CHAPTER 5

An Overview of OASS

5.1 General description of OASS

The cysteine biosynthetic pathway is a platform for the assimilation of inorganic sulfur into organic compounds in bacteria, plants, and parasitic protists, including *E. histoytica*, *T. vaginalis*, and *Trypanosoma cruzi*, which is the main causal agent of dreaded disease of Chagas' disease (American trypanosomiasis) Nozaki et al. 1998; 1999; 2005; 2000; 2001; Westrop et al., 2006). The pathway has been thoroughly analysed in bacteria and plants (Hell et al., 1994; Ogasawara et al. 1994; Saito et al., 1992). In plants, the whole pathway involves more than one compartments of the cell (Saito et al., 1994). In *E. histolytica*, where typical eukaryotic cell organelles like mitochondria and chloroplasts are absent, the pathway exists exclusively in the cytosol. In case of *T. vaginalis and T. cruzi* the two enzymes in the pathway does not have organelle targeting sequences so the whole pathway is accomplished in the cytosol. In bacteria and plants, which has the ability to reduce sulfate into sulfide via sulfite, first brings the sulfate from outside the cell by sulfate transporters (Saito et al., 1992; 1994). After sulfate activation and reduction, incorporated sulfate (+6) is reduced to sulfide (-2), which receives an alanyl moiety from a donor molecule. Serine O-acetyltransferase (SAT) (EC 2.3.1.30) is instrumental in the production of the alanyl donor O-acetyl- serine from serine and acetyl-CoA (Nozaki et al., 1999) (fig. 5.3.1). CS [O-acetyl- L-serine (thiol)-lyase] (EC 4.2.99.8) then comes into picture and catalyzes the production of L-cysteine by the transportation of the alanyl moiety from the O-acetylserine to sulfide (Nozaki et al. 1998, 2000). But in case of *E. histolytica* and *T. vaginalis* which do not have sufficient avenues to utilize the sulfate via sulfate reduction pathway, take the help of sulfide derived from the iron-sulfur proteins present in ingested bacteria which is residing in either intestine or vagina. In contrast to these parasites, animals do not have the sulfur-incorporation pathway and thus dependent on the methionine from any external source for the sulfur requirement.

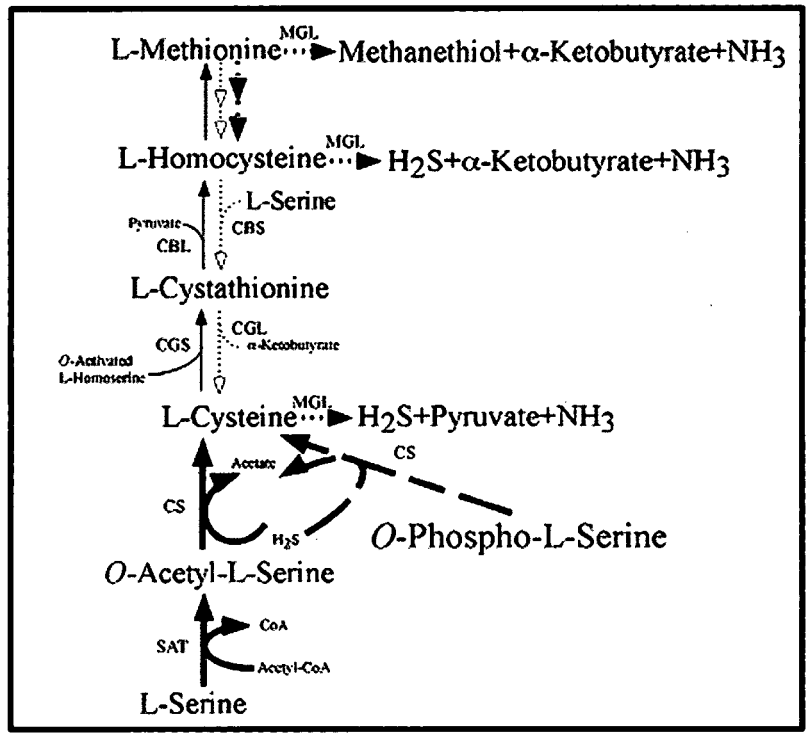

Fig. 5.3.1 General scheme of transsulfuration, cysteine biosynthesis, and sulfur amino acid degradation. The schematic diagram shows all pathways present in bacteria. Open arrows with thin dotted lines depict pathways present in mammals; arrows with thick dotted lines represent pathways present in both *E. histolytica* and *T. vaginalis*; and arrows with thick unbroken lines or thick broken lines represent pathways present only in *E. histolytica* or *T. vaginalis*, respectively. CBS, cystathionine -synthase; CBL, cystathionine -lyase. Adapted from (Vahab et al., 2007)

5.1.1 *Entamoeba histolytica* OASS

Entamoeba histolytica, the causative agent of human amebiasis, is an enteric protozoan parasite, and causes amebic colitis and extraintestinal abscesses (e.g. hepatic, pulmonary, and cerebral) in approximately fifty million inhabitants of endemic areas (The World Health Report, 1995). *E. histolytica* trophozoites which are mainly found in the colonic lumen, which has very less oxygen that is essentially an anaerobic environment, has an access to many bacterial and host derived food. However, the level of oxygen stress they face during tissue invasion, metastasis, and extraintestinal propagation is huge. Despite being an anaerobe *E. histolytica* trophozoites have been proven beyond doubt that it has

some inclination for oxygen and can withstand oxygen tension (Weinbach et al., 1974). Thus, *E. histolytica* must possess mechanisms to detoxify the reactive oxygen species produced by the mammalian host and within their own cells. However, *E. histolytica* does not have well developed antioxidant defense mechanisms present in aerobic or other aerotolerant cells, such as catalase, peroxidase, reduced glutathione, and the glutathione-recycling enzymes glutathione peroxidase and glutathione reductase (Mehlotra et al., 1996). Instead, they have different way of dealing with this kind of situation and have alternative mechanisms for detoxification similar to those known to exist in certain prokaryotes (Bruchhaus et al., 1995).

The biological significance of cysteine synthesis in "amitochondriate" protists is still not yet fully understood. It was shown that cysteine is a major intracellular thiol in these organisms (Brown et al., 1993; Fahey et al., 1984; Gillin et al., 1980b; Gillin et al., 1984). L-Cysteine could not be replaced by any other thiols or reducing agents in the growth medium (Gillin F. D., and L. S. Diamond 1981d), which proves that cysteine is indispensable for the existence of *E. histolytica* and *G. intestinalis* (Brown et al. 1993, Gillin et al. 1981c). Moreover, cysteine also takes part in the antioxidative defense of *E. histolytica* (Nozaki et al., 1999). Cysteine has significant contribution to the oxygen defense mechanisms in this glutathione-deficient organism. It has also been experimentally proven that amoebae need a high amount of extracellular cysteine for attachment to matrix, elongation, motility, and growth in vitro (Gillin et al., 1980b, 1981d).

In nature, the cysteine biosynthetic pathway is the platform for the assimilation of inorganic sulfur. They produce cysteine by transporting alanyl moiety of O-acetylserine, to sulfide, which is synthesized by reduction of sulfate via sulfite. This reaction is catalyzed by cysteine synthase (CS) (O-acetyl-L- serine (thiol)-lyase). This pyridoxal phosphate-dependent enzyme exists in a variety of bacteria (Byrne et al., 1988; Ogasawara et al., 1994) and plants (Hell et al., 1994; Noji et al., 1994; Romer et al., 1992; Saito et al., 1992).

From the recent study it has been shown that *E. histolytica* possesses three allelic SAT isotypes and three allelic isotypes of CS (Nozaki et al., 1998; 1999; 2000; 2001). There are several unique features of the amoebic SAT and CS which does not match to any other organism. It has been shown that SAT1 (previously named SAT) is under strict control in the cysteine biosynthetic pathway in *E. histolytica* (Nozaki et al., 1999). SAT1 which has an allosteric site is under the negative feedback control of cysteine. Negative regulation by L-cystine is unique to the amoebic SAT1 enzyme. The other unique finding about the amoebic SAT1 is that there is a lack of protein - protein interaction with CS. In both bacteria and plants, CS and SAT does interact and form a heteromeric complex with a very high molecular mass of several hundred kilodaltons (Droux et al., 1998; 1992; Dan et al., 2000). However, both CS1 (and CS2) and SAT1 form a homodimer, but there is no evidence that they interact with each other under physiological conditions. There has been a lot of biochemical and genetic method to prove that there is no CS - SAT interaction. The methods employed to prove are (i) separation by conventional chromatography during purification from the crude cell lysate, (ii) and to coimmunoaffinity purification of the proteins, and (iii) the yeast two-hybrid system (Nozaki et al., 1999).

Among the three CS isotypes (CS1 to 3), two CS proteins (CS1 and CS2) are identical except for two conservative amino acid changes (Nozaki et al., 1998). CS3 is only among three which is quiet dissimilar from the other two isotypes, with approximately 83% amino acid identity to CS1 and CS2 (Nozaki et al. 1998; 1999; 2000; 2001). While both CS1 and CS2 are present in the cytoplasm of *E. histolytica*, similar to prokaryotic CysK and CysM, the intracellular distribution of CS3 is not clear. One experiment showed that CS1, CS2, and CS3 rescued the growth defect of a CysK- deficient *E. coli* strain, thereby proving that all three isoforms of CS can even act as functional as CysK in a heterologous organism (Nozaki et al., 1998). The non virulent *E. dispar* also has two CS isotypes, CS1 and CS2 (82 to 83% mutual identity) (Nozaki et al., 2000). *E. dispar* CS1 and CS2 matches with its counterpart *E. histolytica* CS1/2 and 3, respectively. After pooling these informations together it can be understood that *Entamoeba* possesses at least two classes of CS isotypes, each with a distinct pI. But one thing is common to the CS isoforms that

is all of these CS isotypes lack signal sequences or organelle-targeting sequences, suggesting that they have only cytosolic presence. The presence of multiple cytosolic CS isotypes even in the non virulent *E. dispar* species gives ample indication that this enzyme is not having direct linkage with pathogenicity of the amoeba but that it plays an important housekeeping role in *Entamoeba*.

Over expression of CS (2- to 3-fold) but not of SAT (13-fold) by introducing multicopy plasmids in the organism lead to 2 times increase in the intracellular thiol content and the hydrogen peroxide resistance (Nozaki et al., 1999).These data indicate that the intracellular concentration of CS1 (or CS2) but not SAT1 mainly affects the thiol content. One of the major unsolved questions related to the pathway is why these protists possess apparently redundant systems, while they have lost many other metabolic pathways by reductive evolution.

5.1.2 *T. vaginalis* OASS

Recently, sulfur-assimilatory cysteine biosynthesis has been elucidated in *T. vaginalis* (Westrop et al., 2006). There is no SAT in *T. vaginalis* where as it has six copies of CS. Enzymological characterization of CS indicates that *T. vaginalis* CS has unique aspect that it can utilize both O-phosphoserine as well as O-acetylserine as an alanyl donor. Since *T.* vaginalis lacks SAT and therefore it does not have capacity to produce O-acetylserine. But *T.* vaginalis also has three copies each of 3-phosphoglycerate dehydrogenase and phosphoserine aminotransferase and these enzymes produce the substrate for CS that is O-phosphoserine from 3-phosphoglycerate taken from glycolysis.

5.1.3 Bacterial OASS

Cysteine is an essential amino acid that performs important aspect in the catalytic activity and structure of many proteins. The joining of two sulphurs that is disulfide bonds between cysteine residues is the most important aspect in the activation of bacterial transcriptional regulators such as OxyR (Zheng et al., 1998) and the molecular chaperone Hsp33 (Jakob et al., 1999). Disulfide bonds are not only required for proper folding but

also for stability of some proteins, particularly those found in extracytoplasmic compartments (Wedemeyer et al., 2000).

In bacteria, cysteine is synthesized from serine by incorporation of sulfide or thiosulfate. Sulfide is obtained by employing two methods that is either inorganic sulfate is transported from the outside and then reduced or from organic sulfonate compounds such as taurine (Van der Ploeg et al. 1996; 1998). The last step in cysteine biosynthesis is catalyzed by either O-acetylserine (thiol)-lyase A or O-acetylserine (thiol)-lyase B, encoded by the genes cysK and cysM, respectively (Fimmel et al., 1977; Hulanicka et al., 1974). The CysK and CysM proteins from *Escherichia coli* are 43% identical. The difference between CysK and CysM is that CysK produces cysteine from O-acetylserine and sulfide, while the CysM protein synthesizes cysteine from thiosulfate instead of sulfide. The reaction between O-acetylserine and thiosulfate produces S-sulfocysteine, which is converted into cysteine by an as yet uncharacterized mechanism (Nakamura et al., 1984). It is suggested that the O-acetylserine (thiol)-lyase B isozyme is preferentially used during specific environment of less oxygen that is during anaerobic growth conditions (Kredich et al., 1996). In *E. coli*, cysteine performs various functions and one of them is that it can be used to donate the sulfur moiety for methionine biosynthesis in a set of reactions known as the trans-sulfuration pathway. This pathway can be reversed in *Bacillus subtilis*, which can therefore use methionine as its sole source of sulfur (Grundy et al., 2002). The genes taking part in cysteine biosynthesis and sulfur incorporation in *E. coli* and *Salmonella enterica serovar Typhimurium* have been well analyzed (Kredich et al., 1996). In recent times, cysteine biosynthesis has been looked into in the gram-positive bacteria *B. subtilis* (Van der Ploeg et al., 2001) and *Lactococcus lactis* , and also in the *archaeon* genus *Methanosarcina* (Borup et al., 2000; Kitabatake et al., 2000). In contrast, cysteine biosynthesis and sulfur assimilation in the gram-positive *Staphylococcus aureus* (human pathogen capable of causing a variety of infections, ranging from minor skin and wound infections to life-threatening diseases (Lowy et al., 1998) have not been well studied.

5.1.4 Plant OASS

In plants making of cysteine from reduced sulphide in chloroplasts, mitochondria, and the cytoplasm is the end product in environmental sulfur assimilation. Cysteine is the metabolic sulfide donor for all cellular components containing reduced sulfur. In bacteria and plants, cysteine is not only necessary for the protein structure but also the starting material for most of the sulfur-containing metabolites such as methionine, glutathione, phytochelatins, iron-sulfur clusters, vitamin cofactors, and multiple secondary metabolites.Two enzymes are instrumental in the production of cysteine in plants and bacteria [fig. 5.3.2 (a)]. Serine acetyltransferase (SAT4 ; EC 2.3.1.30) transporting acetate from acetyl-CoA to serine, thereby producing O-acetylserine. O-Acetylserine sulfhydrylase (OASS or O-acetylserine(thiol)lyase; EC 4.2.99.8) uses pyridoxal 5 prime phosphate (PLP) as a cofactor to synthesize cysteine from O-acetylserine and sulfide. Association of these two enzymes into an assembly which is called the cysteine synthase complex coordinates sulfate assimilation and modulates cysteine synthesis at the cellular level (Kredich et al., 1969; Ruffet et al., 1994; Droux et al., 1998). The whole complex has one SAT hexamer and two OASS dimers (Kredich et al., 1969), and when the two enzymes are present in association SAT activity is enhanced and OASS activity decreases (Droux et al., 1998).

Environmental stresses changes the expression profile and enzymatic activity of OASS. Although OASS is constitutively expressed, there are other factors like sulfur, nitrogen, and carbon starvation conditions and abiotic stresses like salt and heavy metal exposure that modulates (increase) expression profile of OASS in *Arabidopsis thaliana* (AtOASS) (Takahashi et al., 1996; Dominguez-Solis et al., 2001). It has been shown that over expression of OASS in transgenic plants has a lot of positive impact (improves) on heavy metal tolerance (Dominguez-Solis et al., 2001), increases cysteine biosynthesis in response to sulfur stress (Saito et al., 1994), and is a kind of insurance against oxidative stress (Youssefian et al., 2001; Noji et al., 2001). In the plant cells, there are three isoforms, CS-A, CS-B, and CS-C, present in cytoplasm, chloroplasts, and mitochondria, respectively. This was exhibited by molecular cloning of the corresponding genes (Saito et al., 1992; 1993; 1994) as well as physical studies by organelle separation (Fankhauser

et al., 1976). Plant CSs were also shown to catalyze the production of beta-substituted alanines besides cysteine, and are considered to be involved, at least in part, in detoxifying internal toxins like cyanide, pyrazole, and 3,4-dihydroxypyridine (Ikegami et al., 1994). The OASS from different plant organelles share approximately 40% amino acid sequence identity with the bacterial enzymes (Bonner et al., 2005).

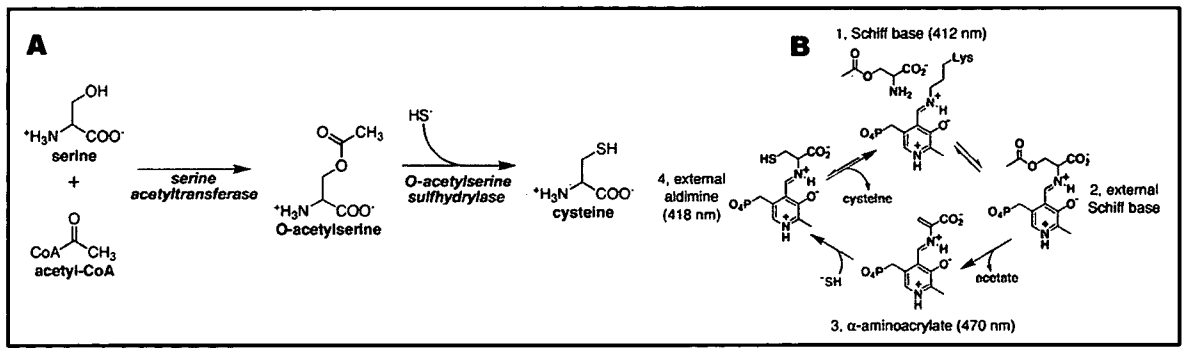

Fig. 5.3.2 Cysteine biosynthesis. Adapted from (Bonner et al., 2005)

The chemical reaction mechanism of OASS is well studied (Tai et al., 2001b). The enzyme active site contains PLP linked to a lysine as an internal Schiff base [fig. 5.3.2 (b), step 1)]. Binding of O-acetylserine displaces the lysine [fig. 5.3.2 (b), step 2)], initiating the first half-reaction yielding alpha - aminoacrylate intermediate linked to PLP [fig. 5.3.2 (b), step 3)]. The second half-reaction involves sulfide addition to the intermediate, thereby generating an external aldimine with the amino acid [fig. 5.3.2 (b), step 4)]. The active site lysine reacts with this intermediate, releasing cysteine and regenerating the Schiff base (Cook et al., 1992; Schnackerz et al., 1995).

5.2 Regulation of OASS

Multienzyme complexes are key themes in a variety of cellular processes, including translation, transcription, gene expression and signal transduction. Organizing macromolecules into complexes offers cells with a way of associating molecular networks and of controlling metabolism by bringing together key enzymes or channeling metabolites between enzyme active sites (Srere, 1987; Winkel, 2004). Kredich et al.,

(1969) is given credit for discovering one of the earliest examples of a macromolecular complex in primary metabolism by isolating the Cys synthase complex but elucidating molecular basis for assembly of this multienzyme complex is only starting up. In plants and bacteria, Cys biosynthesis happens in two stages (Wirtz and Droux, 2005). Ser acetyltransferase (SAT) produces O-acetylserine by transporting acetate from acetyl-CoA to Ser. Next, O-acetylserine sulfhydrylase (OASS) uses pyridoxal phosphate (PLP) as a cofactor to form Cys from O-acetylserine and sulfide.

In plants, strict control of the pathway by two mechanisms keeps intra cellular Cys levels at the required level (Saito et al., 1994). First, feedback inhibition of SAT by Cys can regulate synthesis of the amino acid (Noji et al., 1998). The second level of regulation is done by the association of SAT and OASS to make the Cys synthase complex. The function of the complex is not metabolic channeling, since O-acetylserine freely diffuses out of the complex (Kredich et al., 1969; Cook and Wedding, 1977; Droux et al., 1998). Instead, interaction of SAT and OASS regulates sulfate assimilation and controls Cys synthesis at the cellular level in plants (Hell and Hillebrand, 2001) (fig. 5.3.3). When there is enough amount of sulphur present in cells, the two enzymes associate and form the complex in which SAT activity increases and opposite to this, OASS activity decreases (Saito et al., 1995; Droux et al., 1998). This leads to the formation of O-acetylserine. Under sulphur deficient condition, O-acetylserine keeps piling up because free OASS is unable to produce Cys due to an absence of sulfide. Elevated O-acetylserine levels dissociate the complex, which down regulates SAT. Very high level of O-acetylserine concentration induces genes encoding sulfate transporters, ATP sulfurylase, OASS, and SAT (Hopkins et al., 2005). This results in more of sulfur uptake and reduction. As sulfur levels keep rising up, free OASS catalyzes Cys formation, which reduces O-acetylserine levels. This permits association of SAT and OASS, activation of SAT, and resumption of Cys biosynthesis.The exact analysis in terms of molecular mechanism of how OASS and SAT form the Cys synthase complex are unclear. One SAT hexamer (each Mr of; 180 kDa) and two OASS dimers (each Mr of; 70 kDa) comprise the complex in plants and bacteria (Kredich et al., 1969; Zhu et al., 1998).

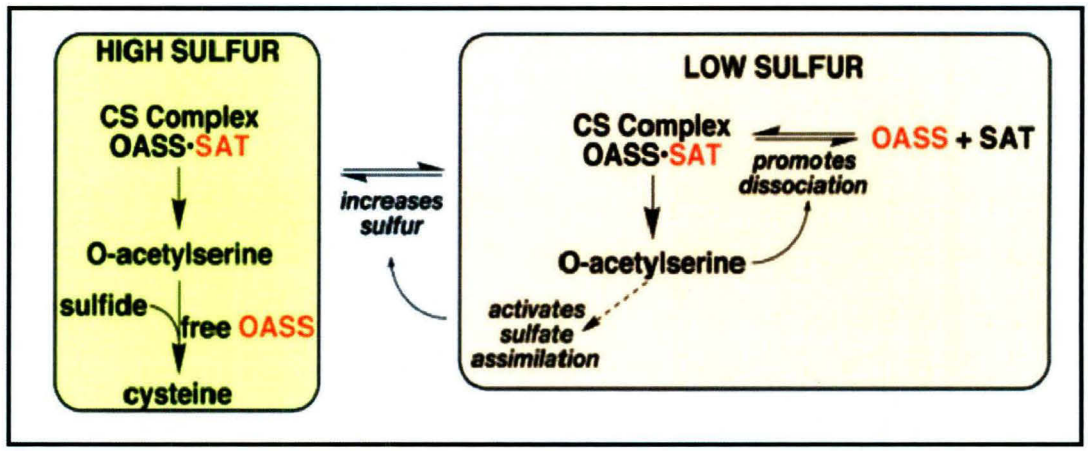

Fig. 5.3.3 Regulation of Cys Synthesis by Formation of the Cys Synthase Complex. Adapted from (Julie et al., 2006)

Demonstration of the interaction between OASS and SAT in plants and bacteria has employed multiple approaches, including size exclusion chromatography, yeast two hybrid analysis, surface plasmon resonance, and fluorescence spectroscopy (Mino et al., 1999; 2000; Wirtz et al., 2001; Berkowitz et al., 2002; Bonner et al., 2005; Campanini et al., 2005).

On further analysing the protein–protein interaction regions in the Cys synthase complex it is found that the C terminus of SAT plays an very important part in association with OASS in plants and bacteria (Mino et al., 1999; 2000; Wirtz et al., 2001). From analysis of the structure of *Haemophilus influenzae* OASS (Hi-OASS) in complex with a peptide (a part of the C terminus of SAT from the same organism), and protein–protein interaction studies of *Arabidopsis* OASS (At-OASS) and At-SAT indicated that the OASS active site is a central theme in the SAT interaction site (Bonner et al., 2005; Huang et al., 2005). Changes in the C termini sequence of SAT and differences in the OASS active site may give evidence in support of the specificity for formation of the Cys synthase complex in different organism like plants and bacteria. These results provide new insights into the molecular mechanism underlying formation of the plant Cys synthase complex.