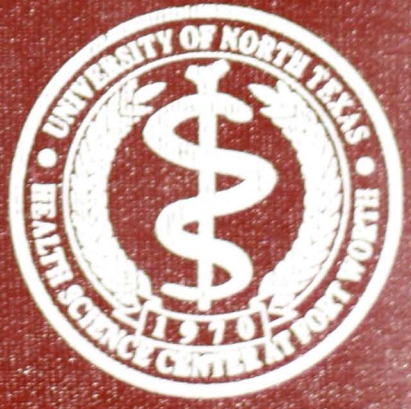
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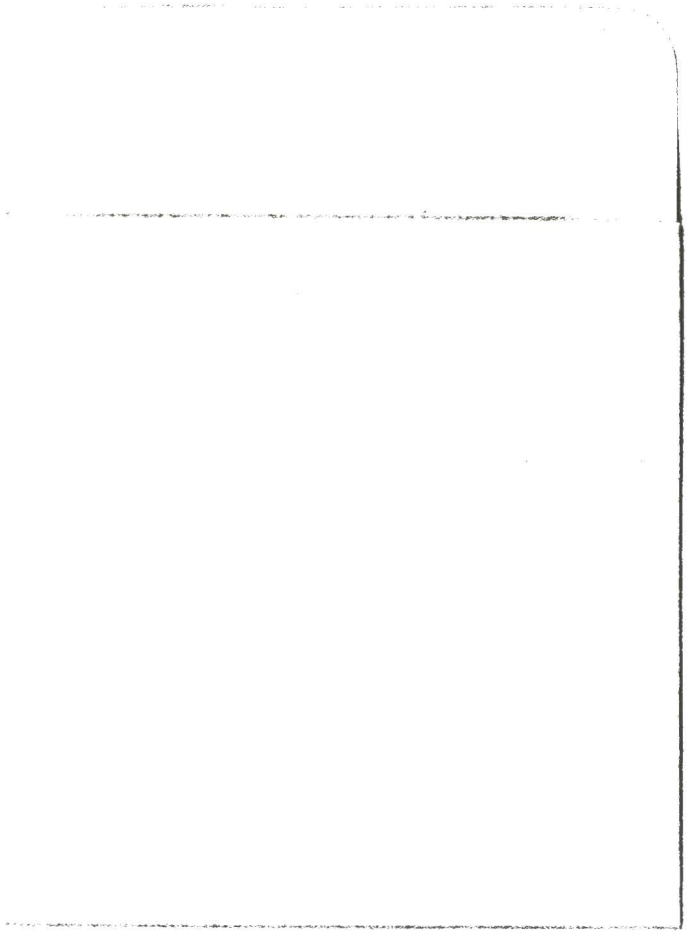

Rege, Vaishali D., Mechanistic Studies of *O*-Acetylserine Sulfhydrylase-A from *Salmonella typhimurium* by Site-Directed Mutagenesis.

Doctor of Philosophy (Biochemistry), July 1996, 94 pp., 3 tables, 27 illustrations, bibliography, 64 titles

O-Acetylserine sulfhydrylase (OASS) is a pyridoxal 5'-phosphate dependent enzyme that catalyzes a β-replacement reaction forming L-cysteine and acetate from *O*-acetyl-L-serine (OAS) and sulfide. The pyridoxal 5'-phosphate (PLP) is bound at the active site in Schiff base linkage with a lysine. In the present study, the Schiff base lysine was identified as lysine 42 and its role in the OASS reaction was determined by changing it to alanine using site directed mutagenesis. K42A-OASS is isolated as an external aldimine with methionine or leucine and shows no reaction with the natural substrates. Apo-K42A-OASS can be reconstituted with PLP suggesting that K42 is not necessary for cofactor binding and formation of the external Schiff base. The apo-K42A-OASS, reconstituted with PLP, shows slow formation of the external aldimine but does not form the α-aminoacrylate intermediate on addition of OAS suggesting that K42 is involved in the abstraction of the α-proton in the β-elimination reaction. The external aldimine formed upon addition of L-ala or L-ser are stable and represent a tautomer that absorbs maximally at 420 nm, while L-cys gives a tautomeric form of the external aldimine that absorbs at 330 nm, also seen in the overall reaction after addition of primary amines to the assay system. The use of a small primary amine such as ethylamine in the assay system or aminoethylation of C43 in apo-K42A-OASS reconstituted with PLP leads to the initial formation of an internal aldimine followed by the slow formation of the α–aminoacrylate intermediate on addition of OAS. Activity could not be fully recovered suggesting a significant rate enhancement resulting from the presence of K42 for transimination and general base catalysis.

Cysteine 43 is the only cysteine in the enzyme OASS-A, and that is next to the internal Schiff base lysine (K42). C43 has been replaced with alanine (C43A), serine (C43S) or threonine (C43T) by site-directed mutagenesis. Also, tryptophan 51 has been replaced with phenylalanine (W51F) or tyrosine (W51Y) by site-directed mutagenesis. Tryptophan 51 is one of the two tryptophan residues in the enzyme, and was thought to be responsible for fluorescence energy transfer to the PLP-internal Schiff base. The growth patterns of the strains carrying mutantions were compared with that of strain DW378 of *S. typhimurium* carrying plasmid pRSM40 with the *cysK* gene encoding OASS-A with no mutation. The growth patterns of the strains with mutations at C43A and C43S were very similar to that of pRSM40 whereas the strains carrying mutations at W51F, W51Y and C43T showed very slow growth. The purification procedures for all of the mutant enzymes were similar to that of the wild type enzyme.

MECHANISTIC STUDIES OF *O*-ACETYLSERINE SULFHYDRYLASE A FROM *Salmonella typhimurium* BY SITE-DIRECTED MUTAGENESIS

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Dean of the Graduate School of Biomedical Sciences

MECHANISTIC STUDIES OF *O*-ACETYLSERINE SULFHYDRYLASE A FROM *Salmonella typhimurium* BY SITE-DIRECTED MUTAGENESIS

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

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in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Vaishali D. Rege

July, 1996

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Scheme Page

LIST OF ABBREVIATIONS

APS	Adenosine-5'-phosphosulfate
BEA	2-Bromoethylamine
CD	Circular dichroism
NAS	*N*-Acetyl-L-serine
OASS	*O*-Acetylserine sulfhydrylase
OASS-A	A isozyme of *O*-Acetylserine sulfhydrylase
OASS-B	B isozyme of *O*-Acetylserine sulfhydrylase
OAS	*O*-Acetyl-L-serine
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PLP	Pyridoxal-5'-phosphate
STA	Serine transacetylase
WT	Wild type

CHAPTER 1

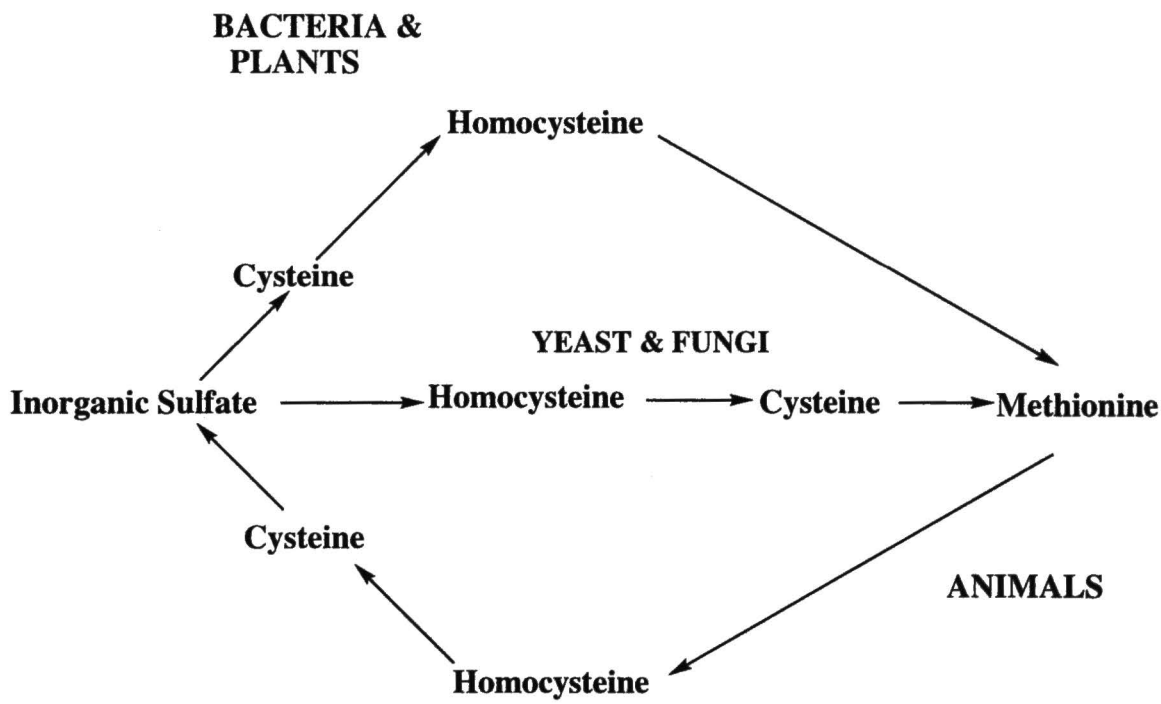
INTRODUCTION

Sulfur Cycle Among Living Organisms. Sulfur being a part of amino acids and many other metabolites is an essential element for living organisms. In nature, sulfur occurs in elemental state as well as in various oxidation states forming sulfates, thiosulfates and other derivatives. Assimilation of sulfur is well developed in living organisms. Microbes and plants have pathways for uptake of inorganic sulfur in various oxidation states and its reduction to sulfide which is followed by utilization of sulfide in various metabolic reactions. Bacteria and plants assimilate more than 4.6×10^{11} kg of inorganic sulfur per annum thereby producing sulfur containing amino acids; cysteine and methionine (Giovanelli, 1987). These amino acids comprise up to 90% of the total sulfur of most plants and bacteria. Methionine and cysteine are predominantly present in proteins, while the nonprotein fraction mainly contains glutathione as the major constituent. Animals, on the other hand, require a dietary source of methionine as a sulfur source which they convert to cysteine and inorganic sulfate. Figure 1 shows the relationship among bacteria, plants, yeast, fungi and animals in terms of assimilation of inorganic sulfur.

L-Cysteine. The amino acid cysteine can serve as an important residue in proteins. In addition to incorporation into proteins, cysteine is metabolized to methionine and glutathione in plants and bacteria. Cysteine participates in a number of biochemical processes that depend on the high nucleophilicity of thiols, important in covalent catalysis. The disulfide bonds of cysteine also stabilize the tertiary structure of proteins. In mammals

Figure 1. Sulfur cycle among bacteria, plants, fungi, yeast and animals.

Bacteria and plants can utilize inorganic sulfate to synthesize L-cysteine. Yeast and other fungi utilize inorganic sulfate to form homocysteine which they convert to cysteine. Animals cannot utilize inorganic sulfate. They require L-methionine as a source of sulfur which they convert into cysteine and inorganic sulfate.


BACTERIA &
PLANTS
Homocysteine
Cysteine
YEAST & FUNGI
Inorganic Sulfate
Homocysteine
Cysteine
Methionine
Cysteine
ANIMALS
Homocysteine

oxidized derivatives of cysteine yield sulfate and taurine, metabolites with important physiological roles in detoxification, bile acid formation, membrane stabilization and neurotransmission (Griffith, 1987).

Cysteine Biosynthesis in Living Organisms. Biosynthesis of cysteine is achieved by different ways in living organisms (Fig. 1). The two most commonly observed routes of cysteine biosynthesis are;

1) transsulfuration of homocysteine and

2) incorporation of the sulfur from inorganic sulfate into cysteine.

Transsulfuration allows interconversion of homocysteine and cysteine with cystathionine as an intermediate (Fig. 2). Cysteine is synthesized in animals via transsulfuration of homocysteine that is derived from methionine (Fig. 2, Giovanelli, 1987). Fungi and yeast are capable of incorporating reduced sulfur into four-carbon esters namely, *O*-succinylhomoserine, *O*-acetylhomoserine, or phosphohomoserine. However, it was recently shown that yeasts and other fungi incorporate sulfide into homocysteine via the four-carbon esters and derive cysteine only by transsulfuration from homocysteine (Fig. 2, Cherest et al., 1993). Bacteria and plants have a pathway for *de novo* biosynthesis of cysteine that involves uptake and reduction of inorganic sulfate, followed by the formation of cysteine. The biosynthesis of cysteine has been studied in various bacteria and plants (Giovanelli, 1987, Chambers and Trudinger, 1971). In plants and bacteria, the two final reactions of the biosynthesis of cysteine are similar and are shown in Fig. 3; acetylation of L-serine is followed by a nucleophilic attack by sulfide to form L-cysteine. It was thought that cysteine biosynthesis in plants takes place in the chloroplasts. However, the demonstration of sulfate incorporation into cysteine in mutants of *Euglena* that lack chloroplasts led to the observation that in *Euglena*, mitochondria possess the capacity for sulfate assimilation and its incorporation into cysteine (Saida et al., 1988). In

Figure 2. Biosynthesis of L-cysteine by transsulfuration of homocysteine.

Animals utilize L-methionine as a source of sulfur to synthesize L-cysteine. Methionine is first converted to homocysteine which is then converted to cysteine by a process called transsulfuration. In fungi and yeast, homocysteine is synthesized from different four-carbon esters and then converted to cysteine in a similar manner.

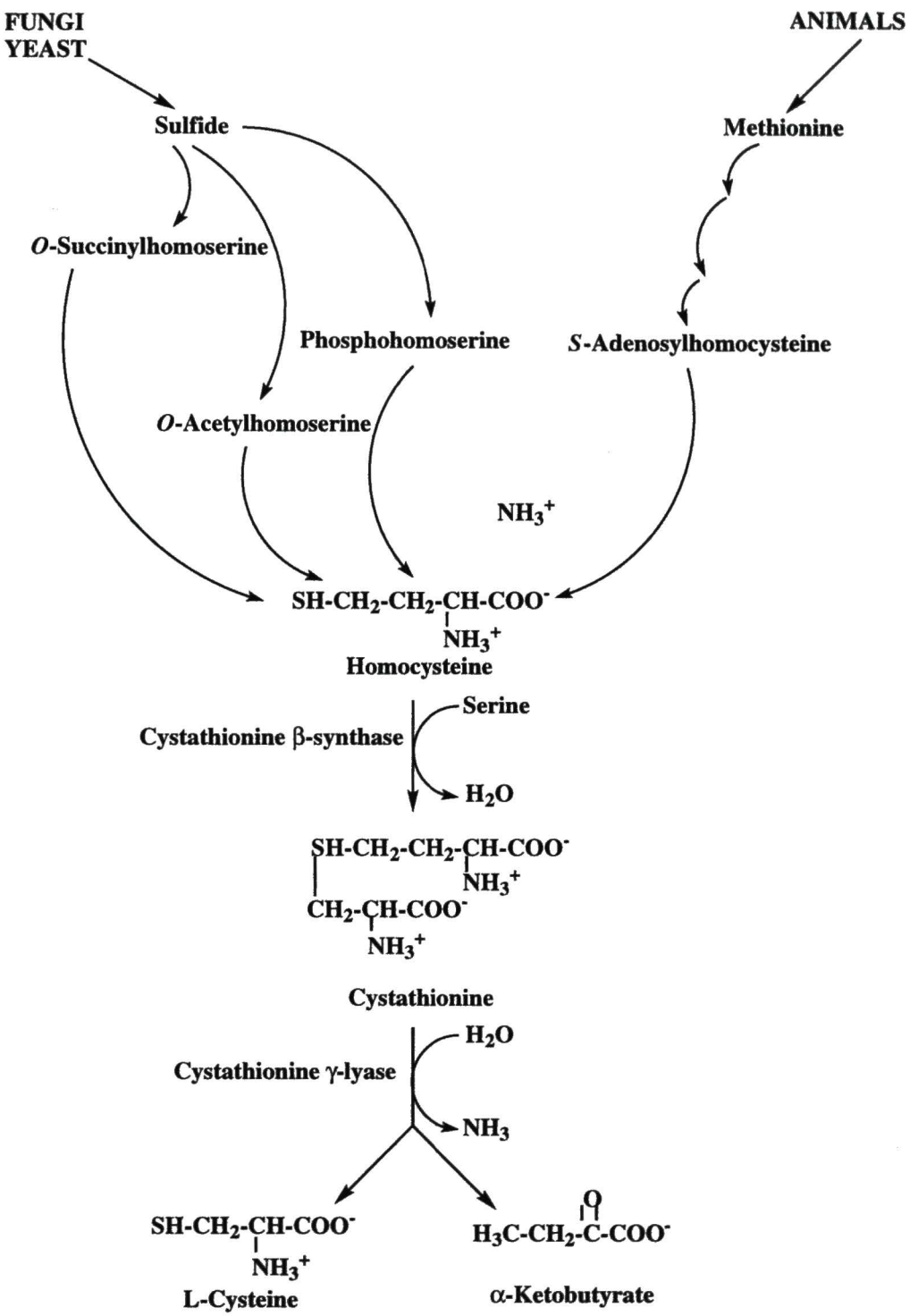
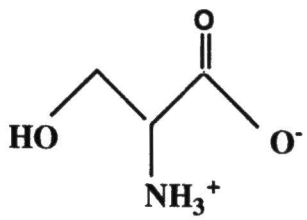
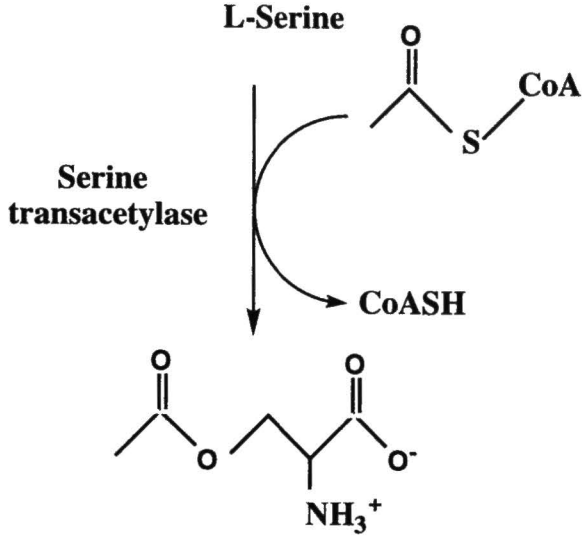

FUNGI
YEAST
ANIMALS
Sulfide
Methionine
O-Succinylhomoserine
Phosphohomoserine
S-Adenosylhomocysteine
O-Acetylhomoserine
NH3+
SH-CH2-CH2-CH-COO-
NH3+
Homocysteine
Serine
Cystathionine β-synthase
H2O
SH-CH2-CH2-CH-COO-
NH3+
CH2-CH-COO-
NH3+
Cystathionine
H2O
Cystathionine γ-lyase
NH3
SH-CH2-CH-COO-
NH3+
L-Cysteine
O
H3C-CH2-C-COO-
α-Ketobutyrate

Figure 3. Biosynthesis of L-cysteine in bacteria and plants.

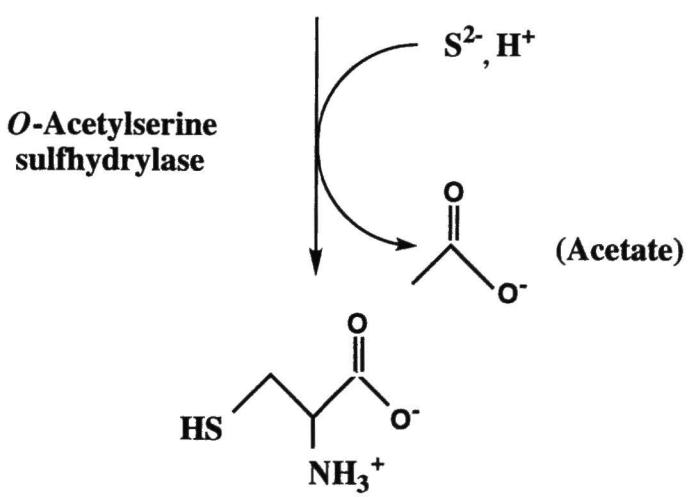
The figure shows the final two reactions in the biosynthesis of L-cysteine that are similar in bacteria and plants. The enzymes, serine transacetylase and *O*-acetylserine sulfhydrylase, form a multienzyme complex known as cysteine synthase.



L-Serine



O-Acetyl-L-serine



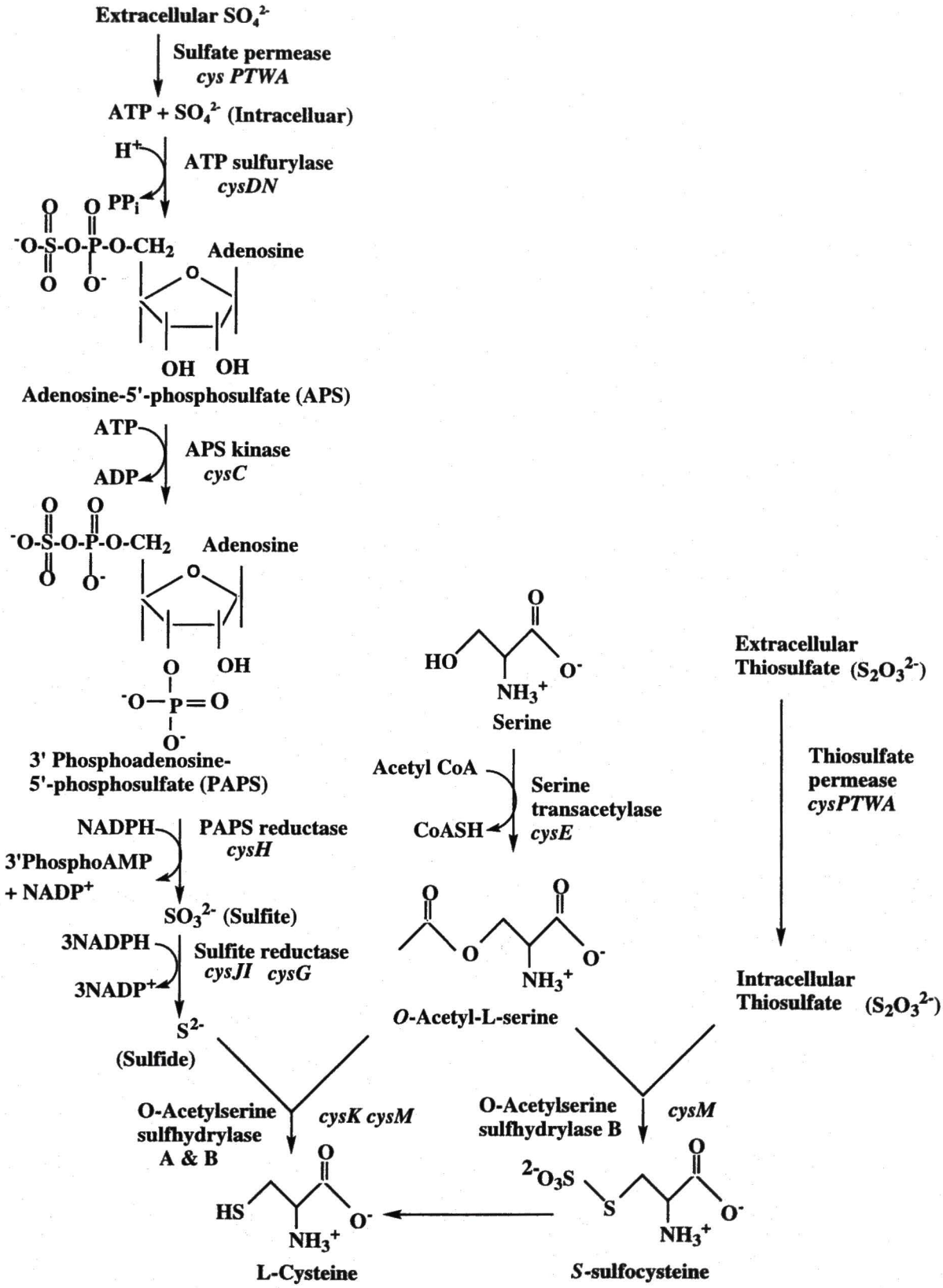
L-Cysteine

higher plants, the incorporation of sulfate, free sulfite and free sulfide into cysteine and the presence of multienzyme complex, cysteine synthase, have been demonstrated in the chloroplast. However, serine transacetylase (STA) activity has been shown to be associated with mitochondria, chloroplasts and the cytoplasm (Droux et al., 1992) and the activity of the enzyme, *O*-acetylserine sulfhydrylase (OASS), has been demonstrated in all three organelles involved in protein synthesis, namely plastids, mitochondria and cytosol (Droux et al., 1992). The studies on yeast show the machinery for cysteine biosynthesis to be present in mitochondria as well as in the cytoplasm.

Cysteine Biosynthesis in E. coli and S. tphimurium. Genetic and biochemical aspects of cysteine biosynthesis have been studied most extensively in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* by Kredich and his colleagues. The original discovery of the *de novo* biosynthesis of cysteine was by Kredich and Tomkins (1966). The pathway for the biosynthesis of cysteine in *E. coli* and *S. typhimurium* is comprised of two arms; one leading to formation of sulfide by reduction of intracellular sulfate and the other leading to the formation of *O*-acetyl-L-serine (OAS) from L-serine and acetyl coenzyme A (Fig. 4). Sulfate uptake is facilitated by sulfate permease which can also take up thiosulfate. The intracellular sulfate is first activated by conversion to adenosine 5'-phosphosulfate (APS), catalyzed by ATP sulfurylase and then converted to a high energy sulfate donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) by APS kinase. PAPS is further reduced to sulfite in a reaction catalyzed by PAPS reductase. Conversion of sulfite to sulfide is then catalyzed by sulfite reductase. Sulfide, thus formed, reacts with OAS, the product of the reaction catalyzed by serine transacetylase (STA), to form cysteine. The formation of cysteine is catalyzed by *O*-acetylserine sulfhydrylase (OASS). A third arm for the biosynthetic pathway of cysteine in Figure 4 shows that thiosulfate can be utilized by *E. coli* and *S. typhimurium,* bypassing reduction

Figure 4. Biosynthesis of L-cysteine in *E. coli* and *S. typhimurium*

The figure shows reactions in the pathway of sulfate reduction as well as formation of L-cysteine in *E. coli* and *S. typhimurium*. The genes encoding each enzyme are shown in italics.

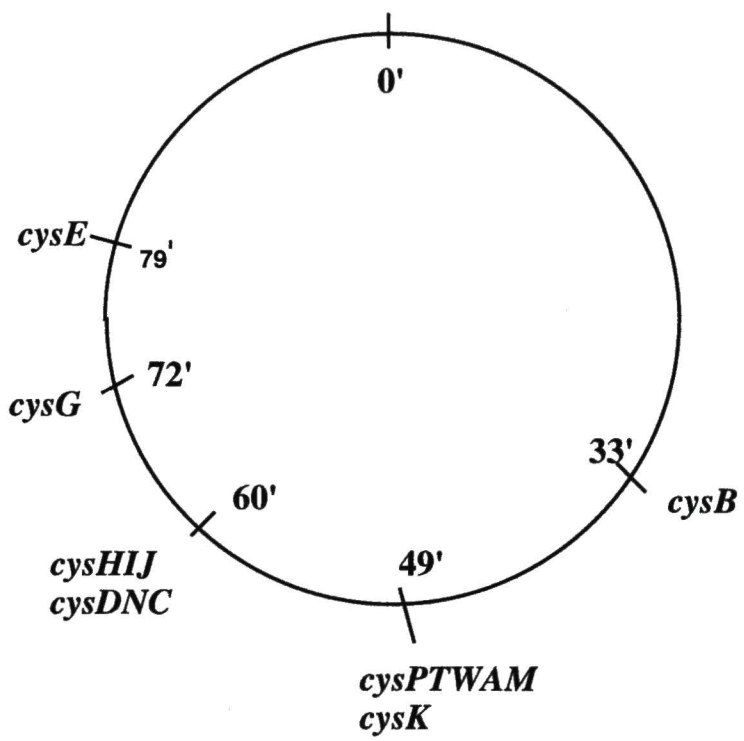

Extracellular SO_4^{2-}
Sulfate permease
cys PTWA
ATP + SO_4^{2-} (Intracelluar)
H^+
ATP sulfurylase
cysDN
PP_i
Adenosine
OH OH
Adenosine-5'-phosphosulfate (APS)
ATP
ADP
APS kinase
cysC
Adenosine
3' Phosphoadenosine-
5'-phosphosulfate (PAPS)
NADPH
3'PhosphoAMP
+ $NADP^+$
PAPS reductase
cysH
SO_3^{2-} (Sulfite)
3NADPH
3$NADP^+$
Sulfite reductase
cysJI cysG
S^{2-}
(Sulfide)
Serine
Acetyl CoA
CoASH
Serine
transacetylase
cysE
O-Acetyl-L-serine
Extracellular
Thiosulfate ($S_2O_3^{2-}$)
Thiosulfate
permease
cysPTWA
Intracellular
Thiosulfate ($S_2O_3^{2-}$)
O-Acetylserine
sulfhydrylase
A & B
cysK cysM
O-Acetylserine
sulfhydrylase B
cysM
L-Cysteine
S-sulfocysteine

of sulfate to sulfide.

Regulation of Cysteine Biosynthesis in E. coli and S. typhimurium. Regulation of cysteine biosynthesis is achieved by several means including gene regulation, feedback inhibition by cysteine and also enzyme degradation (Kredich, 1987).

The genes encoding the enzymes involved in the cysteine biosynthetic pathway of *E. coli* and *S. typhimurium* form a regulon. Figure 5 shows the cysteine regulon of *S. typhimurium*. The cysteine regulon of *S. typhimurium* includes the genes; *cysA* (sulfate permease), *cysC* (APS kinase), *cysD* (ATP sulfurylase), *cysH* (PAPS reductase), *cysI* (sulfite reductase; hemoprotein), *cysJ* (sulfite reductase; flavoprotein), *cysK* (*O*-acetylserine sulfhydrylase A), *cysM* (*O*-acetylserine sulfhydrylase B), *cysE* (serine transacetylase), and *cysB* (regulatory protein CysB). Some of these genes are arranged in groups consisting of positively regulated operons, the negatively autoregulated *cysB* gene, and the *cysE* and *cysG* genes. The gene clusters *cysPTWAM*, *cysJIH*, *cysK* and *cysDNC* are positively regulated by the product of the *cysB* gene which is a transcriptional activator belonging to the LysR family of regulatory proteins. Transcription initiation is facilitated by binding of CysB just upstream at the -35 region of the positively regulated promoters (Kredich, 1992). CysB protein also autoregulates its synthesis by binding to the promoter of the *cysB* gene (Kredich, 1992). The *cysG* gene is not considered a part of the regulon as it is not regulated by the CysB protein. The gene *cysE* is also not regulated by the CysB protein, but it is still considered a part of the regulon as it provides an internal inducer, OAS. High levels of expression (derepression) of the cysteine regulon require three factors; sulfur limitation, the presence of OAS and the CysB regulatory protein (Kredich, 1987). Derepression of the cysteine biosynthetic pathway has been shown with different sources of sulfur. The activities of the enzymes in the pathway decrease progressively with growth on sulfate, sulfide and cysteine or cystine, while

Figure 5. Map of the genes involved in the cysteine regulon of *S. typhimurium*


0'
cysE
79'
72'
cysG
33'
cysB
60'
cysHIJ
cysDNC
49'
cysPTWAM
cysK

djenkolic acid and reduced glutathione have shown maximum derepression of the pathway (Kredich, 1971). Sulfur limited cultures of *cysE* mutants cannot be derepressed for the biosynthetic pathway unless an exogenous source of OAS is provided. *O*-Acetyl-L-serine has also been shown to induce the sulfate reducing enzymes. Therefore, OAS, a direct precursor of cysteine, is considered an internal inducer of the pathway (Kredich, 1987). *O*-Acetyl-L-serine is converted to N-acetyl-L-serine (NAS) nonenzymatically, and NAS has also been shown to induce sulfate reduction. Recent studies on *Klebsiella aerogenes*, have shown that NAS rather than OAS serves as the inducer of the cysteine regulon (Lynch et al., 1994). The combination of feedback inhibition of STA by cysteine and induction of the sulfate reducing enzymes by OAS provides communication between the two branches of the biosynthetic pathway (Kredich 1987).

Formation of L-Cysteine. The final two reactions in the biosynthetic pathway for cysteine are shown in Fig. 3. Acetylation of L-serine to OAS is catalyzed by serine transacetylase (STA) whereas *O*-acetylserine sulfhydrylase (OASS) catalyzes the formation of L-cysteine from OAS and sulfide (Becker and Tomkins, 1969). Serine transacetylase and OASS-A form a soluble multifunctional protein complex that is known as cysteine synthase. About 5% of the total cellular OASS activity is associated with the cysteine synthase complex whereas the remaining 95% is uncomplexed (Kredich and Tomkins, 1969). Acetyl CoA and cysteine were found to be effectors of the cysteine synthase multienzyme complex that influence the K_m values for serine and acetyl CoA (Cook and Wedding, 1976). Cysteine is a potent inhibitor of STA, either as a component of the multienzyme complex or uncomplexed, with a K_i for cysteine of 1 μM at 0.1 mM acetyl CoA (Kredich et al., 1969). In *S. typhimurium*, cysteine synthase is reversibly dissociated in the presence of OAS into one molecule of STA and two molecules of OASS-A (Kredich et al., 1969).

Two isozymes of OASS have been found in enteric bacteria, termed A and B; and both catalyze the formation of cysteine from OAS and sulfide (Becker et al., 1969). In addition to the reaction with sulfide, OASS-B has been shown to be more effective than the A-isozyme in utilizing thiosulfate to form *S*-sulfocysteine which can be reduced to cysteine (Nakamura, 1984). The mechanism of conversion of *S*-sulfocysteine to cysteine is not yet known but may involve hydrolysis of *S*-sulfocysteine to cysteine and sulfate or reduction by glutathione to cysteine and sulfite (Woodin and Segel, 1968). In *E. coli* and *S. typhimurium*, a large excess of OASS-A over OASS-B has been found to be present under aerobic conditions of growth whereas OASS-B is thought to be required for growth under anaerobic conditions (Hulanicka et al., 1979, Filutowicz et al., 1982). Presence of the B isozyme of OASS is thus necessary for the bacteria to grow efficiently under anaerobic conditions with thiosulfate and perhaps other more reduced forms of sulfur as a source, allowing the bacteria to bypass sulfate reduction.

The A and B isozymes of OASS are homodimeric pyridoxal 5'-phosphate (PLP) dependent enzymes with one PLP molecule bound per subunit (Kredich and Tomkins, 1966). The monomeric molecular weight of OASS A is 34,450 (Levy and Danchin, 1988) whereas that of the OASS-B monomer is 27,500 (Nakamura et al., 1989).

OASS From Various Sources. The enzyme, OASS, has been isolated from various plants and bacteria. Most bacteria have been shown to have two isozymes whereas plants have multiple isoforms of OASS (Droux et al., 1992). In plants, the isozymes of OASS have been found in different organelles namely, plastids, mitochondria and cytoplasm (Droux et al., 1992). Figure 6 shows similarity among the active sites of different isozymes of OASS from different species of plants and bacteria. The consensus sequence for the OASS active site is given below:

K L/V E X X X P X X S V **K** D/C R I/A G/A X X M I X X A E

Figure 6. Similarity among the active site of OASS from various sources.

The figure shows similarity among the isozymes of OASS from different plant and bacterial species. The sequence of OASS isozymes are from the following organisms; *Arabidopsis thaliana*, *Capsicum annuum*, *Citrullus lanatus* (watermelon), *Spinacia oleracea* (spinach), *Triticum aestivum* (wheat),, *Escerichia coli*, *Bacillus subtilis*, *Emericella nidulans*, *Haemophilus influenzae*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Flavobacterium* species, and *Synechoccus* species. The lysine in bold is the one in internal Schiff base linkage with PLP. In the figure,

cysK represents a homolog of OASS-A

cysL represents a precursor of OASS in chloroplasts

cysM represents a homolog of OASS-B

CS represents cysteine synthase (OASS without describing the isozyme)

prc represents a precursor

The protein sequences for these enzymes were obtained from Swissprot databank and the alignments were carried out by PIMA (Pattern induced Multiple Alignment program).

cysK	*A. thaliana*	KLEMMEPCSSV**K**DRIGFSMISDAEKKGLIKPG
cysK	*S. oleracea*	KLEGMEPCSSV**K**DRIGFSMITDAEKSGLITPG
cysK	*T. aestivum*	KLESMEPCSSV**K**DRIGYSMISTAEKKGFIVPG
cysK	*E. coli*	KVESRNPSFSV**K**CRIGANMIWDAEKRGVLKPG
cysK	*B. subtilis*	KLEYMNPGSSV**K**DRIGLAMIEAAEKEGKLKAG
cysK	*H. influenzae*	KIEGRNPSYSV**K**CRIGANMVWQAEKDGTLTKG
cysK	*S. typhimurium*	KVESRNPSFSV**K**CRIGANMIWDAEKRGVLKPG
cysK	*Synechoccus*	KIEGRNPAYSV**K**CRIGAAMIWDAEQRGLLGPG
cysL	*A. thaliana*	KLEIMEPCCSV**K**DRIGYSMISTAEKKGLITPG
cysL	*C. annuum*	KLEIMEPCCSV**K**DRIGFSMISDAEEKGLISPG
cysL	*S. oleracea*	KLESMEPCCSV**K**DRIGYSMIDDAEQKGVITPG
cysM	*E. coli*	KLEGNNPAGSV**K**DRAALSMIVEAEKRGEIKPG
cysM	*P. syringae*	KLEGNNPAGSV**K**DRPALSMITRAELRGQIHPG
cysM	*S. typhimurium*	KLEGNNPAGSV**K**DRAALSMIVEAEKRGEIKPG
CS	*C. lanatus*	KLEMMEPCSSV**K**DRIGYSMISDAENKGLITPG
CS	*E. nidulans*	KAEFQNPGGSV**K**DRAALYVVKDAEERGLLKPG
CS	*Flavobacterium*	KLEKSNPGGSI**K**DRIALAMIEDAEAKGLLNKD
CS	*M. leprae*	KVEYFNSGGSS**K**DRIAAKMIDAAEASGQLKPG
CS	*M. tuberculosis*	KLEDRNPTGSI**K**DRPAVRMIEQAEADGLLRPG
(probable)		
CS.(prc)	*S. oleracea*	KQEMMQPTASV**K**DRPALAMIEDAEKKGLISPG
(Mitochondrial)		
CS (prc)	*Z. mays*	KLESMEPCSSV**K**DRIGYSMITDAEEKGLITPG

In the above sequence, X is any amino acid and the K in bold is the lysine in Schiff base linkage with PLP (Saito et al., 1993).

The enzyme, OASS, has recently been found to be important in the detoxification of hydrogen sulfide gas. Increasing utilization of sulfide by introduction of a high copy number plasmid carrying gene for OASS from wheat in tobacco plant has been shown to increase the resistance of tobacco plants to toxic levels of hydrogen sulfide gas (Youssefian et al., 1993). Also, introduction of the gene *cysK* along with *cysE* has been shown to increase wool growth in sheep (Bawden et al., 1995) and prevent hair loss in mice (Ward et al., 1994).

O-Acetylserine Sulfhydrylase-A from Salmonella typhimurium. The enzyme, OASS-A, from *Salmonella typhimurium,* catalyzes a β-elimination reaction forming L-cysteine and acetate from OAS and sulfide (Fig. 7). A Bi-Bi ping pong kinetic mechanism has been proposed for OASS-A (Cook and Wedding, 1976; Tai et al., 1993, Fig. 8). The UV-visible spectrum of OASS-A shows a visible absorption maximum at 412 nm that corresponds to an internal aldimine (Becker et al., 1969 Fig. 9, A). Addition of OAS to the enzyme results in a shift in the absorbance from 412 to 470 nm, indicating the formation of α-aminoacrylate intermediate (Cook and Wedding, 1976; Cook et al., 1992, Fig. 9, B). Addition of sulfide to the α-aminoacrylate intermediate restores the original spectrum with an absorption maximum at 412 nm, corresponding to the internal aldimine (Cook and Wedding, 1976). The time-dependent disappearance of the 470 nm absorbance led to the detection of OAS deacetylase activity of OASS-A in which OAS is degraded to pyruvate, ammonia and acetate (Cook et al, 1992).

Excitation of OASS-A at 298 nm gives an emission spectrum with two maxima, 337 and 498 nm (McClure and Cook, 1994). The 498 nm maximum is observed due to the fluorescence of PLP-Schiff base. The amino acid sequence of OASS-A shows only two

Figure 7. Proposed chemical mechanism for OASS-A (Tai et al., 1995)

In the figure,

I represents an internal aldimine

II represents *gem*-diamine with OAS

III represents an external aldimine with OAS

IV represents α-aminoacrylate

V represents α-aminoacrylate

VI represents an external aldimine with L-cysteine

VII represents *gem*-diamine with L-cysteine

VIII represents an internal aldimine

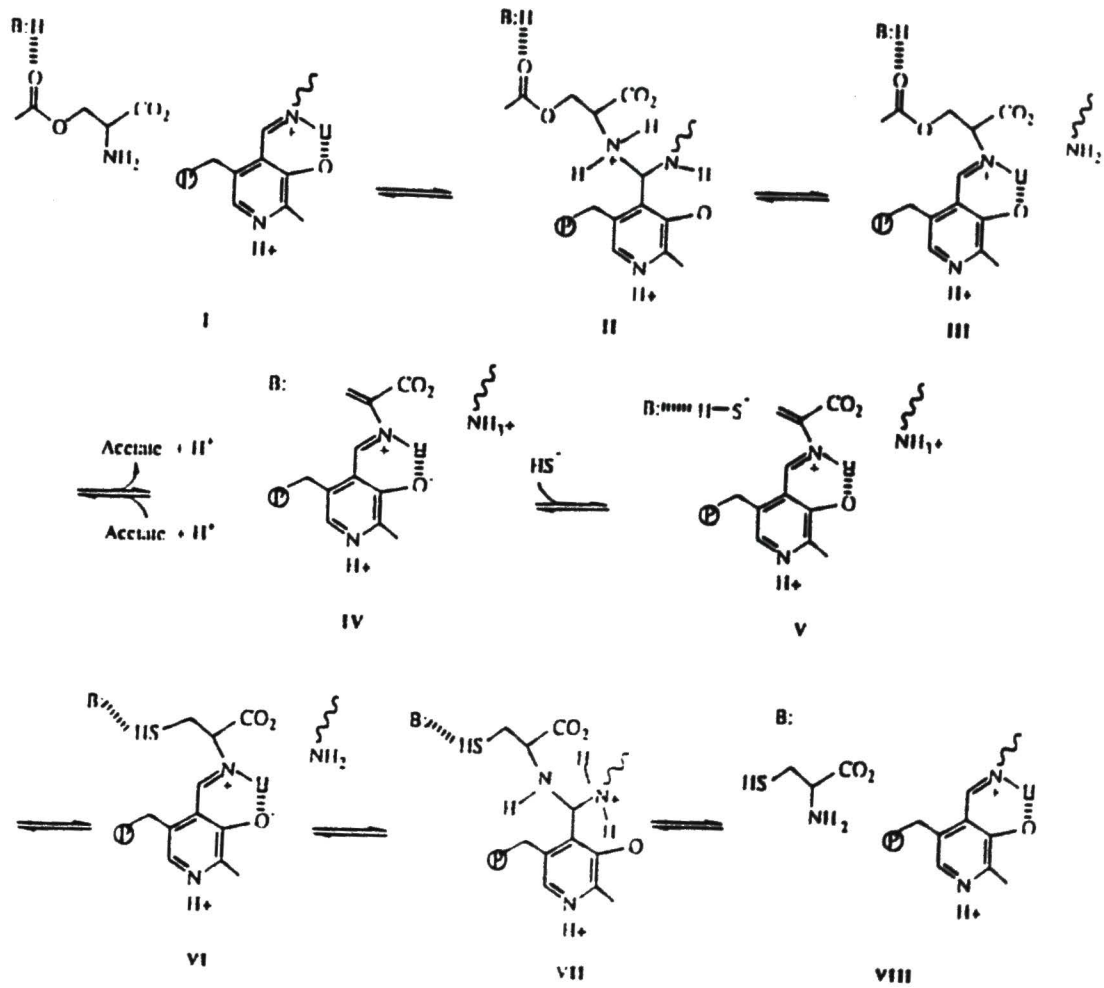
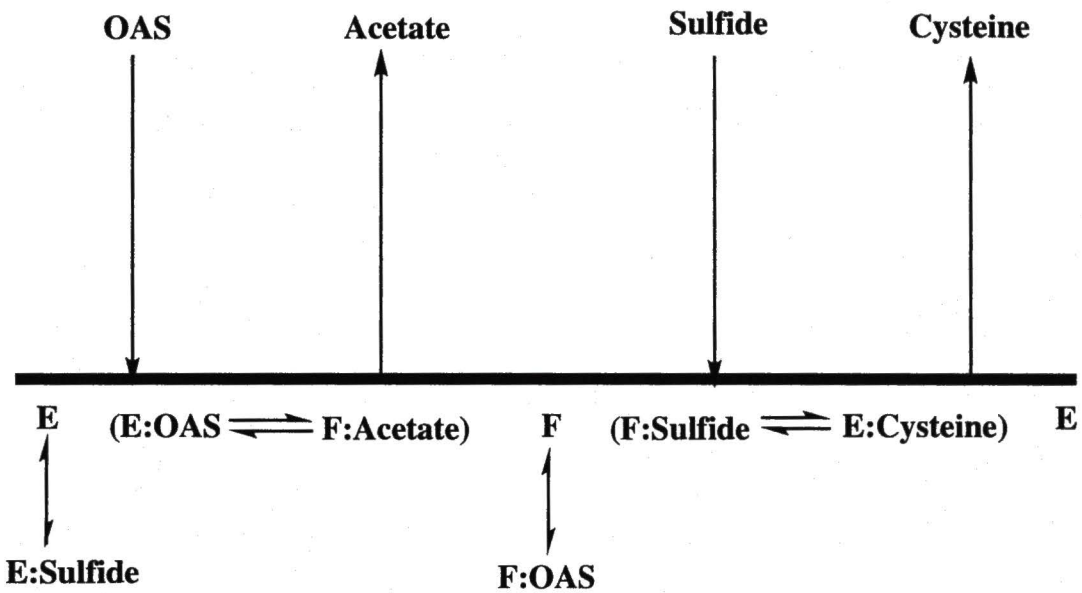


Figure 8. Bi-bi ping-pong kinetic mechanism for OASS-A proposed by Cook and Wedding (1976)


OAS
Acetate
Sulfide
Cysteine
E
(E:OAS ⇌ F:Acetate)
F
(F:Sulfide ⇌ E:Cysteine)
E
E:Sulfide
F:OAS

tryptophan residues at positions 51 and 162 and the 337 nm maximum is the result of exposure of one of these tryptophan residues to aqueous solvent (McClure and Cook, 1994). Apo-OASS-A can be successfully prepared and is stable for at least a month at 4°C (Schnackerz and Cook, 1995). The apoenzyme, when excited at 298 nm, showed on increased fluorescence at 337 nm due to the lack of PLP-Schiff base (McClure and Cook, 1994). These data suggested that PLP quenches the tryptophan fluorescence by 40 to 45% in the holoenzyme. This was also confirmed by Strambini et al (1996). Either of the products, acetate or cysteine, show enhancement of 498 nm fluorescence without quenching fluorescence at 337 nm suggesting energy transfer from one or both tryptophan residues to the PLP Schiff base (McClure and Cook, 1994).

The fluorescence and phosphorescence properties of the two tryptophan residues and PLP-Schiff base of OASS-A have been studied (Strambini et al., 1996). The data on apo-OASS-A suggest that one of the two tryptophan residues is in a relatively polar pocket whereas the other is in a relatively hydrophobic pocket. As mentioned before, reduction in the fluorescence intensity of tryptophan is seen in the holoenzyme due to the quenching by the PLP-Schiff base. The tryptophan phosphorescence of OASS exhibits a single band centered at 405 nm and a broad band around 440-550 nm as a result of delayed fluorescence of the ketoenamine tautomer of the internal Schiff base (Fig. 10). These data also confirm the energy transfer between tryptophan and PLP-Schiff base. Comparison of these data with those obtained from the studies of a W51Y mutant suggest that phosphorescence at 405 nm is derived from W162 while that derived from W51 is quenched by some group on the protein. Addition of OAS to the holoenzyme results in two bands around 406 and 408.5 nm, suggesting changes in the environment of tryptophan residues that result from a conformational change that occurs as the internal Schiff base is converted to α-aminoacrylate Schiff base (Strambini et al., 1996).

Figure 9. UV-visible spectra of OASS-A in the absence and presence of OAS

1. Spectrum of OASS-A with two absorption maxima at 280 and 412 nm, the later being a result of an internal aldimine.

2. Spectrum of OASS-A after addition of the first substrate, OAS. The species absorbing at 470 nm is the α-aminoacrylate intermediate that is formed after the release of acetate.

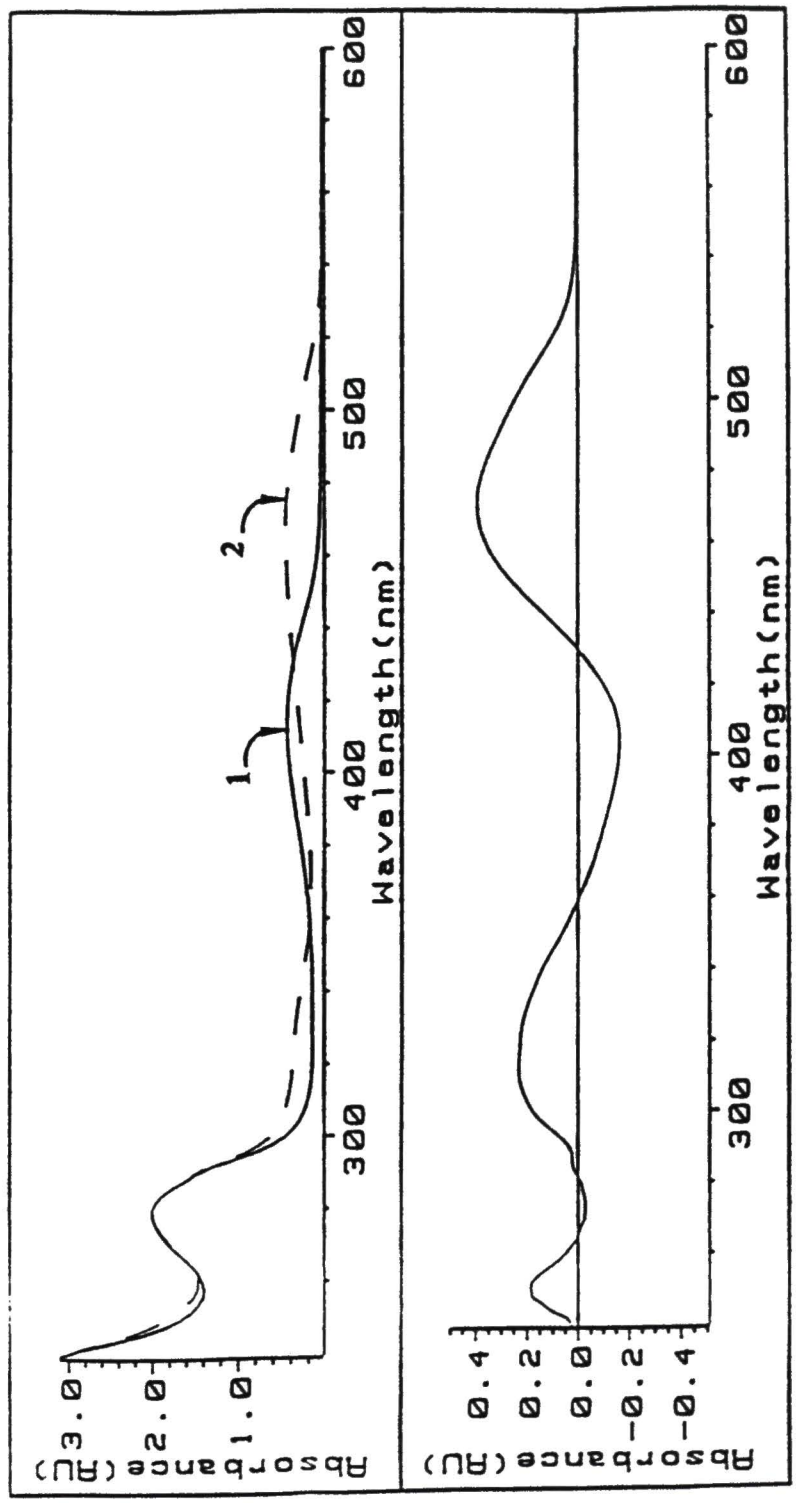
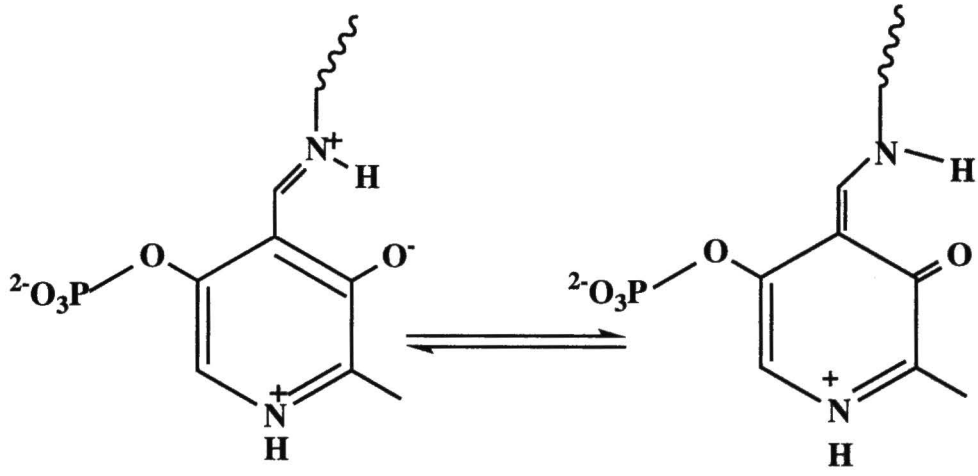

Absorbance (AU)
3.0
2.0
1.0
300
400
500
600
Wavelength(nm)
1
2
Absorbance (AU)
0.4
0.2
0.0
-0.2
-0.4
300
400
500
600
Wavelength(nm)

Figure 10. Ketoenamine and enolimine tautomers of the PLP-internal Schiff base.



Enolimine **Ketoenamine**

Studies on the effects of cofactor analogues were performed to investigate the importance of the different functional groups of PLP in catalysis (Schanckerz et al., 1995). In these studies apo-OASS-A was reconstituted with PLP, 2'-methyl PLP, pyridoxal-5'-deoxymethylenephosphonate and pyridoxal-5'-sulfate. Data suggest that 5'-(phosphate) side chain of PLP has to have a double negative charge for the enzyme to exhibit full activity (Schanckerz et al., 1995).

A chemical mechanism has been postulated based on the pH dependence of kinetic parameters and spectral properties described above. To begin the cycle, OAS binds with its α-amine neutral to facilitate nucleophilic attack on the C4' carbon of the internal Schiff base (Tai et al., 1995, Fig. 7). A proton from the α-carbon of the resulting external Schiff base is abstracted by the ε-amino group of the active site lysine in the β-elimination of acetate from OAS. A protonated enzyme residue is also required to assist in the elimination of the acetate leaving group to form α-aminoacrylate, acting to hold the acetyl group out of the plane of the PLP. After Michael addition of the nucleophilic substrate, a proton is donated to the α-carbon by the ε-amino group of the active site lysine to form the external Schiff base with product. The product is thus released to begin a new cycle. The proposed mechanism has been constructed with the help of spectral titrations with cysteine and serine which form an external Schiff base with OASS (Schnackerz et al., 1995).

The location and limitation of rate-limiting steps for the overall reaction the individual half reactions of OASS-A have been studied using rapid-scanning stopped-flow, and single wavelength absorbance and fluorescence stopped-flow experiments (Woehl et al., 1996). The first half reaction, conversion of OAS to α-aminoacrylate intermediate and acetate, is found to be rate-limiting for the overall reaction. Within the first half reaction, formation of the external Schiff base is observed in the first few milliseconds, followed by its slower conversion to the α-aminoacrylate intermediate (Woehl et al., 1996). Addition

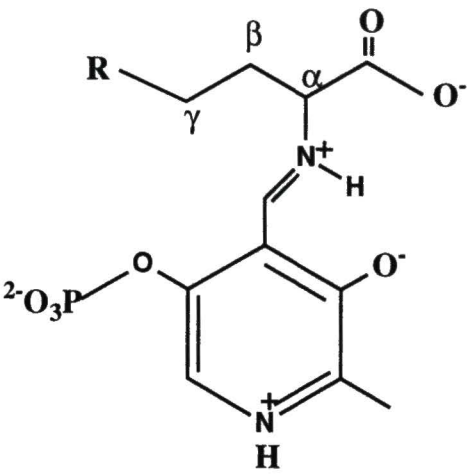
of L-cysteine to OASS-A results in a rapid formation of the external Schiff base, followed by transient formation of α-aminoacrylate intermediate with a slightly lower rate compared to OAS (Woehl et al., 1996). The latter transient formation of the α– aminoacrylate Schiff base is the result of attack by the cysteine thiol on aminoacrylate giving S-(3-L-alanyl)-L-cysteine. Primary and α-secondary kinetic deuterium isotope effects have been measured for OASS-A (Hwang et al., 1996). These data suggest an asymmetric transition state for α-proton abstraction by the active site lysine and the elimination of the acetyl group of OAS to form the α-aminoacrylate intermediate (Hwang et al., 1996).

Pyridoxal 5'-Phosphate Dependent Enzymes: Pyridoxal 5'-phosphate acts as a cofactor for many enzymes catalyzing a wide variety of reactions in the metabolism of amino acids, such as transamination, β-elimination, β, γ-replacement and racemization. The fate of these reactions depend on the chemical changes at either α, β or γ-carbon of the amino acid substrates (Fig. 11). The reactions catalyzed at the α, β and γ-carbon are shown in the Fig. 11.

A comprehensive comparison of amino acid sequences has shown that most of the PLP-dependent enzymes can be assigned to one of the three different groups of homologous proteins; namely α, β and γ families (Alexander et al., 1994). Some examples of the enzymes that are the members of α, β and γ – families are listed in table 1. In all PLP-dependent enzymes, the carbonyl group of the coenzyme binds to an ε-amino group of a lysine residue in the active site forming an internal aldimine. *O*-Acetylserine sulfhydrylase A and the β-subunit of tryptophan synthase are members of the β-family of PLP-dependent enzymes (Alexander et al., 1994, Table, 1). Figure 12 shows similarity among the members of the β-family of PLP-dependent enzymes (Alexander et al., 1994). The lysine in Schiff base linkage with PLP is marked (O) and identical residues appear in boxes in the figure. The protein sequences of OASS-A and the β-subunit of tryptophan

Figure 11. Possible reactions with pyridoxal-5'-phosphate as a cofactor

In the figure, PLP is bound to an amino acid. The α, β and γ carbons of the amino acid are shown. The reactions catalyzed at each of these carbons are listed below the figure.



General amino acid in Schiff base linkage with pyridoxal-5'-phosphate

Reactions at α-carbon: Transamination
Racemization
Decarboxylation

Reactions at β-carbon: Elimination
Replacement

Reactions at γ-carbon: Elimination
Replacement

Table 1. Examples of enzymes that are members of the α, β, γ–families of the PLP-dependent enzymes.

α-family	
	Glycine-acetyltransferase
	5-Aminolevulinate synthase
	Aspartate aminotransferase
	Serine hydroxymethyltransferase
β-family	
	L-Seine dehydratase
	D-Serine dehydratase
	Threonine dehydratase
	Tryptophan synthase β-subunit
	O-Aceytlseine sulfhydryalse, A and B
γ-family	
	O-Succinylhomoserine (thiol)-lyase
	O-Acetylhomoserine (thiol)-lyase
	Cystathionine γ-lyase
	Cystathionine β-lyase

Figure 12. Similarity among the active sites of members of the β-family of PLP-dependent enzymes

The lysine in the Schiff base linkage with PLP is marked (O) in the figure. (Alexander et al., 1994). Amino acid residues in bold are conserved.

(O)

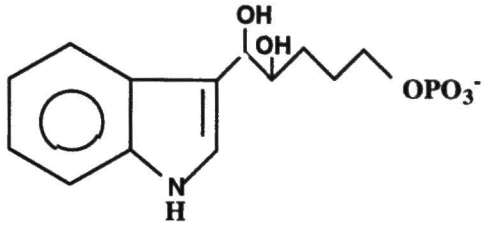
LSerDHT	**K**	M.	**D**.	S	S	Q	**P**	.	S	**G**	**S**	F	**K**	I	**R**	**G**	.	I
ThrDHT	**K**	R	**E**	D	R	Q	**P**	.	V	H	**S**	F	**K**	L	**R**	**G**	A	Y
CysSYN	**K**	V	**E**	S	R	N	**P**	.	S	F	**S**	V	**K**	C	**R**	I	G	.
ThrSYN	**K**	Y	**E**	G	A	N	**P**	.	T	**G**	**S**	F	**K**	D	**R**	**G**	M	V
TrpSYN	**K**	R	**E**	D	L	N	H	.	T	**G**	**S**	H	**K**	I	N	N	A	L
DSerDHT	**K**	K	**D**	S	H	L	**P**	I	S	**G**	**S**	I	**K**	A	**R**	**G**	G	I

synthase share about 14% identity and 40% similarity. The crystal strucutres for the β-subunit of tryptophan synthase and OASS-A also show significant structural similarity (unpublished work of P. Burlehard, G. S. J. Rao, E. Hohenester, P. F. Cook and J. N. Jansonius).

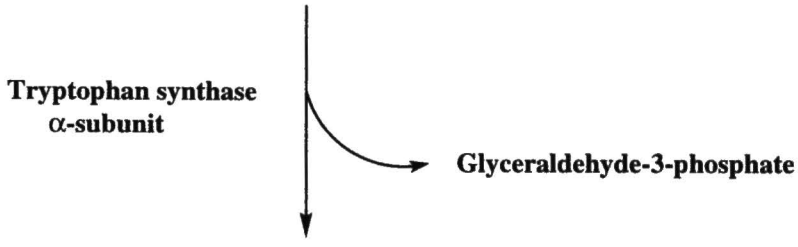
Tryptophan Synthase: The final two reactions of L-tryptophan biosynthesis in bacteria, yeast and plants are catalyzed by tryptophan synthase (Miles et al., 1989). Tryptophan synthase from bacteria exists as a multienzyme complex of two homodimers, $\alpha_2\ \beta_2$, of molecular weight 143,000. The reaction catalyzed by tryptophan synthase $\alpha_2\ \beta_2$ and the individual subunits are shown in Fig.13.

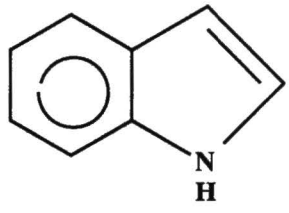
The molecular weight of the α-subunit is 29,000. The β-subunit exists as a dimer of molecular weight 44,000 per monomer and contains two molecules of PLP per dimer. The reactions catalyzed in the multienzyme complex are faster than those catalyzed by individual subunits. In the overall reaction, indole formed at the α-subunit cannot be released but is transported through a tunnel to the β-subunit where it undergoes a PLP-dependent β-replacement reaction with L-serine to give L-tryptophan (Miles et al., 1989). The β-replacement reaction catalyzed by the β-subunit of tryptophan synthase is very similar to that of OASS-A. A proposed chemical mechanism of the β-subunit of tryptophan synthase is shown in Figure 14. In the β-subunit of tryptophan synthase, PLP is bound to lysine 87 (K87) in Schiff base linkage (E in Fig. 14). When L-serine is added to the enzyme, the amino group of L-serine reacts with the internal aldimine to form a *gem*-diamine intermediate (ES I) followed by the displacement of the ε-amino group of K87 in a transaldimination reaction. An external Schiff base with L-serine (ES II) is the product of the transaldimination reaction. The external aldimine is converted to a quinonoid intermediate (ES III) by the loss of α-proton of L-serine which eliminates the hydroxyl group forming the α-aminoacrylate intermediate (ES IV). The aminoacrylate intermediate

Figure 13. Reaction catalyzed by tryptophan synthase α_2 β_2 multienzyme complex.

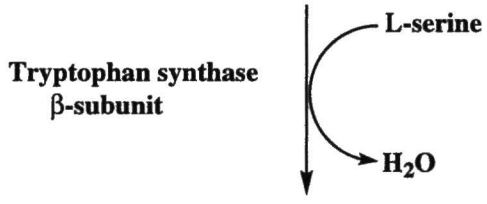


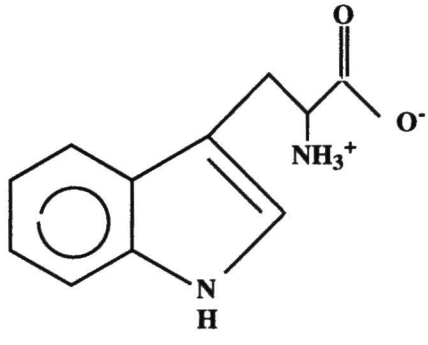
Indole 3-glycerol phosphate





Indole





L-Tryptophan

Figure 14. Proposed chemical mechanism for the β-subunit of tryptophan synthase of *S. typhimurium* (Miles et al., 1989).

Following are the species shown in the figure

E: Internal aldimine

ESI: *gem*-Diamine with L-serine

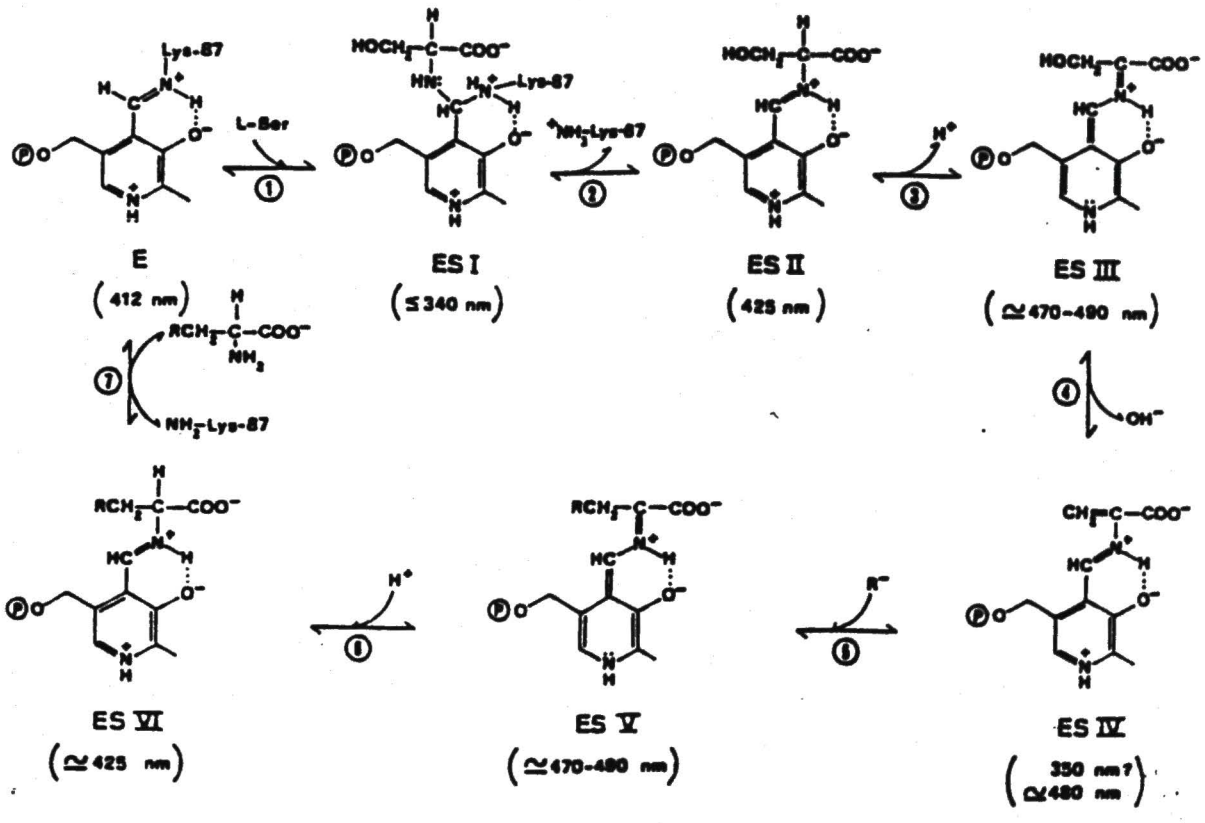
ESII: External aldimine with L-serine

ESIII: Quininoid intermediate

ESIV: α-Aminoacrylate intermediate

ESV: Quininoid intermediate

ESVI: External aldimine with L-tryptophan


E
(412 nm)
ES I
(≤340 nm)
ES II
(425 nm)
ES III
(≃470-490 nm)
ES IV
(350 nm?
≃480 nm)
ES V
(≃470-480 nm)
ES VI
(≃425 nm)
L-Ser
Lys-87
NH₂-Lys-87
OH⁻
H⁺
R⁻

can add a nucleophile, indole in this case, to form external aldimine with L-tryptophan (ES VI). L-Tryptophan is then released with transaldimination by the ε-amino group of K87. The α-aminoacrylate intermediate can also be hydrolyzed to α-keto acid and ammonia (Miles et al., 1989). The steps in the reaction catalyzed by the β-subunit of tryptophan synthase are very similar to those of the reaction catalyzed by OASS-A.

Present Studies. In all PLP-dependent enzymes, the carbonyl group of the coenzyme binds to an ε-amino group of a lysine residue in the active site forming an internal aldimine. In the course of the catalytic reaction, the lysine may be involved in one or more functions including binding of PLP to the enzyme, formation and stabilization of intermediates and/or release of products. The function of the lysine residue in Schiff base linkage have been studied in a number of the PLP-dependent enzymes (Planas and Kirsch, 1987, Miles et al., 1989). The present dissertation describes the experiments employing site-directed mutagenesis to identify the active site lysine, spectroscopic steady state kinetic studies to investigate its role in the OASS-A catalytic reaction. Hypotheses tested were as follows;

1) Lysine 42 forms the Schiff base with PLP

2) Lysine 42 is a catalyst, implicated in transimination and elimination reactions.

CHAPTER 2

EXPERIMENTAL PROCEDURES

Chemicals. 2-Bromoethylamine, ethylamine and guanidinium hydrochloride were purchased from ICN. *O*-Acetyl-L-serine, PLP, L-alanine, L-cysteine, L-serine, Hepes, $NaBH_4$, were purchased from Sigma.

Molecular Biology Reagents. Restriction enzymes were purchased from Promega or USB. The DNA sequencing was carried out by Sanger's method using the kit from USB. For plasmid purification, the Nucleobond AX kit (The Nest Group, Inc.) was used. Oligonucleotides used for mutagenesis and sequencing were prepared using a Biosearch oligonucleotide synthesizer.

Bacterial Strains and Plasmids. The bacterial strains used in these experiments are *Escherichia coli* NM522 (*hsdΔ5, Δ(lac-pro), [F', pro*$^+$*, lacI*^{q}*ZΔM15]*), *Salmonella typhimurium* LB500 (*metA metE551 trpD2 leu hsdLT hsdSA hsdSB* and m^+ for all three modification systems), and *Salmonella typhimurium* DW378 (*trpC109 cysK1772 cysM1770*). The gene, *cysK,* that encodes OASS-A in *S. typhimurium* is cloned in the plasmid pRSM40, (Fig. 15, Monroe and Kredich, 1990). The plasmid, pRSM40, was linearized with *EcoR*I and a 690 bp AvaI fragment containing the codon AAG for K42 was amplified using PCR. The oligonucleotide primers used were

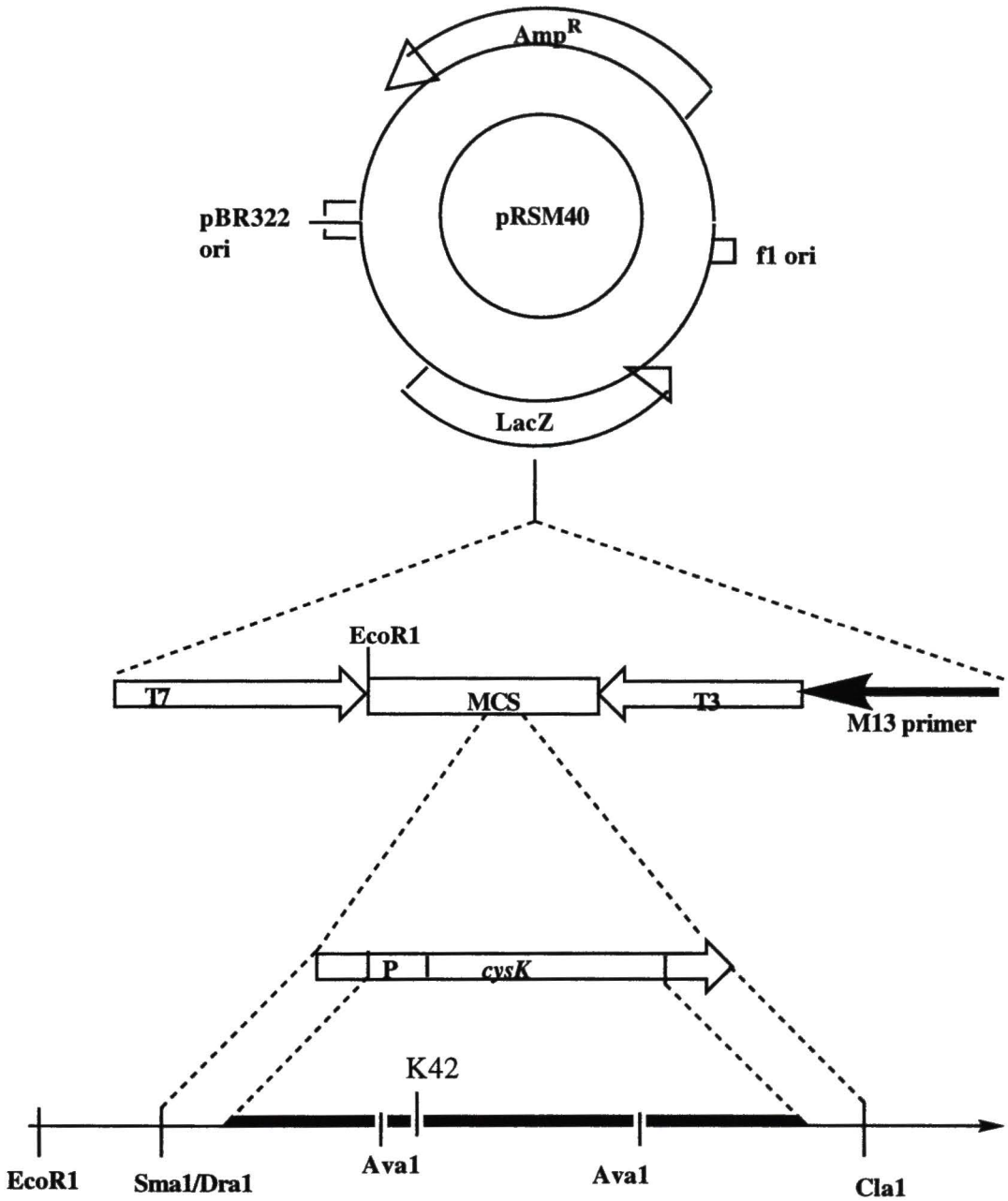
AAG

| | |

(1) 5'-AGTCGCGCAA**CCCGAG**CTTCAGCGTC**GCG**TGCCGTATCGG-3'

Figure 15. Map of the plasmid pRSM40 used for mutagenesis.

The plasmid, pRSM40, contains a 1.48 kb ClaI-DraI fragment of the *S. typhimurium cysK* gene cloned in pT7T3 19U. The 5'-end of the *cysK* gene is towards the SmaI/DraI site.


Amp^R
pBR322
ori
pRSM40
f1 ori
LacZ
EcoR1
T7
MCS
T3
M13 primer
P
cysK
K42
EcoR1
Sma1/Dra1
Ava1
Ava1
Cla1

(2) 5'-AGGTTG**CCCGGG**ATGAAGCCTGCGC-3'

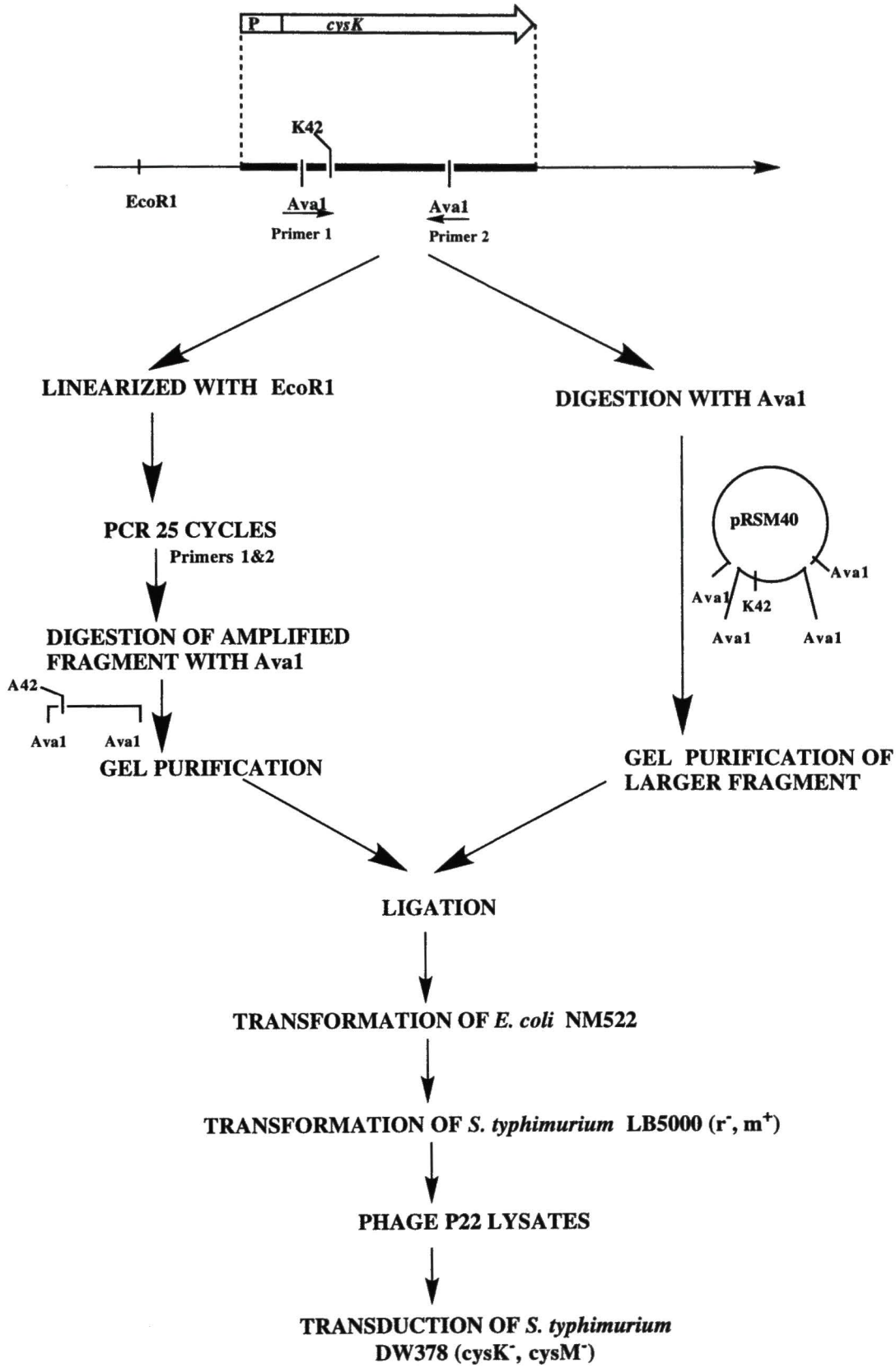
and primer 2 is complimentary to 5'-GCGCAGGCTTCAT**CCCGGG**CAACCT-3' in the DNA sequence of *cysK*.

In oligonucleotide (1), AAG encoding for lysine was changed as shown to GCG encoding for alanine. The bolded sequence CCCGAG is the recognition site for AvaI. In oligonucleotide (2), the sequence CCCGGG is the recognition site for AvaI.

Site-Directed Mutagenesis. The protocol for mutagenesis is shown in Scheme 1. The ligated plasmids carrying the new GCG codon were transformed into *E. coli* NM522 using the $CaCl_2$ method (Sambrook et al., 1988) and purified using the Nucleobond AX kit. The complete *cysK* gene was sequenced with a USB sequencing kit to confirm the mutation and to check for PCR errors. The mutant plasmids were transformed into *S. typhimurium* LB5000 using a modified Hanahan method and stored as phage *P22* lysates (Monroe and Kredich, 1990). Since LB5000 is r^-m^+ for all three restriction-modification systems, the DNA obtained from this strain is not degraded in any other *S. typhimurium* strain.

Expression of the Mutant Enzyme. Expression of the mutant enzyme was carried out in the *cysK*$^-$ *cysM*$^-$ strain (DW378) of *S. typhimurium* as described ahead. Briefly, 2 µl of the phage lysate were added to 100 µl of the DW378 culture grown overnight at 37°C with good aeration. The transductants were selected on LB plates with 100 µg/ml ampicillin in an overnight incubation at 37°C. The plasmid-containing strain was then allowed to grow for 18 hours at 37°C and 250 rpm on Vogel-Bonner medium E (Vogel and Bonner, 1956) supplemented with 0.5% glucose, 1% LB, 40 µM L-tryptophan, 500 µM reduced glutathione and 100 µg/ml ampicillin. Reduced glutathione was the only sulfur source in the medium and was added to derepress the cysteine biosynthesis pathway (Kredich, 1971).

Scheme 1. Procedure for site-directed mutagenesis used to prepare the K42A mutant of OASS-A using polymerase chain reaction.


P
cysK
K42
EcoR1
Ava1
Primer 1
Ava1
Primer 2
LINEARIZED WITH EcoR1
DIGESTION WITH Ava1
PCR 25 CYCLES
Primers 1&2
pRSM40
Ava1
Ava1
K42
Ava1
Ava1
DIGESTION OF AMPLIFIED FRAGMENT WITH Ava1
A42
Ava1
Ava1
GEL PURIFICATION
GEL PURIFICATION OF LARGER FRAGMENT
LIGATION
TRANSFORMATION OF E. coli NM522
TRANSFORMATION OF S. typhimurium LB5000 (r-, m+)
PHAGE P22 LYSATES
TRANSDUCTION OF S. typhimurium
DW378 (cysK-, cysM-)

method of Tai et al (1993). The schematic representation of the purification protocol is shown in scheme 2. UV-visible absorption spectra were recorded for each fraction of the DEAE-5PW and phenyl-5PW column elute. The fractions with an $A_{280/424}$ of 5.0 were pooled and concentrated. Purity of the enzyme was further tested via SDS-PAGE.

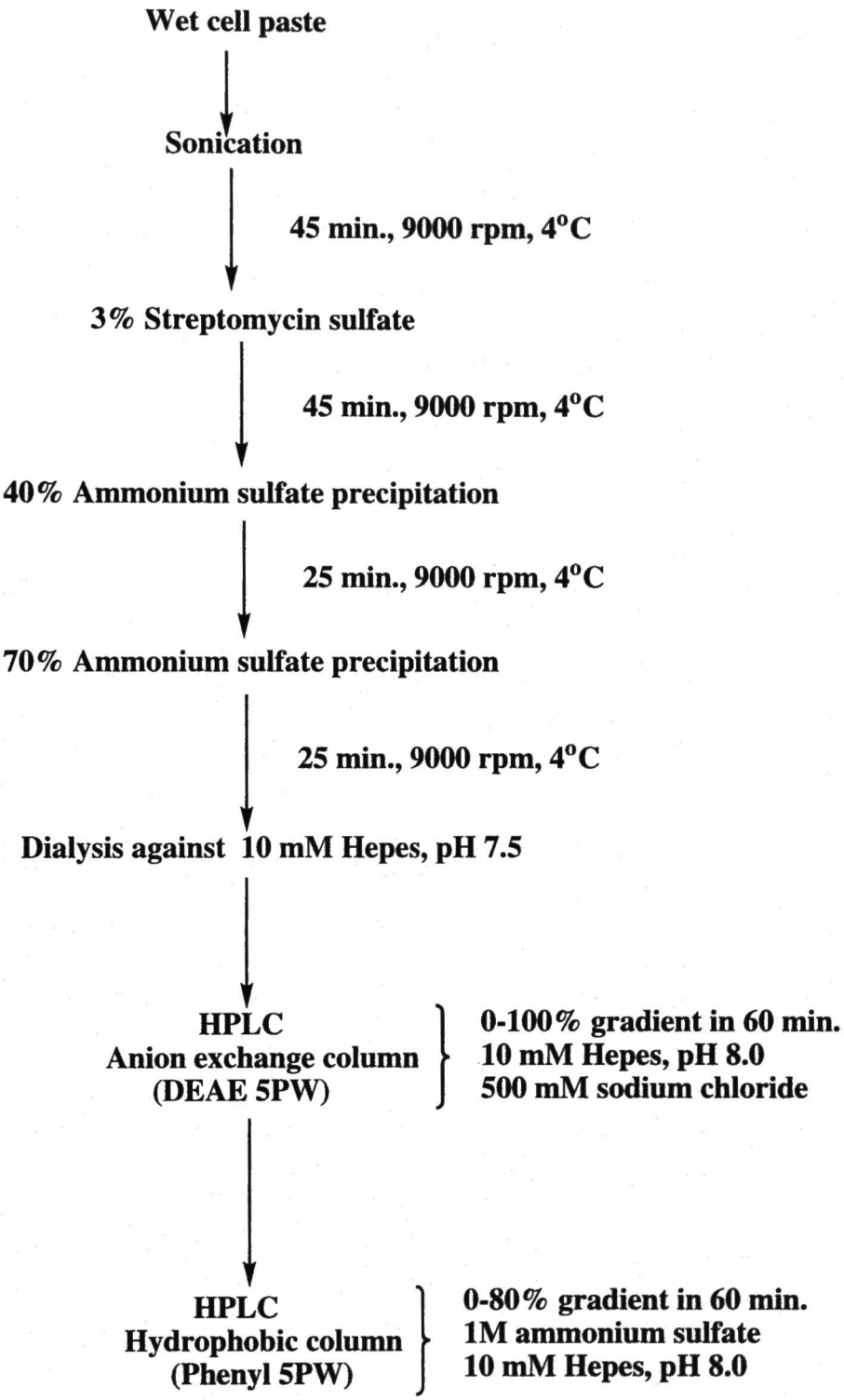
Preparation and Reconstitution of ApoK42A-OASS. The preparation of apoK42A-OASS-And its reconstitution were performed using the method of Schnackerz and Cook (1995) with the exception that OAS was not added prior to dialysis against 5M guanidinium hydrochloride.

Analysis of the PLP-Bound Amino Acids. The enzyme K42A-OASS-A was denatured at 65^{o}C for 20 minutes and then centrifuged at 9000 rpm at 4^{o}C to remove denatured protein. The supernatant that contained PLP-Schiff base was dialyzed against HPLC grade water. The water was then concentrated using a lyophilizer and the samples were sent for amino acid analysis at the Biomolecular Research Facility of the University of Virginia.

Modification of C43 with Bromoethylamine. To modify K42-OASS with BEA, the holoenzyme was first incubated with 5M guanidinium hydrochloride in 50 mM phosphate buffer, pH 7.5 for 15 minutes, 40 mM BEA was added to the apoK42A-OASS and the preparation was incubated for 14 hours at room temperature. Reconstitution with PLP was carried out as described (Schnackerz and Cook, 1995).

Spectral Studies. UV-visible spectra were measured on a Hewlett-Packard, model 8452A photodiode array spectrophotometer. Spectra of apo-K42A-OASS reconstituted with PLP in 10 mM Hepes, pH 7.5 and different concentrations of L-alanine, L-cysteine, O-acetyl-L-serine or L-serine were recorded at wavelengths from 250 to 600 nm using a 1 cm light path. Both buffer and amino acid blanks were subtracted from the spectra. Data were plotted using Cricket Graph III.

Scheme 2. Procedure for purification of the enzyme K42A-OASS-A


Wet cell paste
Sonication
45 min., 9000 rpm, 4°C
3% Streptomycin sulfate
45 min., 9000 rpm, 4°C
40% Ammonium sulfate precipitation
25 min., 9000 rpm, 4°C
70% Ammonium sulfate precipitation
25 min., 9000 rpm, 4°C
Dialysis against 10 mM Hepes, pH 7.5
HPLC
Anion exchange column
(DEAE 5PW)
0-100% gradient in 60 min.
10 mM Hepes, pH 8.0
500 mM sodium chloride
HPLC
Hydrophobic column
(Phenyl 5PW)
0-80% gradient in 60 min.
1M ammonium sulfate
10 mM Hepes, pH 8.0

Fluorescence Studies. Fluorescence spectra of 2.5 μM mutant enzyme were obtained on a Shimadzu RF5000U spectrofluorometer in the absence and presence of amino acids at 25°C. Excitation was at 298 nm and the excitation and emission slit widths were set at 5 nm. Emission was measured over the wavelength range 300 to 550 nm.

Circular Dichroism Studies. CD spectra were collected on an Aviv 62DS spectropolarimeter at 25°C and a pathlength of 0.2 cm. Enzyme concentrations of 100 μg/mL and 4 mg/mL were used for far UV and visible CD spectra, respectively. The buffer used for all spectra was 10 mM phosphate, pH 7.0 and a buffer blank was subtracted from each spectrum.

Enzyme assays. The activity of the reconstituted and the chemically modified enzymes was tested using the following two assay methods. Decrease in sulfide concentration was observed using a computer-assisted sulfide ion-selective electrode assay (Hara et al., 1990), whereas the disappearance in TNB was monitored on a Gilford 2600 spectrophotometer at 412 nm (Ellman, 1959).

^{31}P NMR Spectroscopy. Fourier transform ^{31}P NMR spectra were collected at 121.497 MHz on a Bruker AM300 SWB superconducting spectrometer using a 10-mm multinuclear probehead with broadband ^{1}H decoupling. The NMR tube spinning at 15-20 Hz contained the sample (2 mL) and $^{2}H_2O$ (0.2 mL) as field/frequency lock and was maintained at 20 ± 0.1 °C using a thermostatted continuous air flow. Generally, a spectral width of 2000 Hz was acquired in 8K data points with a pulse angle of 60°. The exponential line broadening used prior to Fourier transformation was 10 Hz. Protein samples were dissolved in 50 mM Hepes buffers containing 1 mM EDTA at the appropriate pH. Changes in pH were performed by dialysis against the desired buffer overnight. pH values of the sample were determined before and after the NMR measurement. Positive chemical shifts in ppm are downfield changes with respect to 85% H_3PO_4.

CHAPTER 3

CHARACTERIZATION OF THE K42A-OASS-A MUTANT ENZYME

Growth and Yield of the Mutant Protein. When cells were grown in shaker flasks overnight, the K42A mutant protein was obtained in a yield of 20 mg/25 g wet cell paste. The mutant protein, as isolated, has λ_{max} values of 280 and 424 nm (Fig. 16 A), and a small amount of activity. Addition of $NaBH_4$ does not affect the absorbance at 424 nm, but eliminates all of the activity. Preparation of larger quantities of the K42A mutant was attempted by overnight growth in a 12 liter fermenter with forced aeration. After a lag of several hours, growth was observed, and cells were harvested after 24 hrs. O-Acetylserine sulfhydrylase was isolated and found to be wild type OASS-A.

Spectral Properties. WT OASS-A, as isolated, shows an absorbance spectrum with two maxima, at 280 and 412 nm (Fig. 16 A, I), the latter corresponding to the internal Schiff base (Cook et al., 1992). The ratio of $A_{280/424}$ is about 5.0 for the K42A mutant protein, compared to a value for $A_{280/412}$ of 3.4 for WT OASS-A. Addition of sodium borohydride alone has no effect on the 424 nm band of the K42A mutant protein as isolated, but in the presence of 5 M guanidinium hydrochloride the absorbance at 424 nm disappears. The absorption spectrum of apoK42A-OASS exhibits a single maximum at 280 nm (Fig. 16 B, I). Reconstitution of apo-K42A-OASS with PLP produces additional absorption bands at 330 and 390 nm (Fig. 16 B, II). The ratio of $A_{280}/A_{(330+390)}$ is around 5.0.

When excited at 298 nm, the fluorescence emission spectrum of WT OASS-A

Figure 16. UV-visible spectra of K42A-OASS-A.

Panel A shows UV/visible spectra of OASS-A (I) and K42A-OASS (II) of equal concentration. Panel B shows apo-K42A-OASS (I) and apo-K42A-OASS reconstituted with PLP (II).

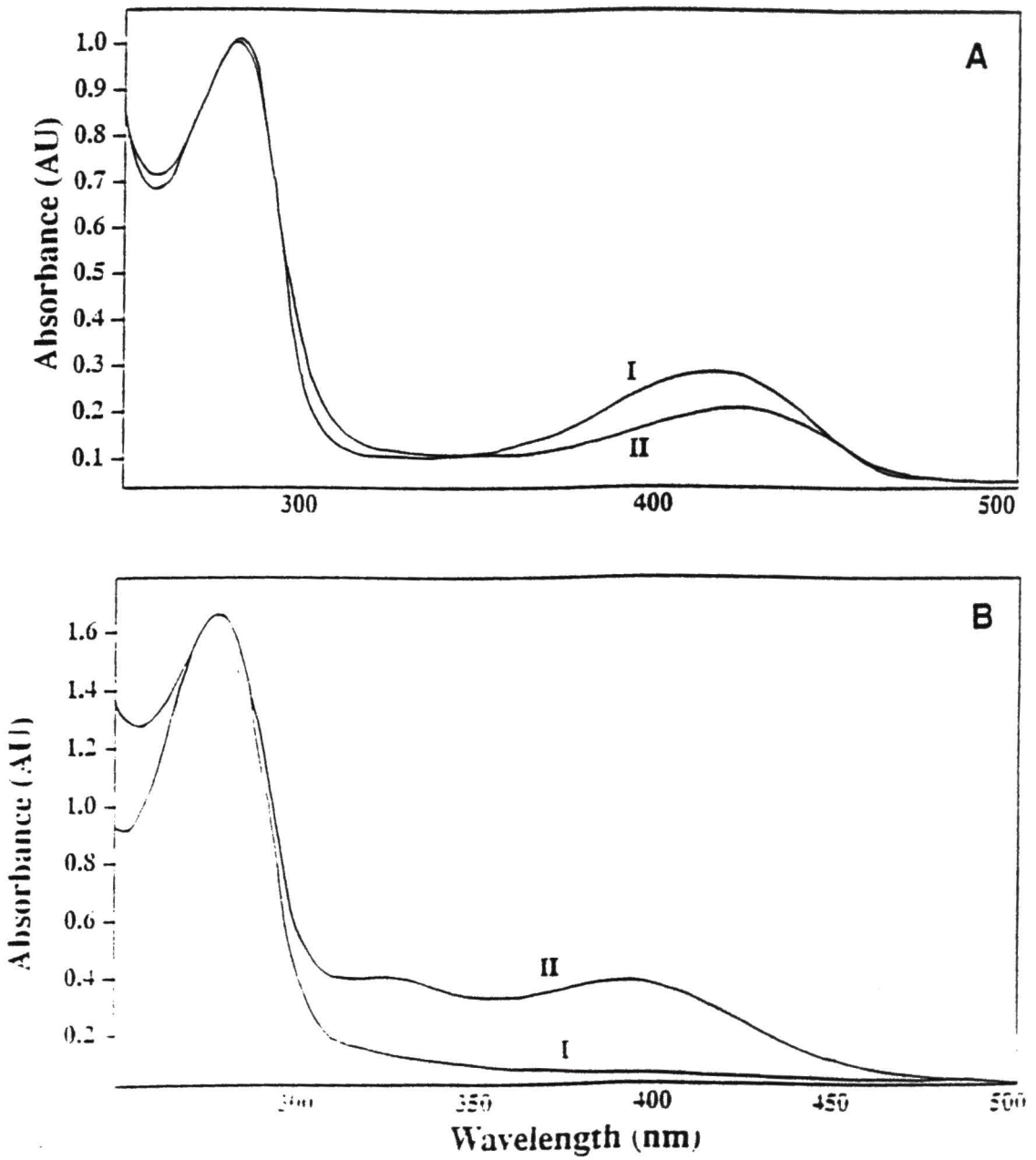
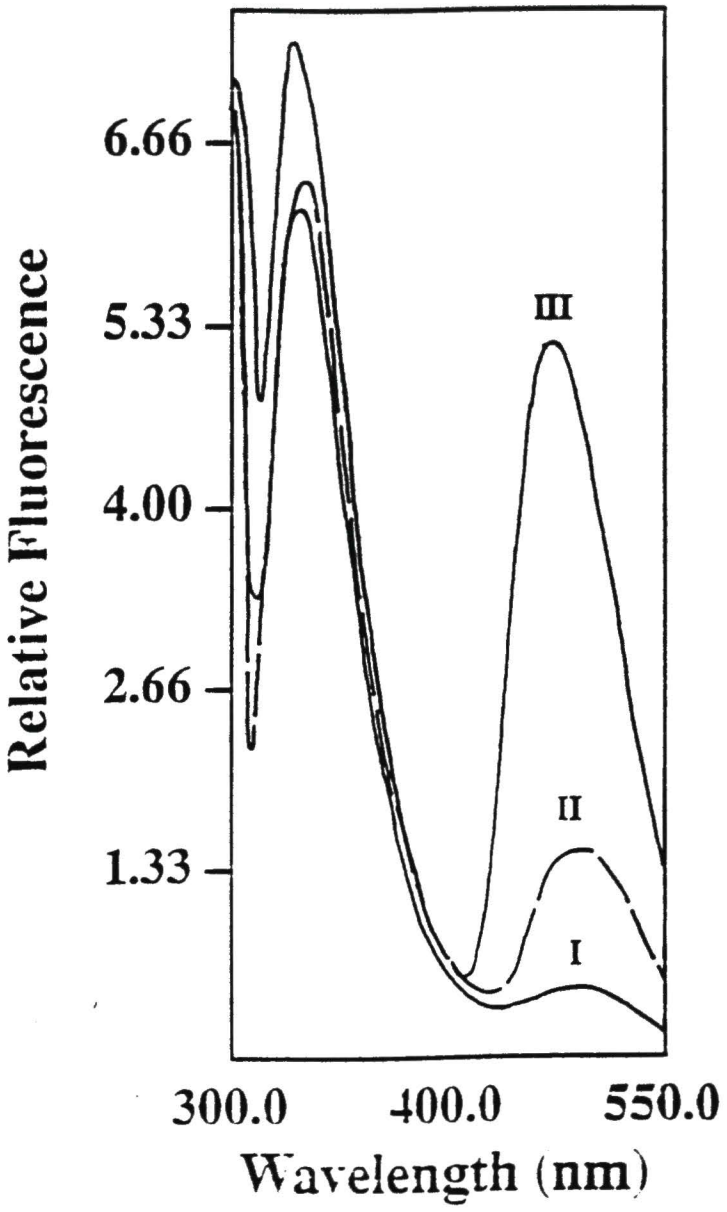

A
Absorbance (AU)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
I
II
300
400
500
B
Absorbance (AU)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
II
I
350
400
450
500
Wavelength (nm)

Figure 17. Fluorescence spectra of OASS-A (I), K42A-OASS (II) and addition of acetate to OASS-A (III).

Each of these spectra were collected at 25°C in 10 mM Hepes, pH 7.5 with an enzyme concentration of 2.5 μM for OASS-A and K42A-OASS.


Relative Fluorescence
6.66
5.33
4.00
2.66
1.33
III
II
I
300.0
400.0
550.0
Wavelength (nm)

shows two maxima at 337 and 498 nm; the ratio of $A_{337/498}$ is 8.0 (Fig. 17, I). The band at a shorter wavelength is a result of intrinsic tryptophan fluorescence whereas the band at the longer wavelength is due to delayed Schiff base fluorescence (McClure and Cook 1994; Strambini et al, 1996). Excitation of the K42A mutant protein at 298 nm shows an emission spectrum with maxima at 337 and 504 nm (Fig. 17, II). The ratio for the K42A mutant ($A_{337/504}$) is around 4.5, which is a value between those observed for the WT enzyme in the absence and presence of acetate (Fig. 17, III, McClure and Cook 1994). ApoK42A-OASS shows no emission around 500 nm (Fig. 18) as also seen for WT apoOASS-A, and the emission at 337 nm is increased in the apo-K42A-mutant, when compared to K42A-OASS.

The emission spectrum of the apoK42A-OASS reconstituted with PLP is not identical to that of K42A-OASS, when excited at 298 nm. The emission band at 500 nm has a lower intensity than that of K42A-OASS as isolated. When excited at 330 nm, the WT enzyme shows a major band with a λ_{max} of 484 nm and a weaker band with a λ_{max} of 362 nm (Strambini et al, 1996). Two bands are also observed in the K42A mutant reconstituted with PLP, but λ_{max} values appear at 444 and 387 nm, respectively (data not shown).

Circular dichroism spectra for K42A-OASS as isolated are identical to those of the WT enzyme in the far UV, suggesting essentially no gross change in the structure of the OASS as a result of the active site mutation. In the visible region, however, K42A-OASS shows a negative Cotton effect with λ_{max} at 424 nm and a molar ellipticity equal to that of the positive Cotton effect of the WT enzyme at 412 nm (Fig. 19). Apoenzyme shows no ellipticity in the visible range whereas enzyme reconstituted with PLP shows positive ellipticity centered around 330 and 390-400 nm (data not shown).

The ^{31}P NMR spectra of K42A-OASS show a single resonance at 5.3 ppm in the

Figure 18. Fluorescence spectra of apoK42A-OASS (I) and K42A-OASS (II).

The apoenzyme does not show any fluorescence around 500 nm whereas exhibits enhanced fluorescence around 337 nm.

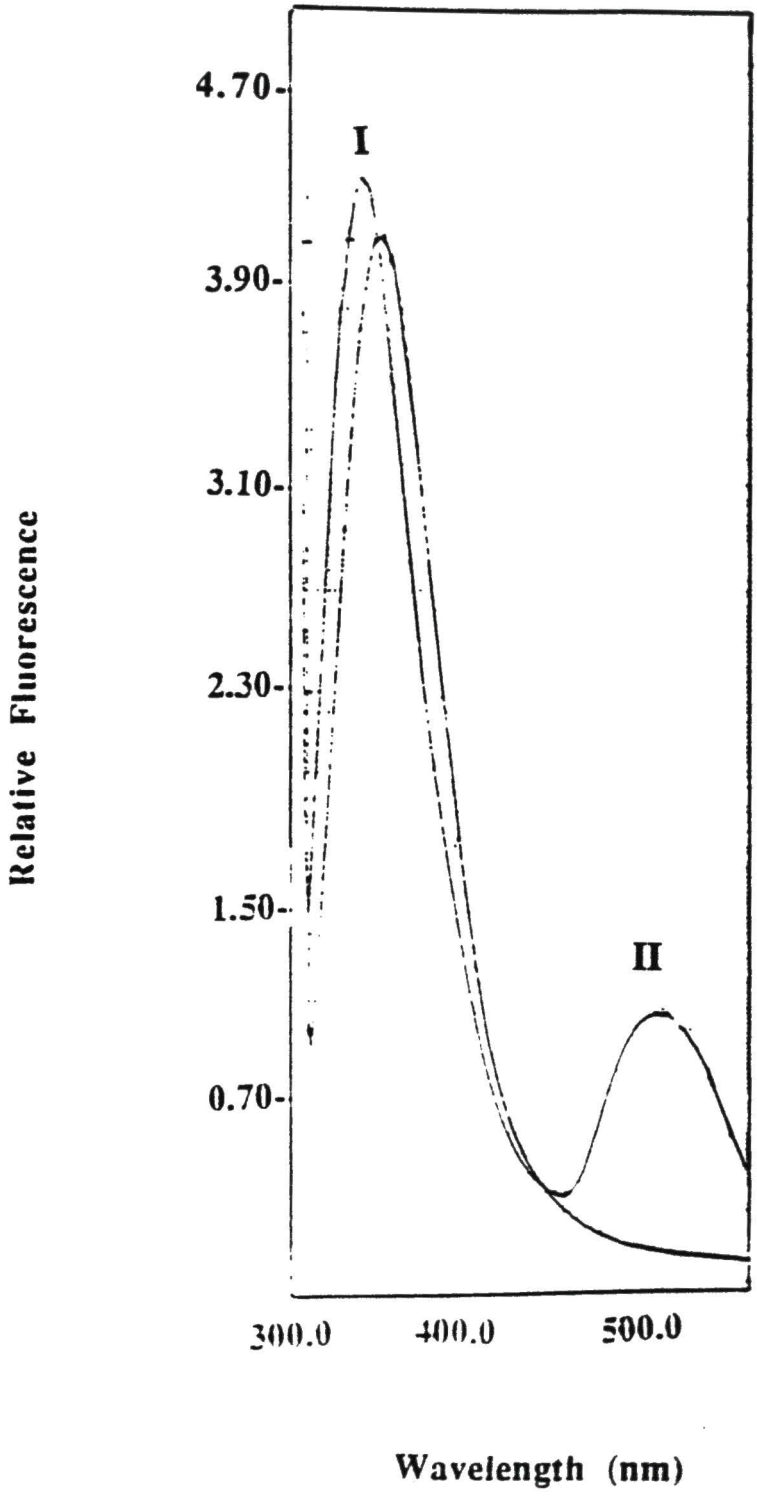

4.70
3.90
3.10
2.30
1.50
0.70
I
II
300.0
400.0
500.0
Relative Fluorescence
Wavelength (nm)

Figure 19. Visible circular dichroism spectra of OASS-A (I) and K42A-OASS (II). These spectra were taken at 25°C with 10 mM phosphate buffer, pH 7.5. The enzyme concentration used was 500 μg for both the spectra.

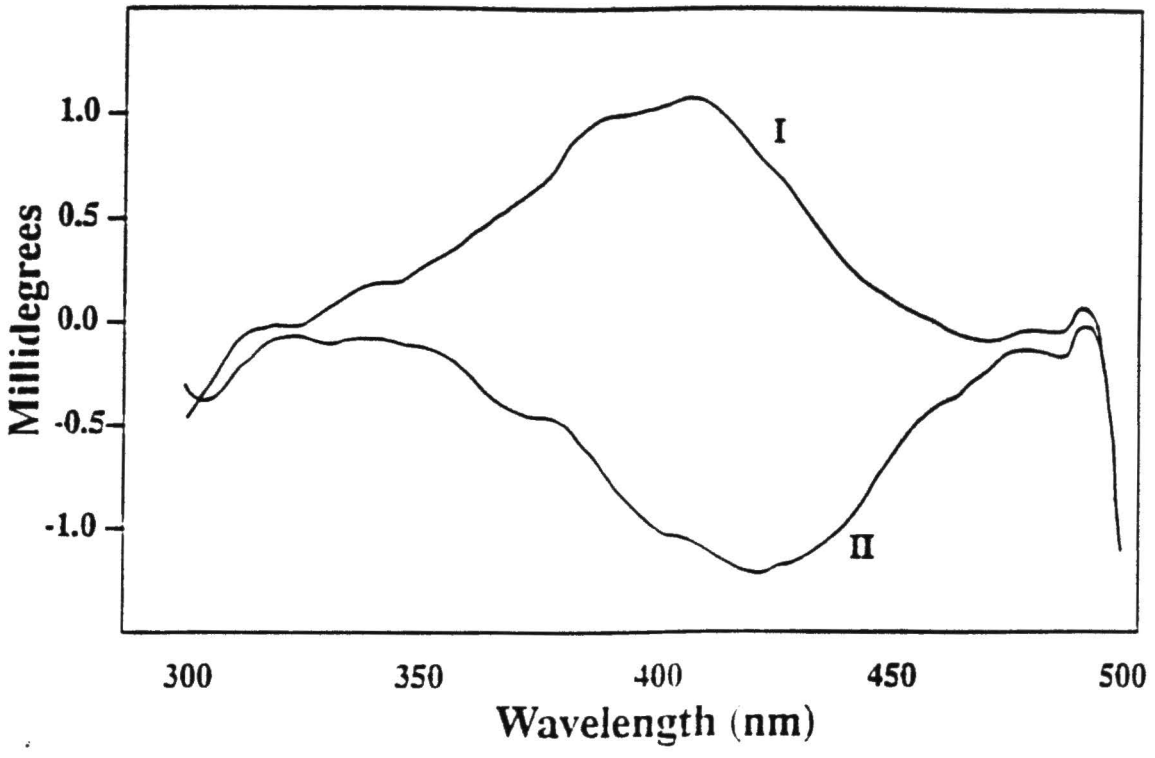
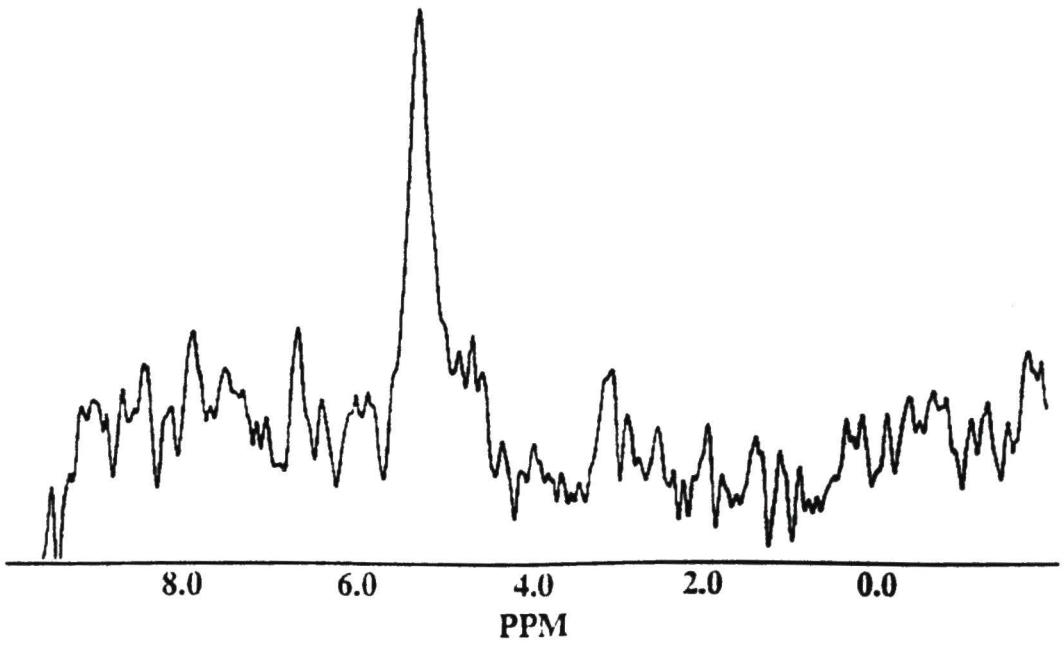

1.0
0.5
0.0
-0.5
-1.0
Millidegrees
I
II
300
350
400
450
500
Wavelength (nm)

Figure 20. ^{31}P NMR spectrum obtained for K42A-OASS

^{31}P NMR spectrum obtained for K42A-OASS at pH 8.5, 100 mM Taps, at 25^{o}C and a protein concentration of 12.5 mg/mL.


8.0
6.0
4.0
2.0
0.0
PPM

pH range 6.5 to 8.5 (Fig. 20) This can be compared to a value of 5.2 ppm for the WT enzyme (Cook et al., 1992).

Formation of External Aldimine. ApoK42A-OASS reconstituted with PLP exhibits spectral changes upon addition of amino acid reactants or analogs. Addition of 20 mM OAS to the reconstituted enzyme leads to a time dependent decrease in the absorbance at 330 nm and a shift in the λ_{max} of the 390 nm band to 424 nm with a concomitant increase in the absorbance at the latter (Fig. 21, A). The reaction is pH independent. Formation of the external aldimine with OAS is complete in about 90 minutes. L-Alanine and L-serine produce similar changes over the same time period and show saturation at 10 mM concentration. L-Cysteine at a concentration of 15 mM produces an increase in the absorbance at 330 nm with a concomitant decrease at 390 nm (Fig. 21, B). A first order plot of the absorbance change for the formation of the L-ala external Schiff base at 310 or 370 nm (wavelengths that produce maximum absorbance changes) vs. time gives a maximum rate constant of 0.048 min^{-1}. Similar data for OAS, serine and cysteine give first order rate constants in the range of 0.035 or 0.04 min^{-1}. The external aldimine species studied above are stable for at least 24 hours. In the above experiments, no species absorbing at or near 470 nm, corresponding to the α-aminoacrylate intermediate was formed (Cook, et al, 1992), suggesting that the ε-amino group of lysine is important in the reaction.

Addition of any of the above amino acids to the reconstituted enzyme gives a decrease in the fluorescence at 337 nm and an increase in the fluorescence at 500 nm; with the λ_{max} at longer wavelength showing a blue shift with time (Fig. 22). The rate of the changes are similar to those obtained from UV/visible spectrophotometry. Formation of the external aldimine with OAS, L-ala, or L-cys was also studied using circular dichroism. Addition of OAS and L-ala to the apoenzyme reconstituted with PLP shows a decrease in

Figure 21. UV/visible difference spectra for external aldimine formation.

Each of these spectra was taken at 25°C with 1 mg of apo-K42A-OASS reconstituted with PLP in the absence and presence of 10 mM OAS (A) or 10 mM L-cysteine (B). Spectra are the difference of those in the presence and absence of amino acid. The reactions were carried out in 10 mM Hepes pH 7.5.

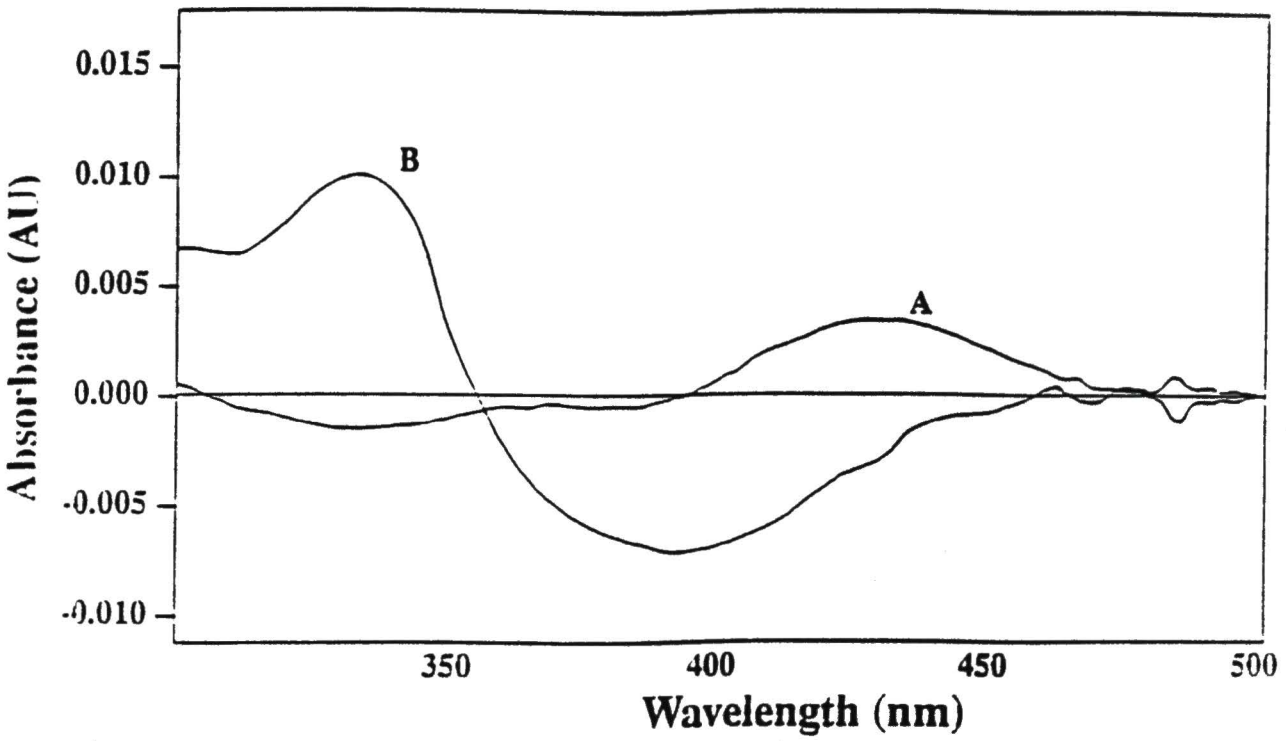

0.015
0.010
0.005
0.000
-0.005
-0.010
Absorbance (AU)
B
A
350
400
450
500
Wavelength (nm)

Figure 22. Fluorescence spectra of external aldimine formation.

The spectra were taken at 25°C with 250 μM of apo-K42A-OASS reconstituted with PLP in the absence and presence of 10 mM Alanine. The reactions were carried out in 10 mM Hepes pH 7.5. Panel A shows the fluorescence spectra in the presence of alanine. Panel B shows only the change in fluorescence maxima around 500 nm.

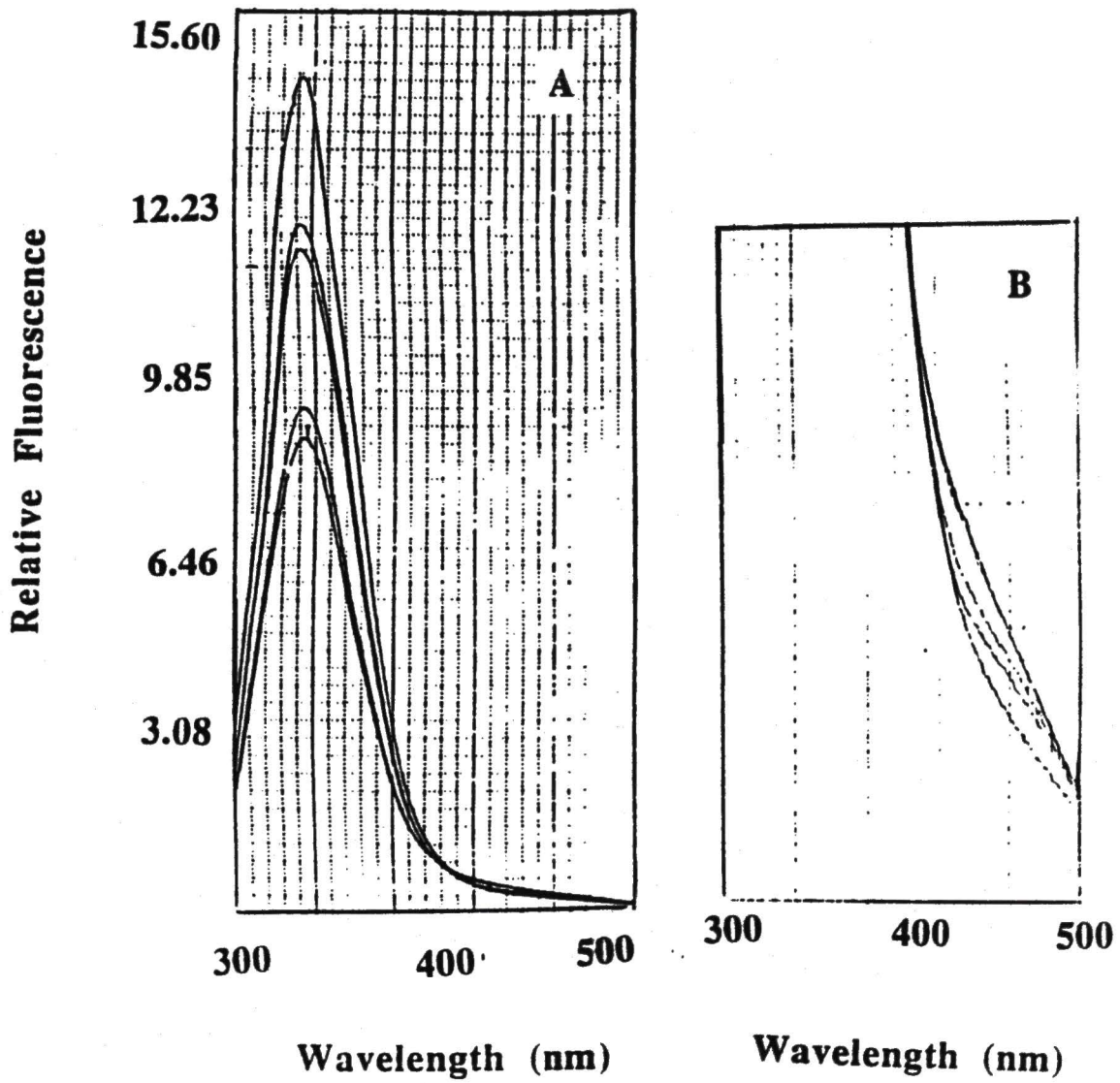
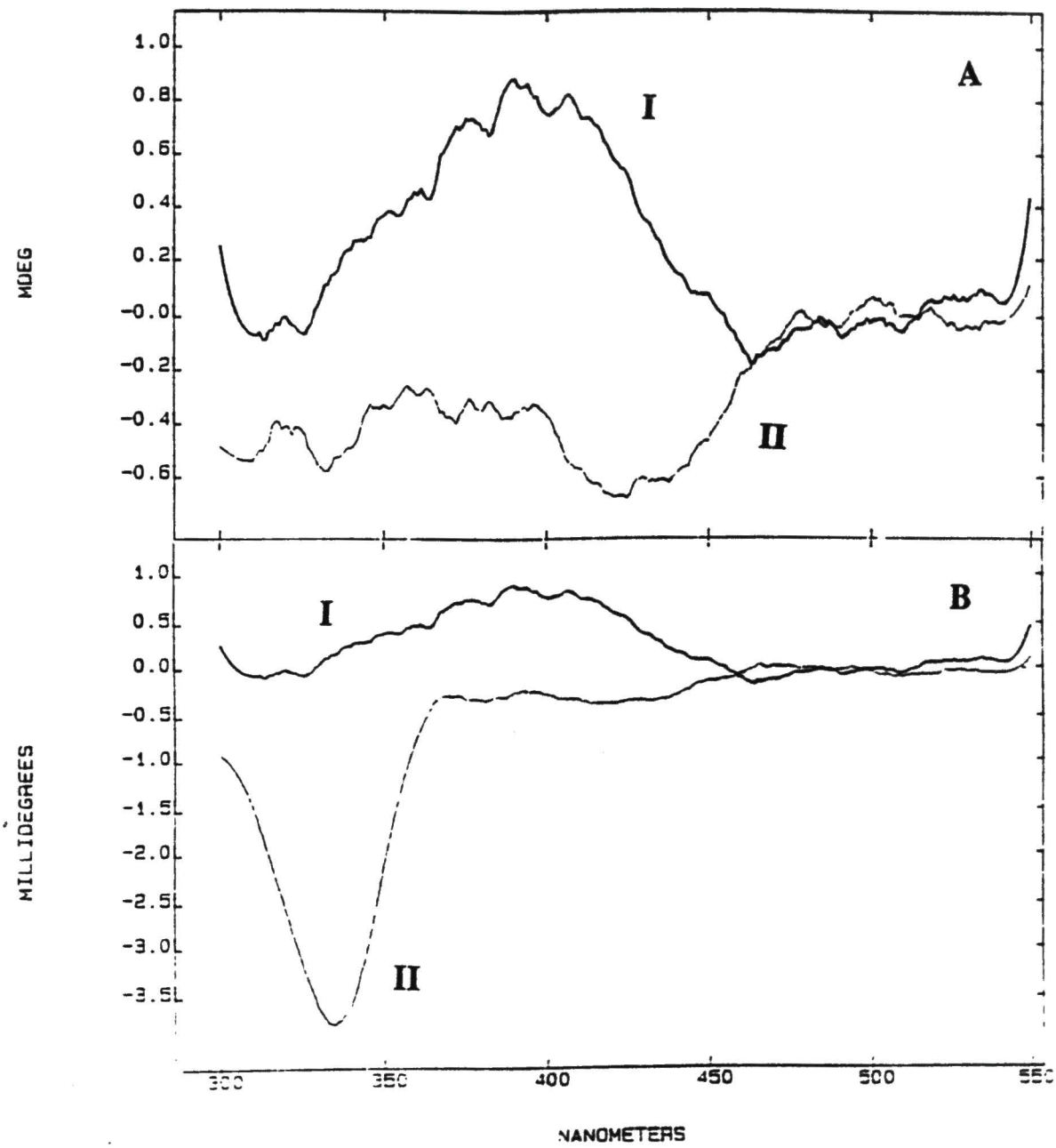

Relative Fluorescence
15.60
12.23
9.85
6.46
3.08
A
300
400
500
Wavelength (nm)
B
300
400
500
Wavelength (nm)

Figure 23. Visible CD spectra of the external aldimine formation

Each of these spectra was taken at 25°C with 4 mg of apo-K42A-OASS reconstituted with PLP in the absence and presence of 5 mM OAS (A) or 2 mM L-cysteine (B). Each spectrum is the overlayed on the spectrum of the apo-K42A-OASS reconstituted with PLP of those in the presence and absence of amino acid. The reactions were carried out in 10 mM phosphate buffer, pH 7.5. Spectrum I in both the panels is that of the K42A mutant enzyme recontituted with PLP whereas spectrum II in Panel A is taken after addition of OAS and spectrum II in Panel B is taken after addition of L-cysteine to the apoK42A OASS-A reconstituted with PLP.


MDEG
MILLIDEGREES
1.0
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
A
I
II
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
B
I
II
300
350
400
450
500
550
NANOMETERS

the ellipticity around 390 nm. Addition of L-cys to the reconstituted apoenzyme, on the other hand, shows a prominent decrease in the ellipticity at 330 nm (Fig 23).

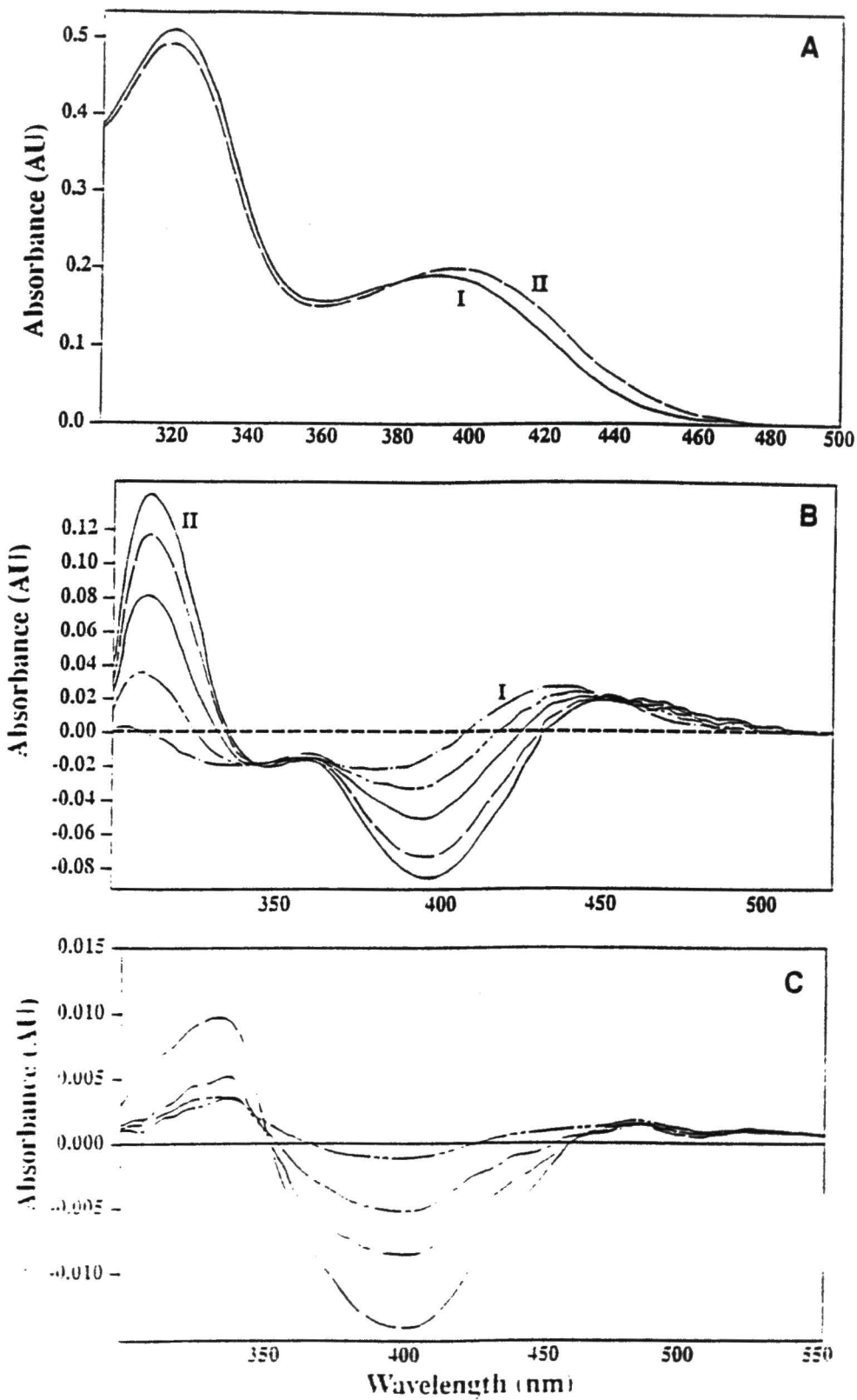
Formation of α-Aminoacrylate. Addition of OAS to the WT enzyme results in the formation of the α-aminoacrylate intermediate accompanied by an absorbance decrease at 412 nm and increases at 330 and 470 nm (Cook et al., 1992). The K42A mutant enzyme, when pre-incubated with 10 mM ethylamine (Fig. 24, A), shows formation of the external aldimine upon addition of OAS, followed by slow formation of the α-aminoacrylate intermediate absorbing at 330 and 470 nm (Fig. 24, B). A maximum first order rate constant of 0.03 min^{-1} is obtained from the time dependence of the absorbance change at either 310 or 470 nm. Addition of sulfide results in an increase in absorbance at 330 nm and decrease in the α-aminoacrylate species that absorbs at 470 nm (Fig. 24, C), giving a spectrum identical to that obtained upon addition of L-cys to the PLP reconstituted apoK42A-OASS. Addition of an increasing concentration of ethylamine gave no change in the species at 330 nm. The resulting 330 nm species can be reduced by $NaBH_4$, giving an increase in the absorbance at 330 nm due to the presence of pyridoxylcysteine.

Circular dichroism experiments performed to study the formation of the external aldimine made use of the apoenzyme reconstituted with PLP after addition of 10 mM ethylamine. Addition of OAS to this enzyme species resulted in CD maxima around 330 and 430 nm with negative ellipticity. Addition of L-cysteine and L-alanine to the enzyme treated with 10 mM ethylamine showed no change in the spectrum.

Rates of L-Cysteine Formation. K42A-OASS as isolated and PLP-reconstituted apoK42A-OASS do not catalyze either the overall reaction, formation of cysteine, with the natural substrates, OAS and sulfide, or the deacetylase reaction, formation of pyruvate, ammonia, and acetate. As discussed above, a small amount of contaminating WT activity was detected in the K42A mutant enzyme as isolated, which could be eliminated by

Figure 24. UV-visible spectra of apo-K42A-OASS-A reconstituted with PLP in the presence of ethylamine, OAS and sulfide.

Panel A shows the UV/visible spectra of apo-K42A-OASS reconstituted with PLP (I) and formation of external aldimine in the presence of 10 mM ethylamine (II). The enzyme concentration used was 500 μg. Panel B and C show the difference between the spectrum in the presence of amino acid and that of free enzyme as a function of time. The reaction is that catalyzed by apo-K42A-OASS reconstituted with PLP in the presence of 10 mM ethylamine. Panel B shows first, the formation of the external aldimine (I), followed by the formation of α–aminoacrylate (II) external Schiff base. Times are 5, 15, 30, 60 and 90 min from top to bottom at 400 nm. Panel C shows the formation of L-cys after addition sulfide to the reaction in Panel B. Times are 5, 15, 30 and 60 min from top to bottom at 400 nm. The reaction was carried out at 25°C with 10 mM OAS and 10 μM sulfide in 10 mM Hepes, pH 7.5. The enzyme concentration used for the reactions was 1 mg.


A
Absorbance (AU)
0.5
0.4
0.3
0.2
0.1
0.0
320
340
360
380
400
420
440
460
480
500
I
II
B
Absorbance (AU)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
350
400
450
500
I
II
C
0.015
0.010
0.005
0.000
-0.010
350
400
450
500
550
Wavelength (nm)

borohydride reduction with no change in the spectrum of the K42A mutant protein. Since the ε-amino group of lysine is absent in the K42A mutant enzyme, two different methods were attempted to restore activity, i.e. addition of ethylamine to the enzyme and chemical modification of C43 with bromoethylamine to form γ-thialysine at position 43. Addition of 10 mM ethylamine to apoK42A-OASS reconstituted with PLP results in an absorbance shift from 394 to 398-400 nm, suggesting the formation of an external aldimine (Fig 24, A). When apoK42A-OASS reconstituted with PLP is preincubated with ethylamine, the rate of α-aminoacrylate formation from OAS (first half reaction) is slow compared to that with the wild type OASS-A. Addition of sulfide to the α-aminoacrylate intermediate causes a disappearance in the absorbance at 470 nm and at 400 nm with an increase in absorbance at 330 nm resulting from the cysteine external Schiff base (Fig. 24, C). Using 9 μM apo-K42A-OASS reconstituted with PLP a single turnover is observed with the catalytic cycle stopping at the L-cysteine external Schiff base. The maximum first order rate of formation of the cysteine external Schiff base is $(3.0 \pm 0.4) \times 10^{-3}$ s^{-1}, 10^5-fold lower than V/E_t for the WT enzyme (Tai et al., 1995). Apparent K values for OAS and sulfide in the formation of the cysteine external Schiff base are 10 mM and 10 μM, respectively, compared to K_m values of 1 mM and 6 μM, respectively, measured for the rate of formation of cysteine by the WT enzyme. In the second case, C43, located next to the active site lysine, was modified with bromoethylamine, giving γ-thialysine which forms an internal Schiff base with an absorbance at 398 nm similar to that of K42A-OASS after addition of ethylamine. Reduction of the γ-thialysine OASS with sodium borohydride results in a bleaching of the 400 nm absorbance and an increase in the absorbance at 320 nm. Denaturation with 4 M guanidinium HCl and dialysis produces no change in the 320 nm absorbance. Again a single turnover appears to occur, and rate data are essentially identical to those obtained with ethylamine. Tables 1 and 2 show the rates of the overall reactions catalyzed by the

apoK42A reconstituted with PLP in the presence of ethylamine and with C43 modified respectively. The alternative substrate, TNB, gave no discernible rate with the reconstituted mutant proteins.

Table 1. Rates of the reaction catalyzed by apo-K42A-OASS reconstituted with PLP in the presence of 10 mM ethylamine.[a]

Sulfide μM	v (μmoles/min)	OAS mM	v (μmoles/min)
(OAS 10 mM)		(Sulfide 25 μM)	
10	7.469×10^{-4}	5	8.243×10^{-4}
25	1.408×10^{-3}	10	7.469×10^{-4}
50	1.072×10^{-3}	20	1.773×10^{-3}

[a]The reactions were monitored with the help of sulfide assay with 300 μg of enzyme per assay in 100 mM Hepes, pH 7.5.

Table 2. Rates of the reaction catalyzed by apo-K42A-OASS reconstituted with PLP after modification of C43 with BEA.[a]

Sulfide μM	v (μmoles/min)	OAS mM	v (μmoles/min)
(OAS 10 mM)		(Sulfide 25 μM)	
10	9.107×10^{-4}	5	8.45×10^{-4}
25	2.59×10^{-3}	10	9.107×10^{-3}
50	2.64×10^{-3}	20	3.245×10^{-3}

[a]The reactions were monitored by sulfide assay with 500 μg of enzyme per assay in 100 mM Hepes, pH 7.5

CHAPTER 4

OTHER MUTANTS

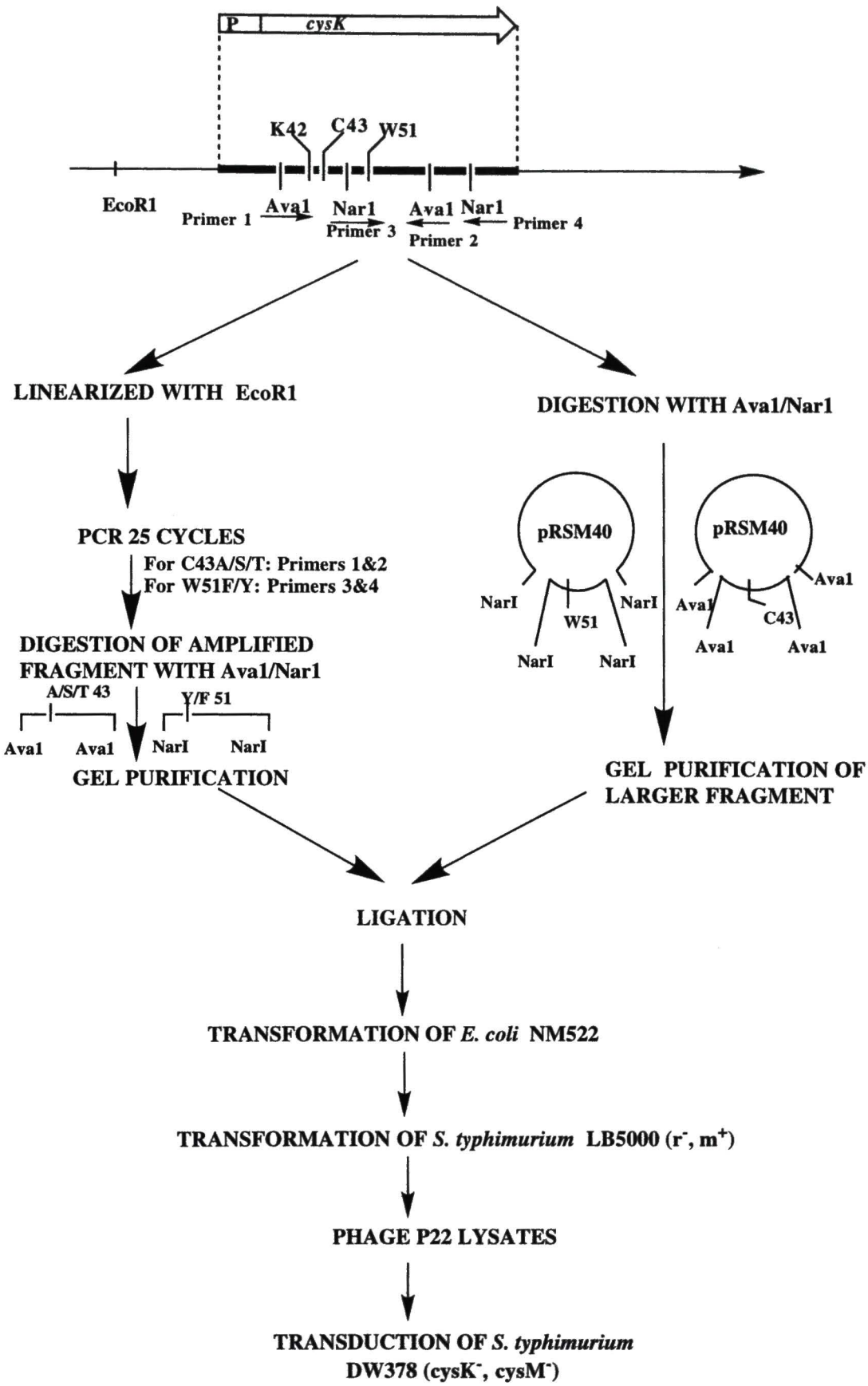
Selection Of The Residues For Mutagenesis. Site-directed mutagenesis with the help of PCR was used to obtain other mutants of OASS A. The residues selected for mutagenesis were cysteine 43 (C43) and tryptophan 51 (W51).

From pH studies, it was suggested that a group with pK_a similar to that of cysteine may be involved in catalysis (Tai et al., 1995). Also, C43 is the only cysteine in the protein and is in the active site, next to the Schiff base lysine. It was, therefore, considered to be a candidate for mutagenesis.

The enzyme OASS-A, when excited at 298 nm, shows two emission maxima at 337 and 498 nm. The maximum at 498 nm is due to the fluorescence of the Schiff base PLP whereas the maximum at 337 nm is a result of the energy transfer between a tryptophan residue and Schiff base PLP (McClure and Cook, 1995). The primary sequence of OASS-A shows the presence of only two tryptophan residues at positions 51 and 162 (Byrne et al., 1988). The residue W51 was therefore selected for mutagenesis to study its involvement in the energy transfer.

Mutagenesis of C43 and W51. The protocol used for mutagenesis of C43 and W51 mutants was very similar to the one used for the K42A mutant of *cysK*. The plasmid, pRSM40 (Fig 15, Chapter 2), was linearized with *Eco*RI. For the production of C43 mutants, a 690 bp AvaI fragment containing the codon TGC for C43 was amplified using PCR. Two oligonucleotide primers were used. Primer used for the 5'-end of the

Scheme 3. Procedure for mutagenesis of C43 and W51 of OASS-A.


P
cysK
K42
C43
W51
EcoR1
Primer 1
Ava1
Nar1
Primer 3
Ava1
Nar1
Primer 2
Primer 4
LINEARIZED WITH EcoR1
DIGESTION WITH Ava1/Nar1
PCR 25 CYCLES
For C43A/S/T: Primers 1&2
For W51F/Y: Primers 3&4
DIGESTION OF AMPLIFIED FRAGMENT WITH Ava1/Nar1
A/S/T 43
Y/F 51
Ava1
Ava1
NarI
NarI
GEL PURIFICATION
pRSM40
NarI
NarI
W51
NarI
NarI
pRSM40
Ava1
Ava1
C43
Ava1
Ava1
GEL PURIFICATION OF LARGER FRAGMENT
LIGATION
TRANSFORMATION OF E. coli NM522
TRANSFORMATION OF S. typhimurium LB5000 (r-, m+)
PHAGE P22 LYSATES
TRANSDUCTION OF S. typhimurium
DW378 (cysK-, cysM-)

amplified fragment is shown as (1) and carries AvaI site as well as the desired mutations. The primer used for the 3'-end is shown as (2) and carries the AvaI site.

TGC

| | |

(1) 5'-AGTCGCGCAA**CCCGAG**CTTCAGCGTCAAG**XCC**CGTATCGG-3'

(X was a mixture of T, A and G)

(2) 5'-AGGTTG**CCCGGG**ATGAAGCCTGCGC-3'

and primer 2 is complimentary to 5'-GCGCAGGCTTCAT**CCCGGG**CAACCT-3' in the DNA sequence of *cysK*.

In oligonucleotide (1), TGC encoding for cysteine was changed as shown to TCC, ACC and GCC, encoding for serine, threonine and alanine, respectively. The underlined sequences 5'-CCCGAG-3' in oligonucleotide (1) and 5'-CCCGGG-3' in oligonucleotide (2), are the recognition sites for AvaI.

For the production of W51 mutants, the plasmid DNA was linearized with *EcoR*I and 600 bp NarI fragment containing the region around W51 was amplified with the help of PCR. The two oligonucleotides used in this case were

TGG

| | |

(3) 5'-GCCGTATC**GGCGCC**AACATGATT**TXT**GATGCCGAAAAGCG-3'

(X was a mixture of A and T)

(4) 5'-GCTGCAACCG**CCGCGG**AAG-3'

In oligonucleotide (3), TGC encoding for cysteine was changed as shown to TAT and TTT, encoding for tyrosine and phenylalanine, respectively. The underlined sequences 5'-GGCGCC-3' in oligonucleotides (3) and (4), are the recognition sites for NarI.

The protocol for mutagenesis is shown in the scheme 3. The ligated plasmids

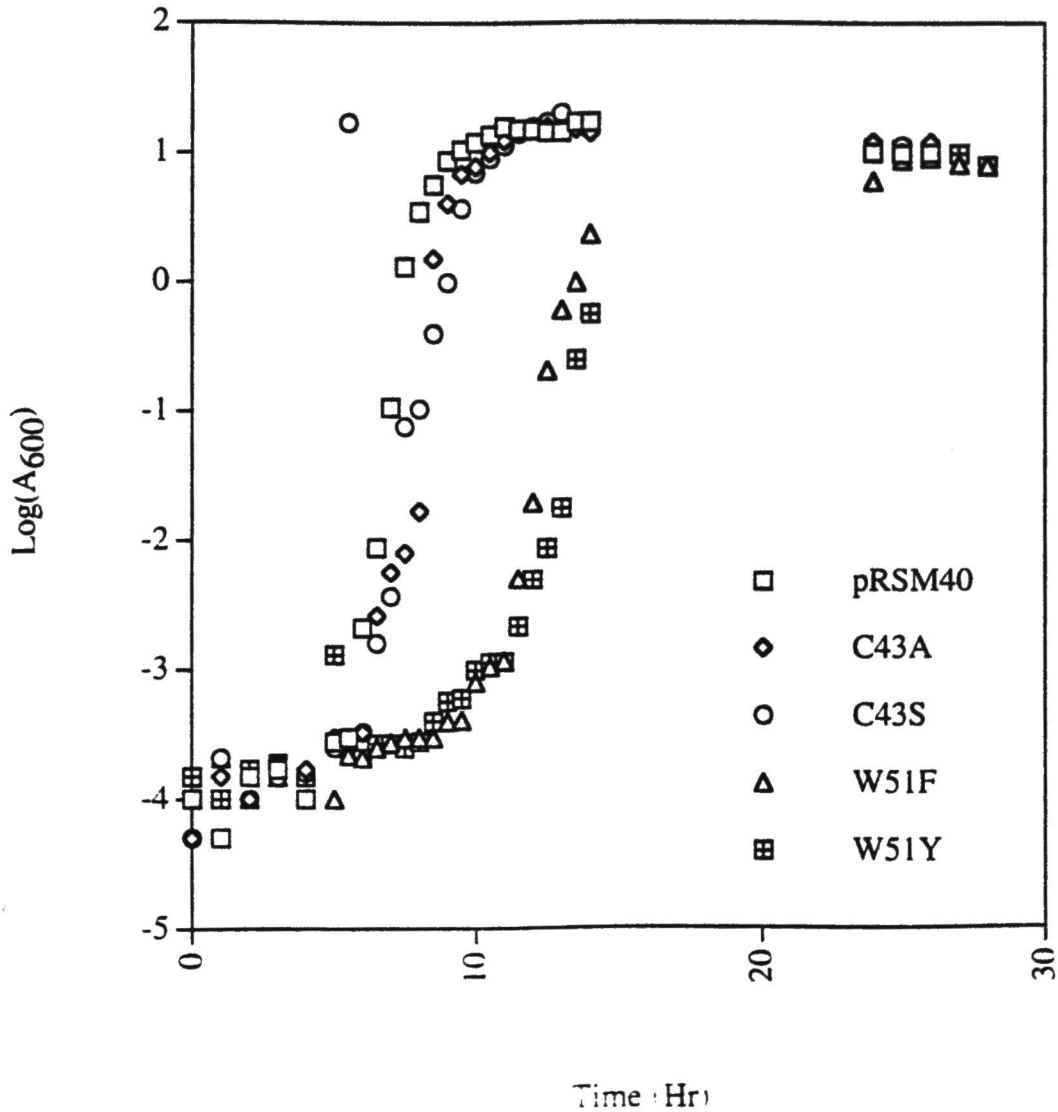
carrying the changed codons were transformed into *E. coli* NM522 using the $CaCl_2$ method (Sambrook et al., 1988) and purified using the Nucleobond AX kit. The initial screening of clones involved restriction digestion by AvaI for the mutants of C43 and that by NarI for W51 mutants. The complete gene was sequenced with a USB sequencing kit to confirm the mutation and to check for PCR errors. The mutant plasmids were transformed into *S. typhimurium* LB5000 using a modified Hanahan method and stored as phage *P22* lysates (Monroe and Kredich, 1990). Since LB5000 is r^-m^+ for all three restriction-modification systems, the DNA obtained from this strain is not degraded in any other *S. typhimurium* strain.

Expression of the mutant enzyme was carried out in the *cysK*$^-$ *cysM*$^-$ strain (DW378) of *S. typhimurium* by transduction with phage *P22* lysates. Briefly, 2 µl of the phage lysate were added to 100 µl of the DW378 culture grown overnight at 37°C with good aeration. The transductants were selected on LB plates with 100 µg/ml ampicillin in an overnight incubation at 37°C. The plasmid-containing strain was then allowed to grow for 18 hours at 37°C and 250 rpm on Vogel-Bonner medium E (Vogel and Bonner, 1956) supplemented with 0.5% glucose, 1% LB, 40 µM L-tryptophan, 500 µM reduced glutathione and 100 µg/ml ampicillin. Reduced glutathione was the only sulfur source in the medium and was added to derepress the cysteine biosynthesis pathway (Kredich, 1971).

Growth Of The Strains Carrying Plasmids With Mutations At C43 And W51. Six strains of *S. typhimurium DW378* with plasmid pRSM40 carrying the cysK gene, five carrying mutations; C43A, C43S, C43T, W51F and W51Y, and one carrying pRSM40 without any mutation as a control were allowed to grow on the minimal media as described above. All the strains were inoculated and allowed to grow at 37°C and 200 rpm. The growth was monitored by withdrawing a sample from each flask at definite time interval.

Figure 25. Growth curves of the mutants of C43 and W51 of OASS-A.

The figure shows growth curves of the strain DW378 of *S. typhimurium* carrying plasmid pRSM40 with and without mutations C43A, C43S, W51F and W51Y.


2
1
0
-1
-2
-3
-4
-5
Log(A600)
0
10
20
30
Time (Hr)
pRSM40
C43A
C43S
W51F
W51Y

Absorbance of each sample was collected at 600 nm. The absorbance was then plotted against time on a logarithmic scale for each strain (Fig. 25). Growth patterns were found to be different for the strains with mutations at C43T, W51F and W51Y from the strain without mutation. For the strains with mutations at C43A and C43S, the growth patterns were very similar to the strain DW378-pRSM40 without mutations.

Purification of The Enzyme From The Strains Carrying Mutations. The enzyme from the mutants was purified by the method of Tai et al (1993). The schematic representation of the purification protocol is shown in scheme 3, chapter II. UV-visible absorption spectra were recorded for each fraction of the DEAE-5PW and phenyl-5PW column elute. Enzyme from all the strains showed similar profiles of purification. Elution from the anion exchange column, DEAE-5PW, was around 40% for all the strains. Also, elution from the hydrophobic column, phenyl-5PW, was at about 45% for all the strains. Purity of the enzyme was confirmed via SDS-PAGE. Enzyme from all the strains showed a band comparable to wild type OASS-A. The UV-visible spectra of each mutant enzyme was found to be similar to that of the wild type OASS-A with two absorption maxima at 280 and 412 nm.

CHAPTER 5

DISCUSSION

Location of the Schiff base Lysine. The primary sequence of OASS-A has been published (Byrne et al., 1988). Studies on location of PLP-Schiff base lysine were performed with the use of radiolabelled PLP and active site peptide sequencing (Tai, 1993). Based on the data obtained, lysine residue at position 42 was found to contain the radioactivity from PLP. The similarity among the active site of OASS-A from *S. typhimurium* and other PLP dependent enzymes of the β-group as well as OASS-A from other sources (Figures 6 and 11, chapter 1) also suggested lysine 42 to be involved in Schiff base linkage with PLP.

K42A Mutant Enzyme

Growth. Surprisingly, growth under conditions of forced aeration produced the wild type enzyme. However, the background strain, DW378, is *cysK*$^-$, *cysM*$^-$ as a result of point mutations and eliminates the activity of OASS-A and OASS-B (Dreyfuss and Monty, 1962). In addition, DW378 is a recombinant positive strain. As a result of the selective pressure of growth in the absence of cysteine, homologous recombination likely occurs between the *cysK* gene on the plasmid carrying mutation K42A and the chromosomal *cysK* gene to produce the WT enzyme. As stated in the Results section, K42A mutant protein could be obtained in good yield only by overnight growth in shaker

culture.

Spectral Properties of the K42A-OASS in the Absence of Amino Acids. The K42A mutant enzyme, as isolated, shows absorbance maxima at 280 and 424 nm, the latter corresponding to the external aldimine of PLP with a mixture of free methionine and leucine based on an amino acid analysis of isolated K42A that was heat-denatured and dialyzed. Similar spectra have been observed for the external Schiff bases of OASS with L-cysteine or L-serine (Schnackerz et al., 1995). The external aldimine of K42A-OASS as isolated cannot be reduced with sodium borohydride under conditions normally used for the reduction of internal and/or external aldimines of PLP. Only in the presence of 5 M guanidinium hydrochloride is the reduction with borohydride feasible, indicating that the external aldimine of K42A-OASS as isolated may be in a closed conformation. It is very difficult to remove PLP from K42A-OASS, even though the mutant enzyme lacks Lys-42, which in WT OASS-A binds PLP covalently via an internal aldimine linkage. The presence of 5 M guanidinium hydrochloride is necessary to open the PLP binding site to facilitate resolution of the cofactor to produce apoK42A-OASS. Reconstitution of the apoenzyme with PLP results in two absorption bands at 330 and and 390 nm. Free PLP absorbs at 388 nm with a shoulder at 325 nm at neutral pH (Peterson and Sober, 1954). The spectrum of reconstituted apoK42A-OASS is very similar to that of free PLP at neutral pH, confirming the presence of the free aldehyde at the active site.

The fluorescence emission spectrum of WT OASS exhibits maxima at 337 and 500 nm when excited at 298 nm (McClure and Cook, 1994; Strambini et al., 1996). The 337 nm band results largely from intrinsic tryptophan fluorescence, while the 500 nm band results from delayed fluorescence of the internal Schiff base (Strambini et al., 1996). Addition of L-cysteine to WT OASS to form the external Schiff base (or acetate which binds to the α-carboxylate subsite, mimicking cysteine), results in a significant

enhancement of the long wavelength band and a blue shift in its λ_{max} to 490 nm (McClure and Cook, 1994; Schnackerz et al., 1995; Strambini et al., 1996). The fluorescence spectrum of K42A-OASS as isolated, also shows maxima at 337 and 504 nm, when excited at 298 nm. The ratio of $A_{337/504}$ for K42A-OASS as isolated is about halfway between that of the WT OASS and the cysteine external Schiff base, suggesting the presence of about 50% occupancy of K42A with an external aldimine between PLP and a free amino acid (methionine and leucine as indicated in the Results). ApoK42A-OASS, on the other hand, shows fluorescence emission only at 337 nm, in agreement with results on WT apoOASS (McClure and Cook, 1994). In apoK42A-OASS, the fluorescence emission at 337 nm is increased when compared to K42A-OASS, likely the result of quenching upon binding of PLP to the apoenzyme, as is also observed for WT OASS and its apoenzyme (McClure and Cook, 1994; Strambini et al., 1996). Similar results are found for D-serine dehydratase (Schnackerz et al., 1973; Federiuk and Shafer, 1983); tryptophanase (Tokushige et al., 1980); and tryptophan synthase (Strambini et al., 1992). The emission band of PLP reconstituted apoK42A-OASS at 500 nm has a lower intensity than K42A-OASS, as isolated, when excited at 298 nm. Reconstituted apoK42A-OASS exhibits two emission bands at 444 and 387 nm, respectively, when excited at 330 nm, qualitatively similar to WT OASS-A which shows maxima at 484 and 362 nm, respectively. For the WT OASS, which exists as an internal aldimine, the major band centered at 484 nm is characteristic of a ketoenamine tautomer, whereas the weaker band around 362 nm is typical for an enolimine tautomer (Strambini et al., 1996). In the case of the PLP-reconstituted apo-K42A-OASS, the 444 nm band may represent the ketoenol form of free PLP, while the weaker 387 nm band may represent the enolaldehyde form of free PLP. The fluorescence data support the presence of an external aldimine in K42A-OASS as isolated.

The CD spectrum of K42A-OASS as isolated, shows a negative Cotton band with a

maximum centered at 424 nm in the visible region. This can be compared to a visible band centered at 412 nm for the WT enzyme (Schnackerz et al., 1995). Addition of L-cysteine to the WT enzyme causes a red shift in both the visible absorbance and CD bands. The visible CD band has a positive sign and is of equal intensity to that observed in the absence of L-cysteine (Schnackerz et al., 1995). The K42A-OASS as isolated exists as a mixture of methionine and leucine Schiff bases. Since these external Schiff bases have a visible Cotton Band opposite in sign to that of the WT cysteine external Schiff base, there are two possible explanations. The difference in chemical structure of met, leu, and cys is responsible for the opposite sign, or the opposite face of the external Schiff base interacts with the protein surface in the cysteine external Schiff base compared to the met/leu external Schiff bases. Since the K42A-OASS as isolated cannot be reduced, these possibilities cannot be distinguished at this time. In the PLP-reconstituted apoK42A-OASS CD bands with positive ellipticity around 330 and 390-400 nm (centered on the visible absorbance bands) are observed. The ^{31}P signal of the internal aldimine of the WT OASS is 5.2 ppm, independent of pH (Cook et al., 1992), while that of the cysteine external Schiff base is shifted slightly downfield to 5.3 ppm (Schnackerz et al., 1995). The ^{31}P NMR spectra of K42A-OASS, as isolated, show a single resonance at 5.3 ppm, consistent with the occurrence of an external aldimine in K42A-OASS as isolated.

The Schiff base lysine has been replaced in three other PLP-dependent enzymes: K258A in aspartate aminotransferase (Toney and Kirsch, 1993), K145A in D-amino acid aminotransferase (Nishimura et al., 1991), and K87T in $\alpha_2\beta_2$ tryptophan synthase (Miles et al., 1989). The most closely related is K87T in $\alpha_2\beta_2$ tryptophan synthase which is isolated as an external aldimine with free L-serine. The spectral properties of the apoK87T-tryptophan synthase reconstituted with PLP are identical to those of the apoK42A-OASS reconstituted with PLP. The Schiff base lysine mutants of the aminotransferases,

however, are isolated with the free aldehyde of PLP bound. From the data obtained with K42A-OASS and Schiff base lysine mutants of other enzyme it is clear that replacement of the Schiff base lysine does not affect the binding of PLP to the enzyme.

Spectral Properties of the K42A-OASS in the Presence of Amino Acids. The external Schiff base forms slowly upon addition of OAS, L-cys, L-ser, or L-ala to the PLP-reconstituted mutant enzyme. The final equilibrium mixture of species is similar to that observed for the WT OASS in the case of L-ser, that is a mixture of tautomers with λ_{max} values at 330 and 418 nm, but differs in the case of L-cys with the 330 nm tautomer observed for K42A-OASS compared to a 418 nm tautomer observed for the wild type enzyme (Schnackerz et al., 1995). The slow formation of the external Schiff base was also observed for K87T $\alpha_2\beta_2$-tryptophan synthase (Lu et al., 1993) and K258A-aspartate aminotransferase (Toney and Kirsch, 1993).

Regeneration of Activity. All amino acids tested, OAS, L-cysteine, L-serine, and L-alanine, with PLP-reconstituted apo-K42A-OASS form an external Schiff base slowly (0.04 min^{-1}) compared to WT OASS (700-1000 s^{-1}; Woehl et al., 1996). Cordes and Jencks (1962) demonstrated that the rate constants for reactions in solution of imines of PLP with semicarbazide are greater than those for the parent aldehyde alone with semicarbazide. The present results are consistent with the suggestion of Cordes and Jencks that the rate constant for formation of the external aldimine from the WT OASS internal aldimine should be greater than that for enzyme-bound free aldehyde of PLP. Thus, significant rate enhancement is realized as a result of K42 allowing transimination to occur in the catalytic cycle.

Addition of 10 mM ethylamine to reconstituted apoK42A-OASS in the presence of OAS shows formation of external aldimine with λ_{max} at 398 nm, followed by the slow formation of the α-aminoacrylate intermediate. Addition of sulfide results in an increase in

absorbance at 330 nm and a decrease in the α-aminoacrylate intermediate, giving a spectrum identical to that obtained upon addition of cysteine to reconstituted apoK42A-OASS. Similar spectral changes in the presence of OAS were observed for K42A-OASS modified at position 43 to a γ-thialysine. The restored activity is 10^5-fold lower than that of WT enzyme and only one turnover is carried out in both half reactions. The spectral changes observed suggest that the reaction is terminated with the formation of the cysteine external Schiff base and the cysteine cannot be displaced, a prerequisite to start a new cycle. Data are consistent with the above conclusion that K42 is important for transimination, not only to form the external Schiff base, for example with OAS, but also to form the internal Schiff base and release the amino acid product, for example cysteine. The lack of ability of ethylamine to displace cysteine may be entropic, since ethylamine lacks the advantage of being locked into place, or may be geometric resulting from ethylamine being bound at the active site in such a way that it is unable to carry out the displacement effectively. Planas and Kirsch (1991) have suggested that the decreased basicity of the γ-thialysine group in K258-γ-thialysine-aspartate aminotransferase is considered to be principally responsible for the 14-fold lower V_{max} value compared to WT aspartate aminotransferase. The 10^5-fold lower activity of the C43-γ-thialysine derivative of K42A is likely a result of geometric considerations, since C43 is one amino acid removed from the normal Schiff base lysine position.

In conclusion, lysine-42 of OASS-A facilitates the formation and dissociation of the OAS and L-cysteine external Schiff bases, allowing more facile transimination. It is not required for cofactor binding but participates as a general base in the first half reaction, abstracting the α-proton of OAS, and as a general acid in the second half reaction, donating a proton to the α-carbon.

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