*Please note these web pages are part of an assignment for a graduate course in Advanced Biochemistry and Molecular Biology BCMB8010 at the University of Georgia. Questions should be directed to kkeegan@uga.edu.*

# O-Acetylserine Sulfhydrylase

*A Key Enzyme in the Pathway of L-Cysteine Biosynthesis in Plants and Microorganisms*

## Introduction (Abstract)

Sulfur plays a vital role in both the structure and chemical function of many biological molecules [1]. In proteins, it is essential in forming the disulfide bridges that contribute to their three-dimensional structures [1]. In biomolecules such as ferredoxin, sulfur is also critical to catalytic and electrochemical activity [2]. Higher plants, and some bacteria and archaea, have the ability to reduce inorganic sulfur from the environment to sulfide and to incorporate the reduced sulfur into organic compounds [3]. Notable among these compounds is the amino acid L-cysteine, which is central to the production of a variety of other sulfur-containing metabolites [1,2]. O-Acetylserine sulfhydrylase (OASS, EC 2.5.1.47, formerly EC 4.2.99.8) is the enzyme that catalyzes the final step in cysteine biosynthesis [4]. OASS is a homodimeric pyridoxal 5-P′-phosphate (PLP)-dependent enzyme that uses O-acetylserine (OAS) and sulfide as its substrates [5]. OAS is produced by acetylation of the β hydroxyl group of serine by acetyl-CoA, a reaction catalyzed by the enzyme serine acetyltransferase (SAT, EC 2.3.1.30) [6]. In the cysteine biosynthesis reaction, OAS makes an external aldimine linkage with the PLP cofactor in the active site of OASS. This causes the enzyme to undergo a global conformational change from the native open position to a closed position. In the closed position, only small molecules such as sulfide (the second reactant) or acetate (the first product) can access the active site [5]. Within the active site of OASS, the acetate side-chain of OAS is removed and replaced with a thiol group from sulfide, yielding acetate and cysteine as the products of the reaction [7]. In addition to the closed conformation induced by binding of OAS, the binding of chloride ions at an allosteric site on the dimer interface of OASS causes the enzyme to assume an inhibited conformation that prevents OAS from forming a stable external aldimine linkage in the active site. Chloride may be an analog of sulfide, which is the physiological inhibitor of OASS [8].

## Background

### *There Are Two Mechanisms by which Organisms Synthesize Cysteine*

Cysteine is regarded as the terminal metabolite of sulfur assimilation and the starting point for the production of many different biomolecules, including: methionine, glutathione, vitamin B7, CoA, iron-sulfur clusters, and a variety of secondary metabolites [1]. Cysteine-containing molecules such as glutathione and thioredoxin also play a major role in maintaining an intracellular reducing environment, which mitigates the effects of oxidative stress on the cell [2]. There are two known routes through which organisms make cysteine: they either synthesize it from a compound that already contains reduced

sulfur, or they assimilate sulfur from the environment and incorporate it directly into cysteine with the aid of OASS [6].

Figure 1 (all figures can be found at the end of the paper) was created using Microsoft PowerPoint and is based on a diagram from the 2003 paper by Mino and Ishikawa [6]. It shows the steps of cysteine synthesis in mammals as it is understood today. In short, mammals convert methionine to homocysteine, homocysteine to cystathionine, and finally cystathionine to cysteine, the end product. Important to note is that cysteine synthesis in animals begins with L-methionine, which like cysteine is a sulfur-containing amino acid. Animals, as well as some plants and microorganisms (such as *Saccharomyces cerevisiae* and *Lactobacillus lactis*), do not have a mechanism for the assimilation of inorganic sulfur and cannot synthesize *de novo* sulfur-containing amino acids [2,6]. This is why methionine is considered an essential amino acid [9].

In contrast to the previous model, some plants (e.g. *Arabidopsis thaliana* and *Spinicia oleracea*) and microorganisms (e.g. *Escherichia coli*, *Salmonella typhimurium*, *Azospirillum brasilense*, and *Aeropyrum pernix*) have a pathway that enables them to assimilate inorganic sulfur directly from their surroundings [2,9,10]. Figure 2 was created using ChemSketch and Microsoft PowerPoint. It is based on a diagram from the 3rd edition of *Biochemistry*, by Voet and Voet [9], and shows the 8-electron reduction of sulfate to sulfide in *E. coli*. The sulfur is shown in red for ease in tracking its position throughout out the steps in the pathway. The reduction of sulfate to an activated form, APS, is the common gateway through which sulfur enters sulfur-assimilating organisms. The reaction equilibrium of ATP sulfurylase actually favors the breakdown of APS into sulfate and ATP, so the products of the forward reaction (pyrophosphate and APS) must be immediately metabolized [1]. This pathway is highly conserved in sulfur-assimilating organisms. In higher plants, however, the enzyme APS reductase reduces APS directly to sulfite, bypassing the need for further activation of APS to PAPS [1]. Sulfur-assimilating organisms synthesize cysteine from serine and sulfide in two steps. In the first step, SAT converts serine and acetyl-CoA to the activated sulfide acceptor OAS. The second step is the insertion of sulfide into OAS in a β-replacement reaction the yields cysteine and acetate. This final step is catalyzed by OASS [11].

## *Discovery of OASS Activity*

In 1948, Sofia Simmonds performed a study on the utilization of sulfur-containing amino acids by two mutant strains of *E. coli*. The *methionineless* strain was unable to synthesize the amino acid methionine, while the *cysteineless* strain lacked the capacity to synthesize the amino acid cysteine [12]. It had already been established that animals used homocysteine and its precursor, methionine, to synthesize cysteine [13], so it was not surprising that the *methionineless* strain of Simmonds' experiment grew in the presence of methionine, homocysteine, and cystathionine. The *cysteineless* mutant, however, could only grow when provided with cysteine; it could not grow when restricted to methionine, homocysteine, or cystathionine. Simmonds concluded that if the metabolism of methionine and cysteine in all *E. coli* strains is the same, then the formation of cysteine from methionine must follow a different pathway than that used in the conversion of cysteine to methionine [12]. This was the first clue that bacteria may have a means of

synthesizing cysteine that differs from the pathway of cysteine synthesis utilized in animals.

Nearly two decades after Simmonds' experiment, Kredich and Tomkins used spectrophotometry assays to characterize enzymatic activity along the pathway of cysteine biosynthesis in *E. coli* and *S. typhimurium*. Based on their results as well as on prior research, Kredich and Tomkins proposed a two-step model for the conversion of L-serine to L-cysteine. In the first step, an enzyme which they referred to as serine transacetylase (now known as SAT) catalyzes the transfer of an acetyl group from acetyl-CoA to L-serine, producing O-acetyl-L-serine and CoA. In the second step, another enzyme which they named O-acetylserine sulfhydrylase catalyzes the deacetylation of OAS and the addition of a sulfide, resulting in the end product, L-cysteine [13].

Figure 3 presents an updated version of Kredich and Tomkins' original hypothesis. It is adapted from a similar figure in the 2005 paper by Bonner, *et al.*, and was created using ChemSketch and Microsoft PowerPoint [10].

## *Methods of Isolation and Characterization*

OASS was originally isolated from the enterobacteria *E. coli* and *S. typhimurium* [13]. It has since been isolated from such diverse organisms as *A. pernix* (a hyperthermophilic archae) [6,14], *Haemophilus influenzae* (the flu virus) [15], *Arabidopsis* [10], and *A. brasilense* (a plant pathogen) [2]. The most common expression system for the enzyme is E. coli [10]. The enzyme is typically isolated from crude extract by salting out with ammonium sulfate followed by three rounds of column chromatography: anion exchange (e.g. DEAE-Sephacel column), gel filtration (e.g. Sephadex G-100 column), and hydrophobic interaction (e.g. phenyl-Sepharose column) [2]. Alternatively, OASS may be hybridized with an N-terminal His tag and purified on a $Ni^{2+}$-NTA column instead of using the salting out method [10].

Site-directed mutagenesis has also been an important tool in the characterization of OASS. It been used to identify the active site residues involved in forming the internal aldimine with PLP (lysine) and in stabilizing the pyridoxal ring during the reaction (serine) [10]. It has also been used to elucidate the differences in the active sites of the isoforms OASS-A and OASS-B found in enteric bacteria [3].

OASS is well-suited to study by spectrophotometry due to the presence of the coenzyme PLP at its active site. Like other PLP-dependent enzymes, there is a correlation between OASS conformational changes and catalytic intermediates [16]. When the enzyme is in open conformation without bound substrate, PLP is in an internal Schiff base linkage with the active site lysine residue and OASS shows an absorption band centered at 412nm, with a small shoulder at ~330nm. This pattern has been attributed to the ketoenamine and enolimine tautomers of the bound PLP, respectively [17]. Binding of OAS disrupts the internal Schiff base, and sulfide addition to the intermediate generates an external Schiff base linkage with PLP [10]. This causes the enzyme to undergo a global conformational change from an open to a closed species [5]. The rate of appearance of the closed species can be detected via spectroscopy by measuring the

change in absorbance due to the tautomers of the internal Schiff base [16]. The figure at left shows the tautomers of the internal aldimine. Figure 4 was created using ChemSketch and Microsoft PowerPoint. The chemical structures were adapted from the 2007 paper by Speroni, *et al.* [16]. The complete catalytic mechanism of OASS is discussed in the "Structural Biology" section of this paper.

X-ray crystallography has of course been invaluable in understanding the structure and different conformations of OASS as it catalyzes the final step in cysteine biosynthesis [5,7,8,14,16,19]. Initial crystallization is achieved using the hanging-drop vapor diffusion method. The small initial crystals (~0.5mm in the longest dimension) are then used to seed sitting drops. This procedure can yield large crystals measuring up to 1.5mm x 0.8mm x 0.6mm in size [7].

## Biological Aspects

### *Isoforms and Localization*

OASS is found as a variety of isoforms in plants and bacteria [10,16]. In plants, OASS isoforms are localized to specific organelles. For example, *Arabidopsis* expresses three different OASS isoforms: one is expressed in the cytosol, another in plastids, and the third in mitochondria [10]. These isoforms have about a 40% amino acid sequence homology to the OASS-B isoforms found in *S. typhimurium* [10]. Bacteria express two known isoforms of OASS, OASS-A and OASS-B. The B-isozyme is expressed in S. typhimurium under anaerobic conditions, and appears to be less selective than OASS-A [16]. OASS-A and OASS-B have about a 40% sequence homology [3]. The catalytic mechanism of OASS-B has not been as well studied as that of OASS-A, but it is presumed to be very similar to OASS-A [16].

### *Regulation*

Figure 5 illustrates the pathway of cysteine biosynthesis in bacteria and its regulation. It was created using Microsoft PowerPoint and is adapted from the 2008 paper by Campanini, *et al.* [15]. Cysteine biosynthesis in bacteria is regulated in a variety of ways, but they share a common theme in that they are all dependent on the cellular concentration of OAS, an OASS substrate. OASS is known to form complexes with both SAT and ATP sulfurylase. Whether the OASS/SAT complex (known as cysteine synthase) or the OASS/ATP sulfurylase complex is more abundant in the cell appears to be a function of the abundance of OAS [15]. Sulfur limitation in the cell leads to the accumulation of OAS because without sulfide OASS cannot convert OAS to cysteine. Overabundance of OAS leads to the dissociation of the OASS/SAT complex, which is otherwise prevented by the association of sulfide with the complex. Free OASS can then complex with ATP sulfurylase. This interaction results in a 1000-fold increase in the activity of ATP sulfurylase [15]. The overall result is that the cell responds to a sulfur-deprived environment by increasing the activity of ATP sulfurylase, which increases the rate of APS production and leads to an increase in the production of cysteine. When OAS turnover is rapid due to a high availability of sulfur, it cannot cause the dissociation of the OASS/SAT complex [15]. SAT interacts with OASS in a way that substantially inhibits its activity [11,15, 19,20]. Binding of the ten C-terminal peptides of SAT seems to block access to the catalytic center of OASS [11]. Thus when sulfur is abundant, OASS activity

is retarded and cysteine biosynthesis slows [15]. The structure of the decapeptide tail of SAT in OASS is shown in Figure 6. It was created in Pymol from PDB file 2ISQ [19]. The SAT tail is shown in sticks, PLP is shown as spheres, and the OASS monomer is shown as a cartoon. Another means of regulation that takes place when the cell has a steady supply of sulfur is that the abundance of cysteine serves to competitively inhibit SAT, thereby reducing the amount of OAS produced [15]. In this way, feedback inhibition spurred by the formation of cysteine inhibits the synthesis of new cysteine molecules. OAS rapidly and spontaneously converts to *N*-acetylserine (NAS) at pHs above 7.0. Thus when sulfur levels in *E. coli* are low and OAS accumulates beyond the amount that OASS can accommodate, NAS is produced in the cell. NAS functions as an inducer of the transcription of the cysteine regulon (represented in the figure by *CysB*, a regulatory protein of the regulon). Transcription of genes in the regulon produces more sulfur uptake machinery and more molecules of OASS, allowing the cell to convert the accumulated OAS to cysteine [15].

### *OASS-catalyzed Synthesis of Nonproteinaceous Amino Acids*

Nonproteinaceous amino acids are attractive to the pharmaceutical industry because they can be utilized as building blocks for ingredients that contain chiral centers and as substances that have a variety of biological activities [21]. At present, however, the artificial synthesis of such nonproteinaceous amino acids is difficult and expensive compared to that of natural amino acids [21]. In 2003, Zhao, *et al.* performed experiments with cysteine synthase in *E. coli* as a system for the production of the nonproteinaceous amino acids β-(pyrozol-1-yl)-L-alanine (β-PA) and β-(triazol-1-yl)-L-alanine (β-TA). β-PA specifically interacts with brain glutamate receptors and β-TA can be used as a histidine analogue. Higher plants use various nucleophiles and mercaptans in place of sulfide to synthesize nonproteinaceous amino acids from OAS [21]. Building on this observation, Zhao, *et al.* used OASS and SAT from recombinant *E. coli* to synthesize β-PA and β-T. They were successful in getting E. coli to excrete OAS by adding glucose to the media. By adding free OASS-A and the appropriate nucleophiles to the excreted OAS, they were able to synthesize the target nonproteinaceous amino acids [21]. The next step in this line of research might be to use isoforms of OASS with differing specificities to produce other nonproteinaceous amino acids. Until recently, only very small amounts of OASS-B have been isolated and purified [16]. Because of its lower specificity [16], OASS-B may prove to be better at synthesizing nonproteinaceous amino acids.

## Structural Biology of OASS-A

### *General Structure of OASS-A without Bound Substrate*

Figure 7 is a representation of the open conformation of OASS-A. It was created in Pymol using PDB file 1OAS and labeled in Microsoft PowerPoint [7,15,16]. The figure shows two subunits of OASS associated in a homodimer, which is how they are normally found in nature [5]. Each subunit has a mass of 34.45kDa [7]. The molecule of PLP bound in each active site is shown in spheres and the enzyme is shown as a cartoon. Each monomer of OASS consists of a large domain and a small domain. The large domain is made up of an interior β-sheet comprised of five parallel β-strands and one antiparallel β-strand. The interior β-sheet is flanked by α-helices on one side and three α-helices on the

other. The small domain is comprised of a β-sheet structure containing four parallel β-strands, which is surrounded by four α-helices. Two beta turns, one from each monomer, overlap to form part of the homodimer interface [16]. There are two allosteric regulation sites situated at the dimer interface [22]. When chloride is bound at these sites, the activity of OASS is lowered, demonstrating allosteric inhibition [8]. The active site of each subunit is situated between the large and the small domain of each subunit, where the coenzyme PLP is bound [22].

## *OASS-A Active Site*

Figure 8 is a view of the active site of OASS-A with PLP bound. It was created in Pymol using PDB file 1OAS and labeled in Microsoft PowerPoint [7]. PLP forms an internal aldimine (Schiff base linkage) with Lys41in the active site. This structure is displayed in sticks. Amino acid residues containing at least one atom within 4Å are shown in lines. Two different colors have been used for these amino acids to make them easier to distinguish from one another. Lys41 and Ser272 (labeled in orange) both contribute to the reaction catalyzed by OASS. Lysine interacts directly with PLP through the Schiff base linkage, and the serine helps to stabilize the pyridoxal ring during the reaction [10]. The active site of OASS-B is very similar to that of OASS-A, although the presence of two ionizable groups, Asp281 and Cys280, indicate a less hydrophobic site in general. Asp281 replaces Pro299 in OASS-A, and Cys280 replaces Ser300 [16]. The pyridoxal ring of PLP, as well as Tyr305 to some extent, are responsible for the spectroscopic activity of OASS [15].

## *OASS Catalytic Mechanism*

OASS is a β-replacement enzyme, meaning that it is an enzyme that catalyzes the substitution of one functional group for another at the β-carbon of an amino acid. β-Replacement enzymes are part of a larger group known as the β-family. All enzymes in the β-family are PLP-dependent [7]. Figure 9 shows the catalytic mechanism of OASS-A. The figure was created in ChemSketch and Microsoft PowerPoint, and is adapted from the 2005 paper by Rabeh, Alguindigue, and Cook [4]. The conversion of OAS to cysteine proceeds via a ping-pong Bi Bi kinetic mechanism. The first half of the reaction (1-3 in the figure) comprises the rate-limiting step of the overall reaction, with a rate constant of $280s^{-1}$ [4]. It begins with the nucleophilic attack of the negatively charged OAS on the imine of the internal Schiff base (1). Specifically, it attacks C4′ of PLP. This results in the formation of an external Schiff base linkage between PLP and OAS (2). The stable α-aminoacrylate (AA) intermediate is produced via α,β-elimination, and acetate is released (3). The second half of the reaction (4-6) begins with the AA in the active site undergoing nucleophilic attack by the second substrate, bisulfide (4). The bisulfide adds to the $C_\beta$ of the AA intermediate to form the external Schiff base with cysteine. The last step is a transimination step (5) in which PLP releases cysteine in favor of reforming the internal Schiff base linkage with the active site lysine. At this point, a new molecule of cysteine has been synthesized and the OASS enzyme is reconstituted and ready to accept to next molecule of OAS (6). The second half of the reaction occurs very quickly, with a rate constant of $>1000s^{-}$ [4]. Because of this, it is thought that the disulfide reactant simply diffuses into the active site of OASS and forms the product upon contact [4].

### *OASS-A Closed Conformation*

During catalysis, OASS changes from an open (no substrate bound) to a closed (substrate bound) and back to an open conformation (product released, native enzyme reconstituted) [5]. The trigger for the global conformational change in the presence of bound OAS is the interaction of the substrate α-carboxyl group with a substrate-binding loop, which is comprised of residues 67-70. One of the largest rearrangements within the substrate-binding loop is that of Asn69. Asn69 shifts about 7Å to make two hydrogen bonds with the α-carboxyl moiety. This shift causes the movable domain to change location, closing the active site. This has the effect of not only preventing access by other molecules of OAS, but also provides stability to the external aldimine and contributes to the proper alignment of functional groups in the substrate with functional groups on the enzyme [8]. Figure 10 comparing the open (left) and closed (right) conformations of OASS-A. The figure was generated in Pymol using PDB files 1OAS and 1D6S and was labeled in Microsoft PowerPoint [7,5,22]. OASS is shown in light-colored cartoon, the substrate-binding loop is shown in pink sticks, and PLP and the substrate analog (in the OASS monomer on the right) are shown in spheres. The orange circles show how the movable domain cuts off the active site in the closed conformation. The orange labels point to the specific structural elements that shift position as a consequence of OAS binding [22].

### *OASS-A Inhibited Conformation*

In 2000, Burkhard, *et al.* crystallized OASS in complex with chloride ions. X-ray diffraction of the crystals revealed a conformation that was neither open nor closed. Each chloride ion binds to an allosteric site on a monomer at the dimer interface, inducing a cascade of side-chain reorientations that ends with the resituating of the entire movable domain (labeled in orange on the preceding figure). Burkhard termed this form of the enzyme the “inhibited conformation”. This is supported by evidence from inhibition studies showing that chloride ions are non-competitive inhibitors of OAS and sulfide [8]. Figure 11 shows an OASS-A homodimer in the inhibited conformation with chloride ions bound to the allosteric sites. The enzyme is shown in light-colored cartoon, the chloride ions are shown as teal spheres, the substrate-binding loop is shown as pink sticks, and PLP is shown as spheres. The figure was generated in Pymol using PDB file 1FCJ [8]. Although in this experiment chloride ions were complexed with OASS, it is unlikely that chloride is a physiological inhibitor of OASS due to its high dissociation constant of about 40mM. Intriguingly, there has been no evidence of an obvious sulfide binding site on OASS, although sulfide is known to be a competitive inhibitor of the enzyme, with a dissociation constant of 50μM. Because the sizes of sulfide (170pm radius) and chloride (167pm radius) are nearly identical, it is thought that sulfide may be the anion that associates with the allosteric binding site of OASS *in vivo* [8].

## Abbreviations Used

AA, α-aminoacrylate; APS, adenosine-5′-phosphosulfate; NAS, *N*-acetylserine; OAS, *O*-acetylserine; OASS, *O*-acetylserine sulfhydrylase; β-PA, β-(pyrozol-1-yl)-L-alanine; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PLP, pyridoxal 5-P′-phosphate; SAT, serine acetyltransferase; β-TA, β-(triazol-1-yl)-L-alanine.

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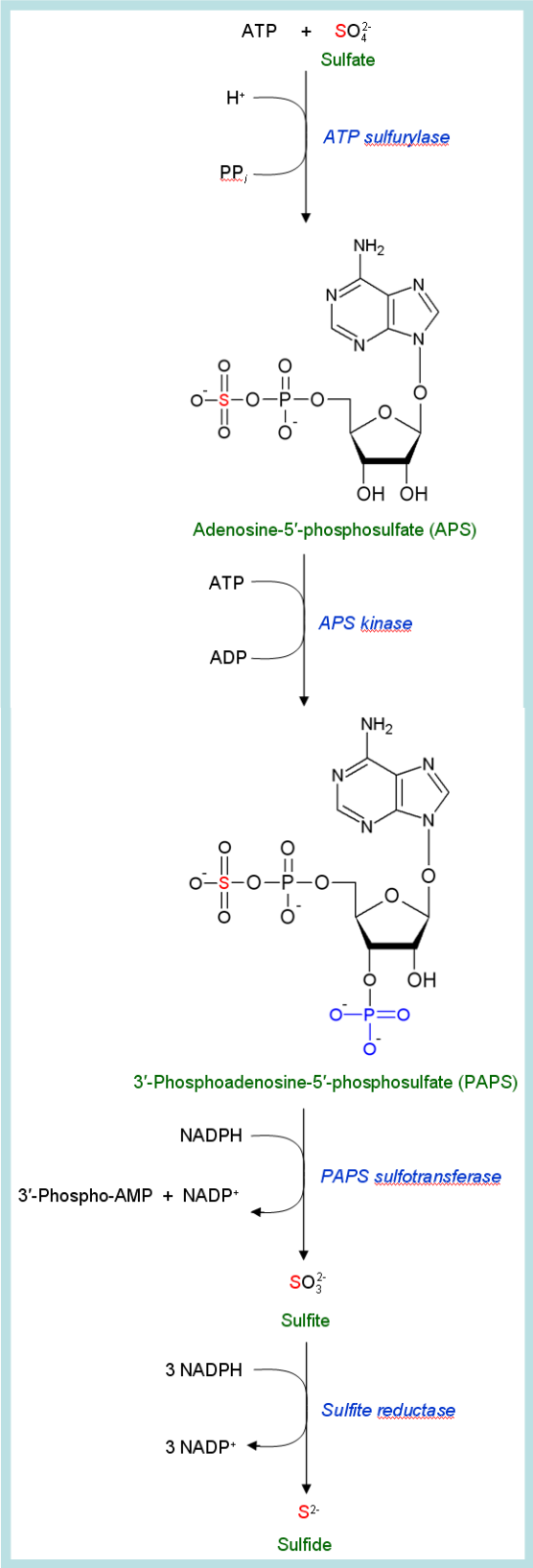
Figure 1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$H^+$
$PP_i$
ATP sulfurylase
Adenosine-5′-phosphosulfate (APS)
ATP
ADP
APS kinase
3′-Phosphoadenosine-5′-phosphosulfate (PAPS)
NADPH
3′-Phospho-AMP + $NADP^+$
PAPS sulfotransferase
$SO_3^{2-}$
Sulfite
3 NADPH
3 $NADP^+$
Sulfite reductase
$S^{2-}$
Sulfide

Figure 2

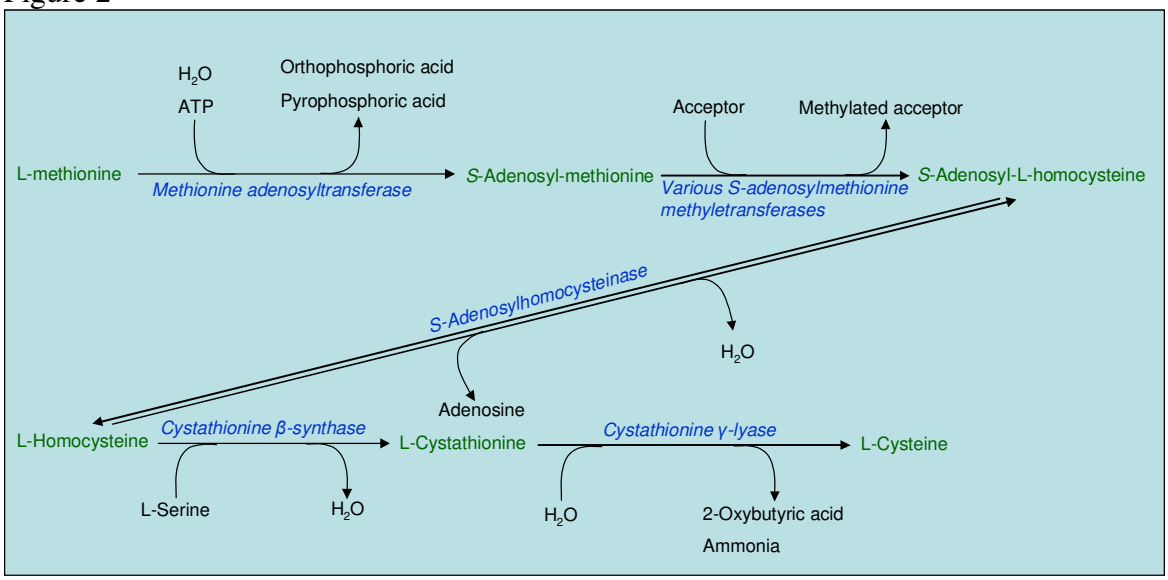


Figure 3

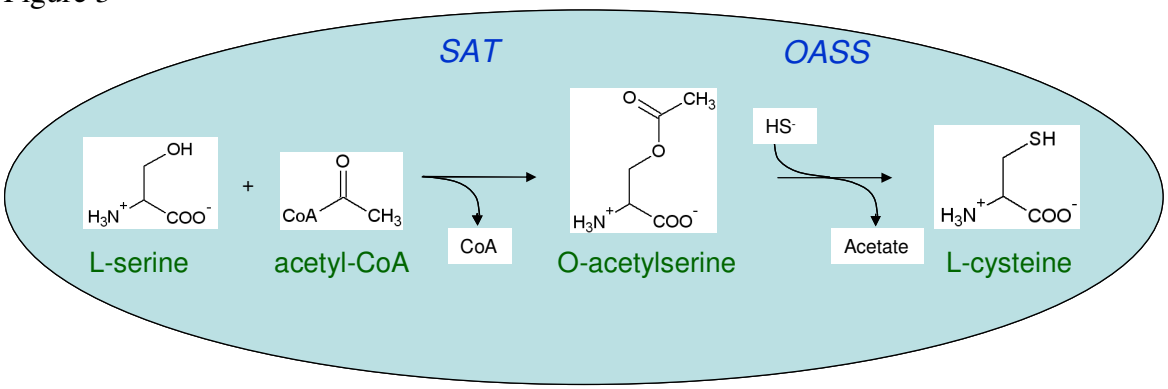


Figure 4

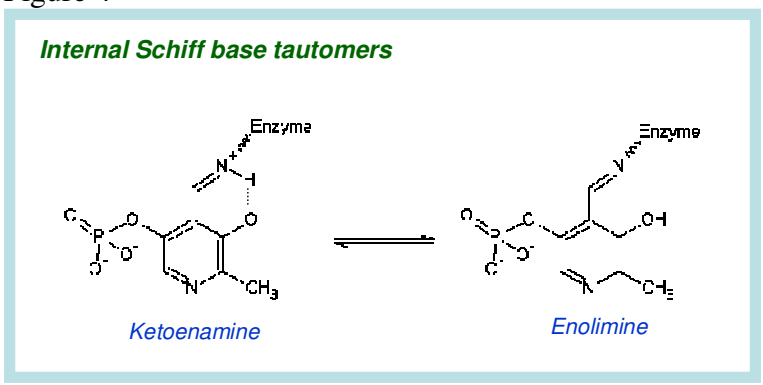

Figure 5

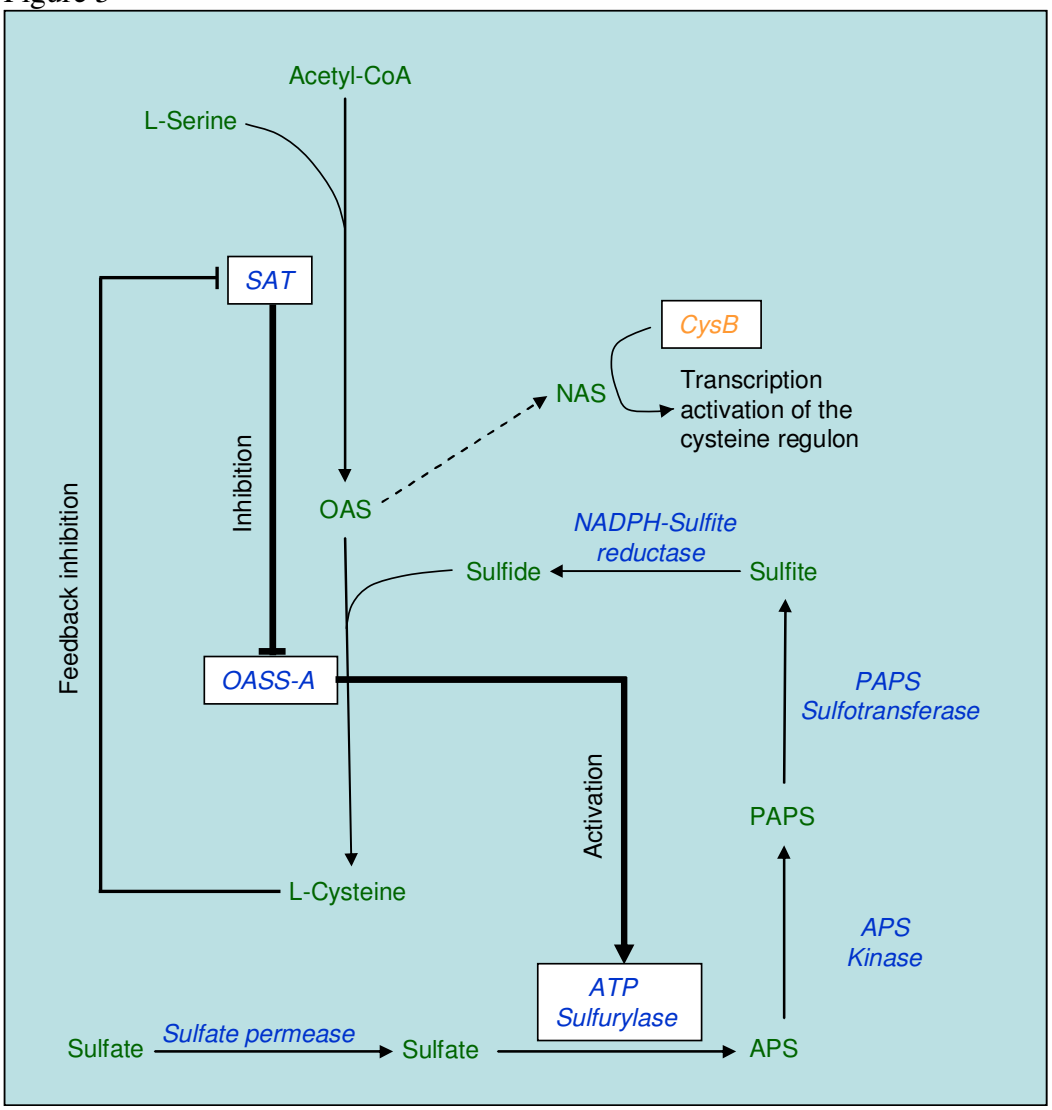
Acetyl-CoA
L-Serine
SAT
CysB
NAS
Transcription activation of the cysteine regulon
Inhibition
Feedback inhibition
OAS
NADPH-Sulfite reductase
Sulfide
Sulfite
OASS-A
PAPS Sulfotransferase
Activation
PAPS
L-Cysteine
APS Kinase
ATP Sulfurylase
Sulfate
Sulfate permease
Sulfate
APS

Figure 6

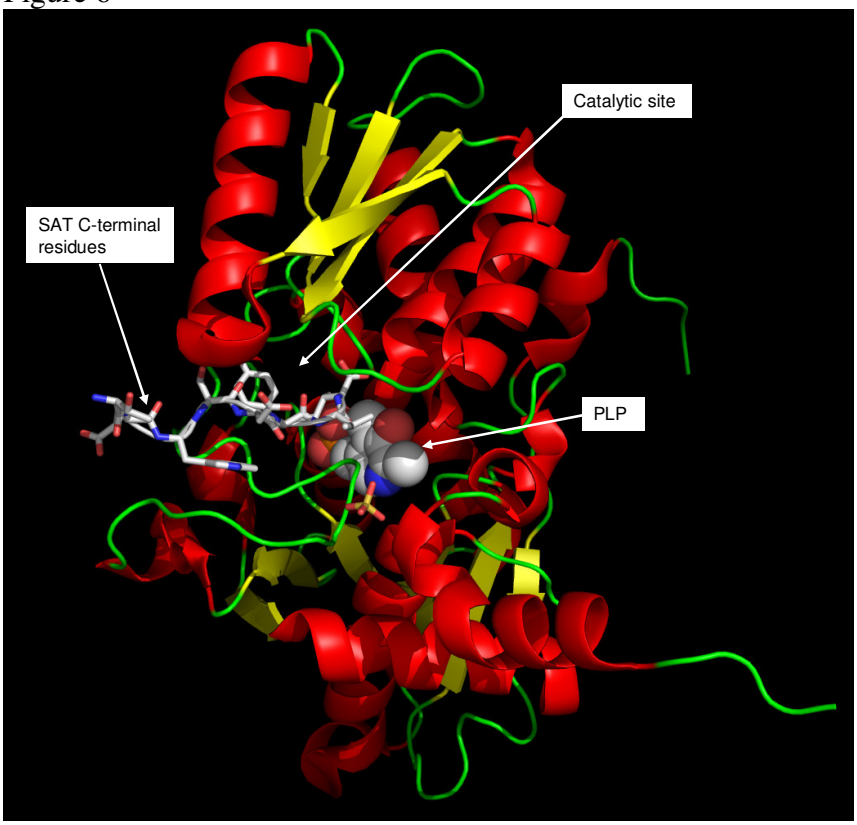

Figure 7

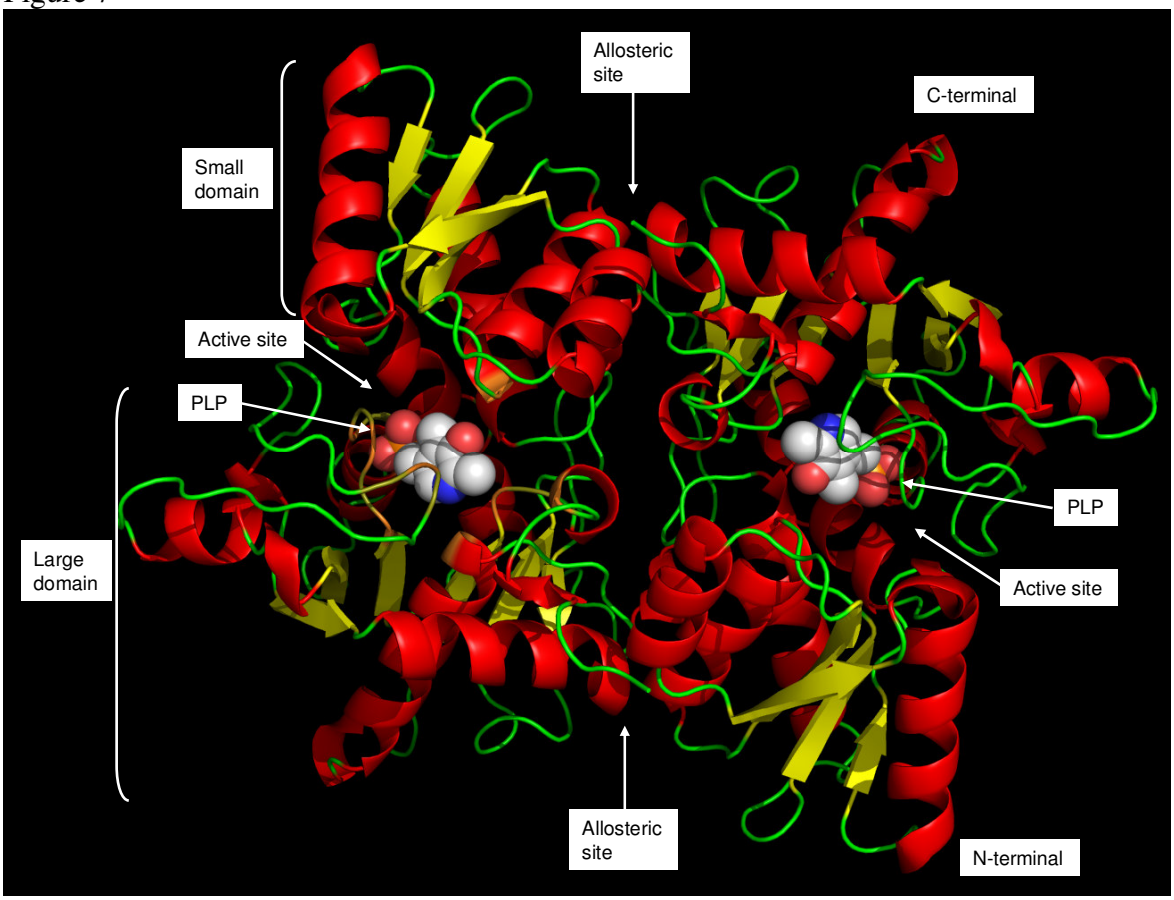


Figure 8

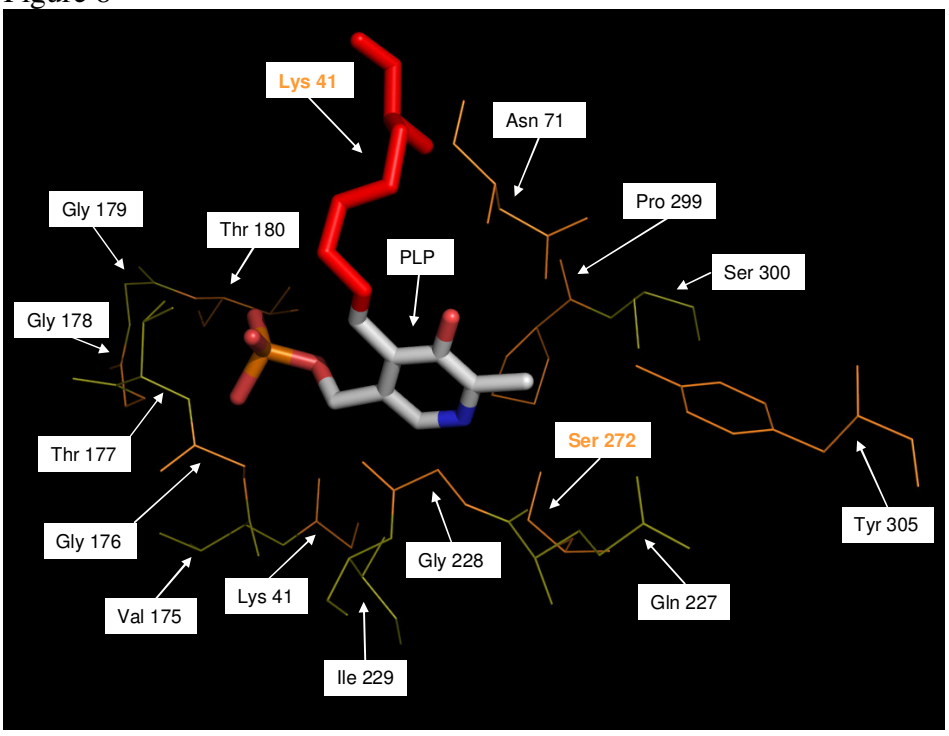

Figure 9

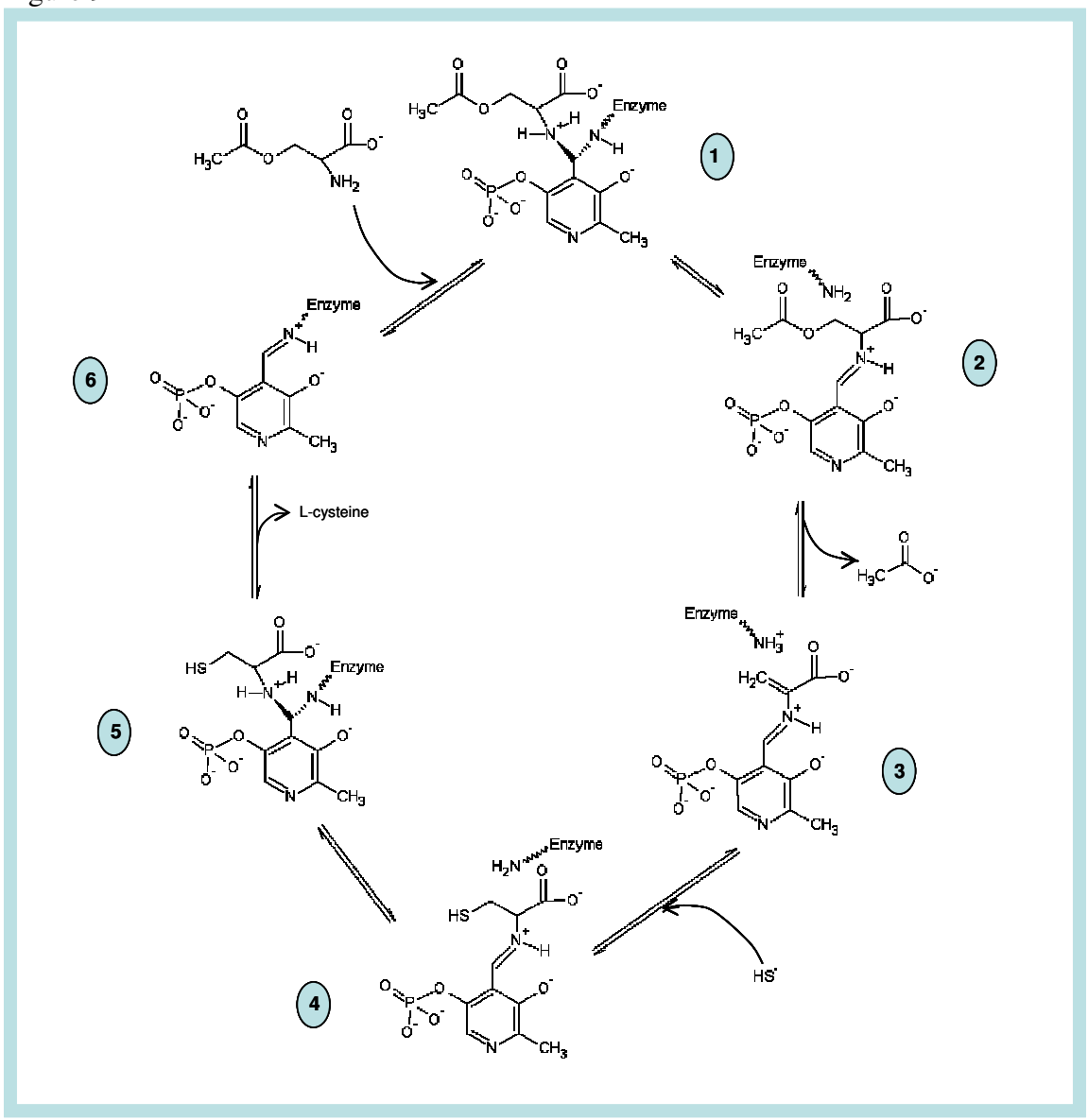

Figure 10

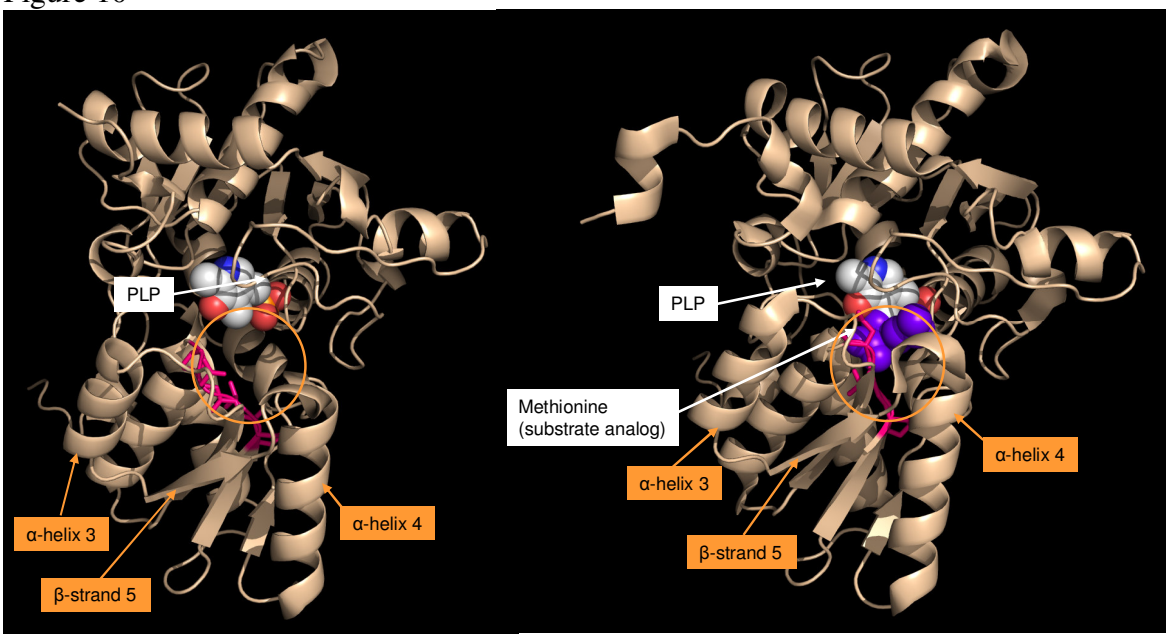


Figure 11