg, H., Garris, J. B., Mattsson, C., Palm, M., Griggs, T., and Read, M. S.,** Purified human factor VIII procoagulant protein: comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs, *Proc. Natl. Acad. Sci. U.S.A.,* 82, 8752, 1985.
155. **Kandel, E.,** Nerve cells and behavior, in *Principles of Neuroscience,* 2nd ed., Kandel, E. and Schwartz, J., Eds., Elsevier, New York, 1985, 13.

156. **Grafstein, B. and Forman, D. S.,** Intracellular transport in neurons, *Physiol. Rev.*, 60, 1167, 1980.
157. **Perry, G. W., Burmeister, D. W., and Grafstein, B.,** Fast axonally transported proteins in regenerating goldfish axons, *J. Neurosci.*, 7, 798, 1987.
158. **Perry, G. W., Burmeister, D. W., and Grafstein, B.,** Effect of target removal on goldfish optic nerve regeneration: analysis of fast axonally transported proteins, *J. Neurosci.*, 10, 3439, 1990.
159. **Wenthold, R. J. and McGarvey, M. L.,** Different polypeptides are rapidly transported in auditory and optic nerves, *J. Neurochem.*, 39, 27, 1982.
160. **Stone, G. C., Hammerschlag, R., and Bobinski, J. A.,** Fast-transported glycoproteins and nonglycosylated proteins contain sulfate, *J. Neurochem.*, 41, 1085, 1983.
161. **Stone, G. C., Hammerschlag, R., and Bobinski, J. A.,** Fast axonal transport of tyrosine sulfate-containing proteins: preferential routing of sulfoproteins toward nerve terminals, *Cell Mol. Neurobiol.*, 4, 249, 1984.