Isolation and Characterization of Glyoxalase I from *Escherichia coli*

by

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Abstract

Glyoxalase I (S-lactoylglutathione methylglyoxal lyase, EC 4.4.1.5) from *Escherichia coli* was overexpressed using DF502/pDM7 and a purification scheme developed to allow purification of the enzyme to near homogeneity. The enzyme from this source was found to be homodimeric with a molecular weight of approximately 30 kDa. The pH optimum was determined to be greater than pH 8.0. Preliminary experiments have indicated an isoelectric point for this enzyme to be between pH 4.15-5.0. Metal-chelation studies indicate that the rate of loss of enzyme activity is buffer-dependent. Attempts to identify metal ions capable of activating *E. coli* glyoxalase I indicated that Ni^{2+} most effectively activated the enzyme followed by Mn^{2+}, Co^{2+}, Ca^{2+} and Mg^{2+}. Surprisingly, zinc was found not to increase the activity of the enzyme in contrast to what has been observed for several other glyoxalase I enzymes. Amino acid modification studies showed that aspartic acid and/or glutamic acid may be crucial for catalytic activity or substrate binding. The K_m and V_{max} for the hemimercaptal for methylglyoxal and glutathione was found to be 69 μM and 0.753 μmol/min, respectively. The enzyme was found to be unstable in several buffer systems but the presence of glycerol aided stabilization of glyoxalase I.

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I wish to thank Dr. John Honek for his guidance and patience. It's been a pressing 4 years as we tried to overproduce and study this enzyme.

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"D'oh!"

Homer Simpson

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ATP	adenosine triphosphate
A. niger	*Aspergillus niger*
BICINE	N,N-bis(2-hydroxyethyl)glycine
BSA	bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
cm	centimeter
dA	dalton
DEPC	diethyl pyrocarbonate
DHAP	dihydroxyacetone phosphate
DMSO	dimethyl sulfoxide
EC	enzyme classification (number)
E. coli	*Escherichia coli*
ESMS	electrospray mass spectrometry
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	N,N,N′,N′-ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(2-aminoethyl ether)-N,N′-tetraacetic acid
EPR	electron paramagnetic resonance
EXAFS	extended x-ray absorption fine structure
FPLC	fast protein liquid chromatography

g	gravitational force
G3P	glyceraldehyde-3-phosphate
GSH	glutathione, reduced
HR	high resolution
H. sapiens	*Homo sapiens*
hr	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC_{50}	50% inhibition concentration
k_{cat}	catalytic turnover constant
kDa	kilodalton
K_m	Michaelis-Menten constant
L	litre
LB	Luria-Bertani growth media
MG	methylglyoxal
MGR-I, MGR-II	methylglyoxal reductases
MG^R	methylglyoxal resistance
μg	microgram
μL	microlitre
μM	micromolar
mA	milliamperes
mg	milligram
mL	millilitre

mm	millimetre
mM	millimolar
M	molar
ε_M	molar extinction coefficient
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
nm	nanometre
NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
P. putida	*Pseudomonas putida*
S. cerevisiae	*Saccharomyces cerevisiae*
s	second
SDS	sodium dodecyl sulfate
TEMED	N,N,N′,N′-tetramethylethylenediamine
TNM	tetranitromethane
TIM	triosephosphate isomerase
TRIS	tris(hydroxymethyl)aminomethane
V_{max}	maximal enzyme velocity

λ	wavelength

CHAPTER 1

INTRODUCTION

A number of investigators have speculated on the possible role(s) of the ubiquitous glyoxalase system and it is currently believed that the two enzymes (glyoxalase I and glyoxalase II) work in tandem to transform cytotoxic α-ketoaldehydes to noncytotoxic α-hydroxycarboxylic acids (Racker, 1951) (Figure 1). Glyoxalase I (S-*D*-lactoylglutathione methylglyoxal lyase (isomerising), EC 4.4.1.5), the first enzyme of the system, serves to transform nonenzymatically formed hemimercaptals of glutathione and α-ketoaldehydes into thioesters while glyoxalase II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6) serves to hydrolyze these thioesters into α-hydroxycarboxylic acids with the subsequent result that glutathione is regenerated (Uotila, 1973). To better comprehend why Nature requires the glyoxalase enzymes, a discourse of the biosynthesis and catabolism of α-ketoaldehydes (with emphasis on methylglyoxal production) as well as the enzymes involved in these processes is presented.

1. Methylglyoxal: Biosynthesis and Catabolism

Methylglyoxal (2-oxopropanal, pyruvaldehyde, MG) is an aliphatic α-ketoaldehyde that inhibits cell growth at approximately 0.4 mM and is cytostatic at higher concentrations (Szent-Gyorgi and Egyund, 1967; French and Freedlander, 1958).

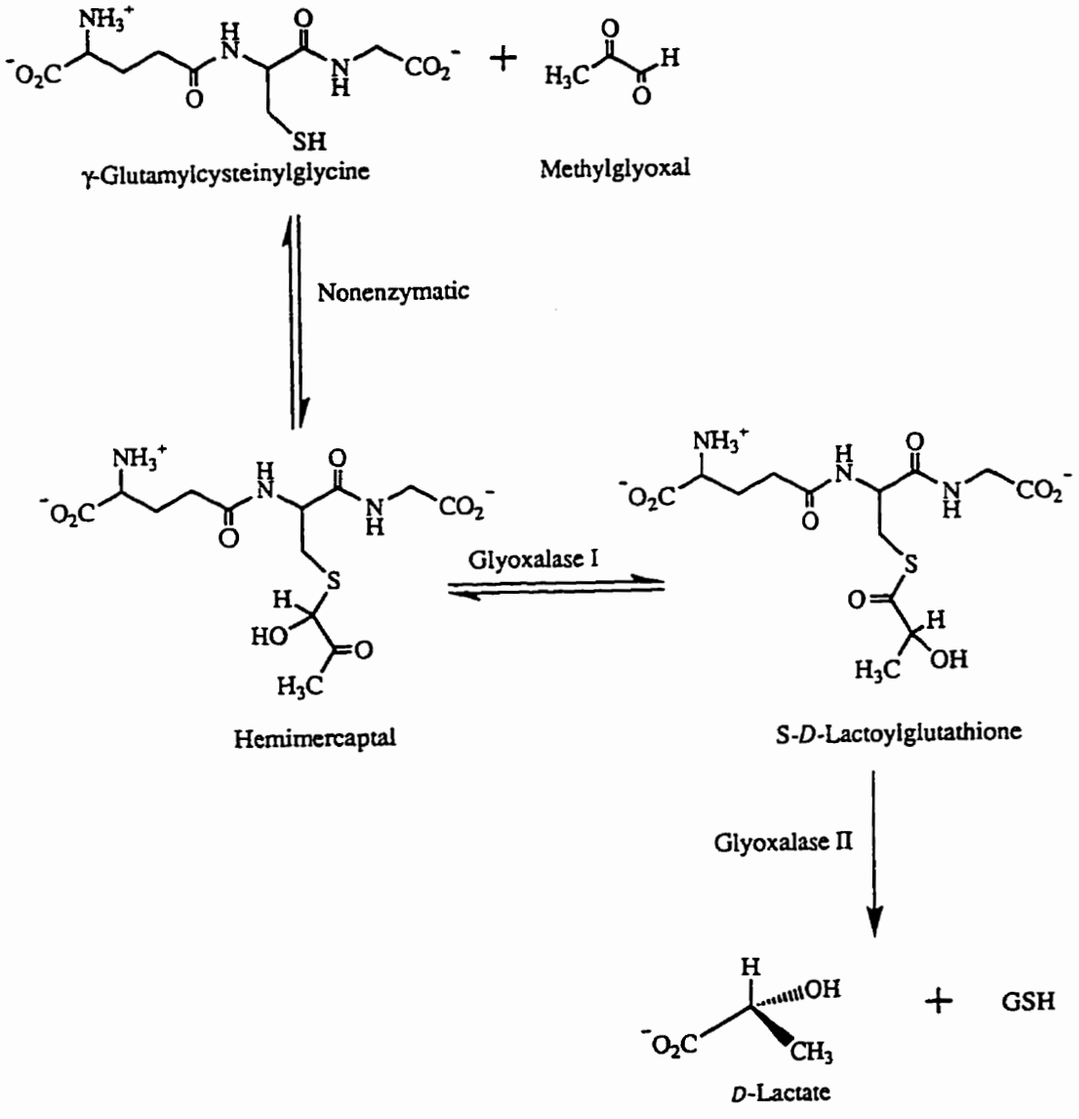

Figure 1. The glyoxalase system.

The interaction of biomolecules with methylglyoxal would appear to be rather nonspecific as mitochondrial enzyme activities (Canuto *et al.*, 1985), plasma membrane adenylate cyclase activity (Paradini *et al.*, 1985) and putrescine-activated S-adenosylmethionine decarboxylase activity (Pegg, 1973) are all affected by the presence of methylglyoxal possibly due to modification of sulfhydryl groups (Paradini *et al.*, 1985)). Inhibition of protein synthesis by methylglyoxal at the tRNA level possibly due to polysome degradation has also been reported (White and Rees, 1982). α-Ketoaldehyde toxicity is not limited to organisms with advanced cellular structures as viral stability is also affected by methylglyoxal toxicity (de Beck *et al.*, 1957).

Approximately 60 years ago methylglyoxal was believed to have been a key intermediate in the process of glycolysis in plants, animals and microbes (Dakin and Dudley, 1913; Neuberg, 1913). This rationale was justified at the time due to the fact that an ubiquitous enzyme system existed that converted methylglyoxal to lactic acid (Dakin and Dudley, 1913; Neuberg, 1913). It is now known that this is due to the glyoxalase system which produces *D*-lactate in contrast to *L*-lactate which is produced during glycolysis. Once it was determined that *L*-lactate was in fact being produced and not *D*-lactate, the role of methylglyoxal in glycolysis was dismissed (Meyerhof, 1948).

In the early 1950's, studies on iodoacetate-poisoned *Pseudomonas saccharophila* indicated that methylglyoxal was being produced from glyceraldehyde-3-phosphate (G3P) (Entner and Doudoroff, 1952) but as no obvious enzymatic reaction appeared to be producing methylglyoxal from glycohydrolytic intermediates, further reports concerning methylglyoxal and glycolysis did not reappear until 1964 (Wang *et al.*, 1964) and later in the 1970's (Cooper, 1975).

Studies performed on a triosephosphate isomerase-deficient mutant strain of *Escherichia coli (E.coli)* demonstrated that such strains could produce dihydroxyacetone phosphate (DHAP) from glycerol (Cooper and Anderson, 1970 ; Hopper and Cooper, 1972). However since these mutants lacked triosephosphate isomerase (TIM) (EC 5.3.1.1) they could not produce G3P. It was noted that methylglyoxal could be produced from DHAP and it was established that methylglyoxal was being formed due to the presence of methylglyoxal synthase (EC 4.2.99.11) (Hopper and Cooper, 1971 ; Freedberg *et al.*, 1971). Methylglyoxal synthase has been found in rat and goat liver (Sato *et al.*, 1980; Ray and Ray, 1981) but this enzyme has yet to be found in humans (Figure 2).

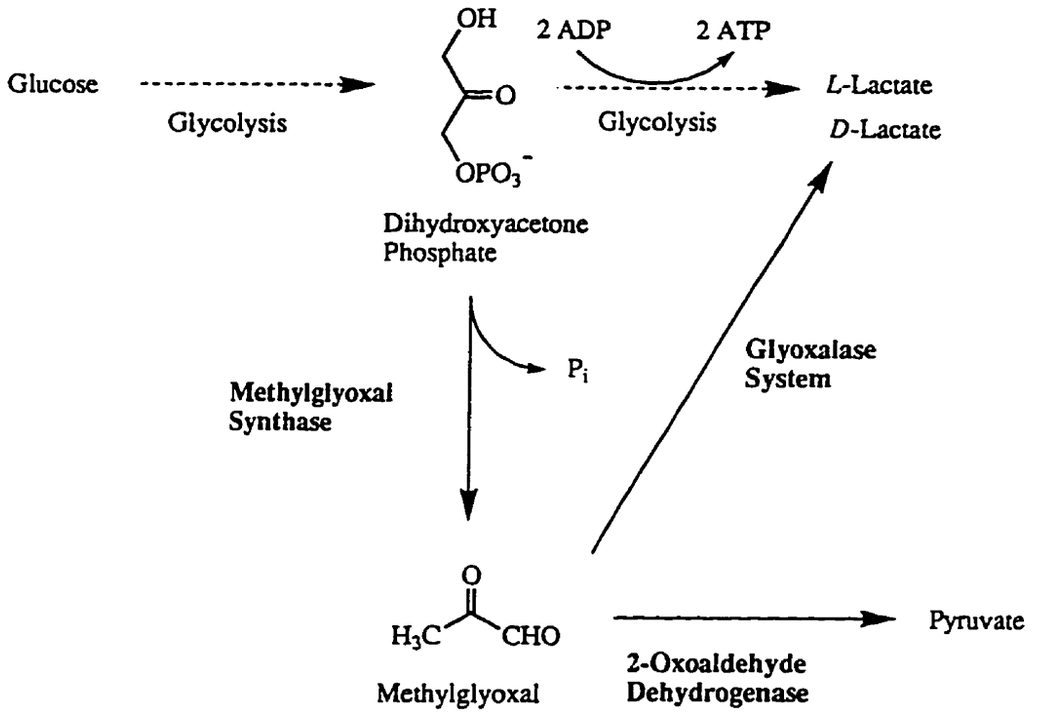

Figure 2. Enzymatic synthesis of methylglyoxal.

2. Methylglyoxal Bypass of Glycolysis

A number of bacterial systems are known to possess glyoxalase activities (Neuberg and Gorr, 1925; Widmann, 1929; Still, 1941) as well as *D*- and *L*-lactate dehydrogenases (EC 1.1.2.4 and EC 1.1.2.3, respectively) that are capable of forming pyruvate from lactate (Kline and Mahler, 1965). Formation of pyruvate from methylglyoxal as opposed to formation of pyruvate from phosphorylated 3-carbon intermediates would constitute a bypass of some of the glycolytic pathway. In *E.coli* lacking TIM, the conversion of glycerol to pyruvate may occur by this bypass (Cooper and Anderson, 1970). Bacteria deficient in glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3) or enolase (EC 4.2.1.11) do not undergo sustained growth on glucose or glycerol indicating that the methylglyoxal bypass appears not to be a sustainable alternative to glycolysis in *E. coli* (Anderson and Cooper, 1969; Irani and Maitra, 1974; Hillman and Fraenkel, 1975).

3. Formation of Methylglyoxal

3.1. Methylglyoxal Synthase

Irreversible conversion of dihydroxyacetone phosphate (DHAP) into methylglyoxal and inorganic phosphate is catalyzed by methylglyoxal synthase (Figure 2) (Cooper and Anderson, 1970). It is believed that an enzyme base abstracts a proton from C-3 (hydroxymethyl group) of DHAP to generate an enediol which then loses phosphate to form an enolaldehyde. Tautomerization of the enolaldehyde then generates methylglyoxal (Summers and Rose, 1975). The mechanism is postulated to involve stereospecific proton abstraction of the pro-*S* hydrogen of the hydroxymethyl group; intramolecular transfer of the pro-*R* hydrogen to generate the methyl group was not observed (Summers and Rose, 1975). Proton addition to form the methyl group was nonstereospecific and this led to the hypothesis that an enolaldehyde (keto form predominates in solution) and not enediolphosphate is the enzyme product (Summers and Rose, 1975).

Methylglyoxal synthase has been identified and isolated from yeast (Babel and Hoffman, 1981), bacteria (Hopper and Cooper, 1972; Cooper, 1974; Tsai and Gracy, 1976) and some mammals (Sato *et al.*, 1980; Ray and Ray, 1981). The enzyme from *E. coli* K-12 strain CA244 is of approximately 67 kDa in molecular weight with pH optimum of 7.5. The

enzyme is specific for DHAP (K_m = 0.47 mM) (Hopper and Cooper, 1972).

3.2. Triosephosphate Isomerase

Formation of MG and inorganic phosphate by aldolase and triosephosphate isomerase is known to occur in mammalian tissues but apparently does not occur in yeast (Pompliano *et al.*, 1980; Richards, 1991). Such formation is believed to involve phosphate elimination with the subsequent formation of an α,β-unsaturated aldehyde which then rapidly undergoes tautomerization to methylglyoxal (Figure 3). The conversion of DHAP to methylglyoxal is not the primary enzymatic reaction catalyzed by TIM and is at most a minor side reaction comprising one turnover per million (Richards, 1991).

J.P. Richard has undertaken an analysis of the kinetic parameters for such a conversion for TIM from rabbit muscle and has found that such a process has a catalytic efficiency of only 14 $M^{-1}\cdot s^{-1}$ (k_{cat} = 0.011 s^{-1} and K_m = 760 μM) but given the concentrations of triose phosphates ($4x10^{-5}$ M) and TIM ($1x10^{-5}$ M) in a cell it would be possible to generate approximately 0.4 mM methylglyoxal concentrations in a cell per 24 hour period (Richards, 1991). If these calculations are representative of cellular processes that lead to methylglyoxal formation then the role of glyoxalase I may be to detoxify TIM-produced methylglyoxal.

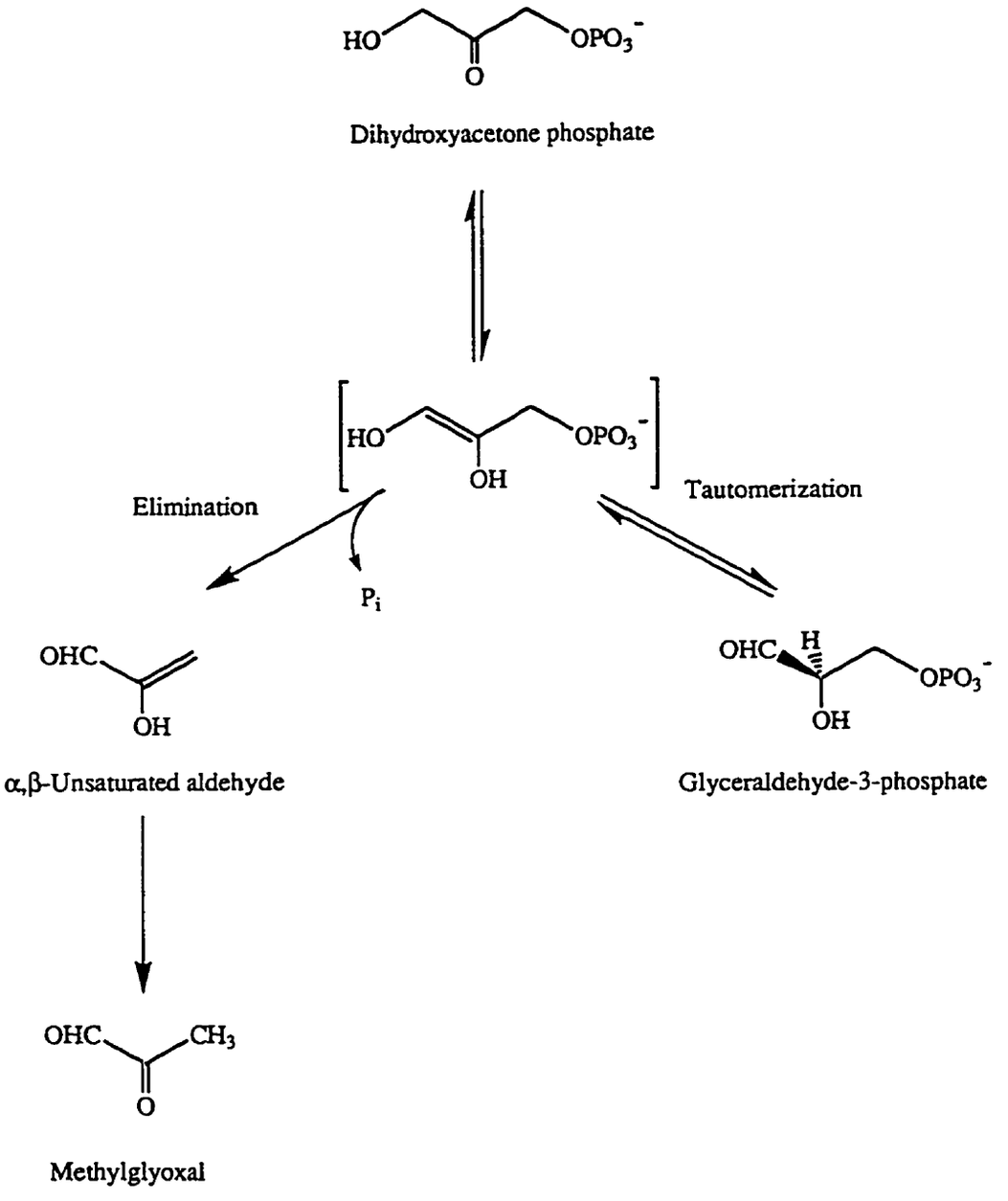

Figure 3. Formation of methylglyoxal from triosephosphate isomerase.

3.3 Acetol Dehydrogenase

Methylglyoxal can be formed from 1-hydroxyacetone (acetol) by the action of acetol dehydrogenase (EC 1.1.1.-) which utilizes NAD^+ as a cosubstrate (Taylor *et al.*, 1980). Little work has been done on this enzyme from *Corynebacterium*, Strain A1 due to its instability (complete inactivation occurs within 24 hours when stored as crude extracts at 3 °C) (Taylor *et al.*, 1980).

3.4. Aminoacetone Oxidase

The production of methylglyoxal from aminoacetone via an amine oxidase (EC 1.4.3.6) (Urata and Granick, 1963; Ray and Ray, 1987)during the catabolism of threonine was proposed in 1959 (Figure 4) (Elliot, 1959). Studies on the amine oxidase from goat liver indicated that it is a homodimer of 186 kDa which is capable of oxidizing aminoacetone, spermidine, spermine, tryptamine, putrescine and ethanolamine and that this enzyme is sensitive to carbonyl group modifying reagents (Ray and Ray, 1987).

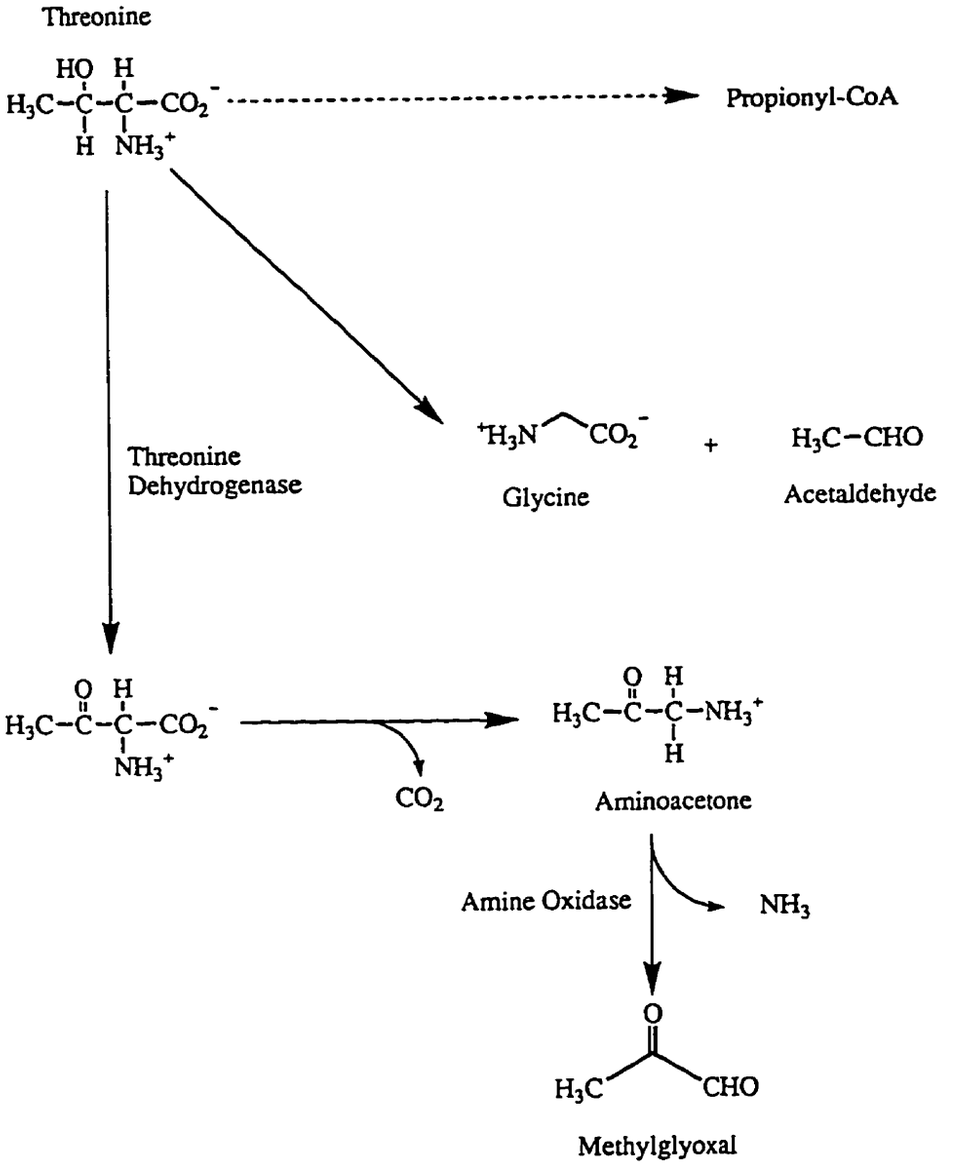

Figure 4. Known pathways of threonine catabolism.

3.5. Nonenzymatic Formation of Methylglyoxal

Work by Phillips and Thornalley have demonstrated the formation of methylglyoxal from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate in Krebs-Ringer phosphate buffer pH 7.4 (Phillips and Thornalley, 1992). At 37 °C the rate of formation from DHAP and G3P was found to be first-order with rate constants of $1.94 \pm 0.02 \times 10^{-5}$ s^{-1} and $1.54 \pm 0.02 \times 10^{-4}$ s^{-1}, respectively (Phillips and Thornalley, 1992). It has yet to be determined if nonenzymatic formation of methylglyoxal from biological small molecules is an important source of methylglyoxal. Nonenzymatic formation of methylglyoxal from DHAP is believed to involve base abstraction of a proton from C-3 (hydroxymethyl group) of DHAP to generate an enediol which then loses phosphate to form an enolaldehyde. Tautomerization of the enolaldehyde then generates methylglyoxal (Summers and Rose, 1975).

4. Methylglyoxal Catabolism

4.1. Methylglyoxal Dehydrogenase

The enzyme methylglyoxal dehydrogenase also termed α-ketoaldehyde dehydrogenase (EC 1.2.1.23) catalyzes the oxidation of methylglyoxal to pyruvate (Figure 5). The enzyme from *Pseudomonas putida* (*P. putida*) has a molecular weight of approximately 42 kDa and will oxidize glyoxal, methylglyoxal, formaldehyde and acetaldehyde and requires NAD^+ as a

cofactor (Rhee *et al.*, 1987). The enzyme from goat liver consists of two different types of enzyme (one which utilizes NAD^+ and the other which utilizes $NADP^+$) each with a molecular weight of approximately 42 kDa (Ray and Ray (1982)). These enzymes are specific only for 2-oxo-aldehydes. Methylglyoxal dehydrogenase has not been found in *Saccharomyces cerevisiae (S. cerevisiae)* (Murata *et al.*, 1985a) nor has it been found in higher plants (Kato *et al.*, 1988).

4.2. Methylglyoxal Reductase

The enzyme aldehyde reductase (EC 1.1.1.21) catalyzes the conversion of α-ketoaldehydes to α-hydroxyaldehydes. Aldehyde reductase exhibiting MG reductase activity (methylglyoxal reductase) has been detected and purified from goat liver (Ray and Ray, 1984), *S. cerevisiae* (Murata et al., 1986; Inoue *et al.*, 1988) and *Aspergillus niger* (*A. niger*) (Inoue *et al.*, 1988). A number of substantial differences were found to exist between the enzyme from yeast and the enzyme from goat liver:

a. the yeast enzyme utilizes NADPH while the goat enzyme uses NADH.

b. the reaction catalyzed by the yeast enzyme is irreversible whereas the goat liver enzyme catalyzes a reversible reaction.

c. the yeast enzyme is specific for 2-oxoaldehydes whereas the

goat liver enzyme has broad substrate specificity and will transform aldehydes and 2-oxoaldehydes.

d. the yeast enzyme is monomeric with a molecular weight of approximately 43 kDa and the enzyme contains 5% w/w carbohydrate while the goat liver enzyme is dimeric with a molecular weight of approximately 89 kDa.

A. niger possesses two different methylglyoxal reductases (MGR-I and MGR-II, respectively). The first enzyme is specific for 2-oxo-aldehydes and has a molecular weight of 36 kDa while the second enzyme utilizes NADPH and has broad substrate specificity and has a molecular weight of 38 kDa. Why *A. niger* requires two methylglyoxal reductases has not yet been determined. The enzymes from yeast and mold require an ordered addition of NADPH and then substrate otherwise irreversible inactivation occurs for these enzymes (Inoue *et al.*, 1988).

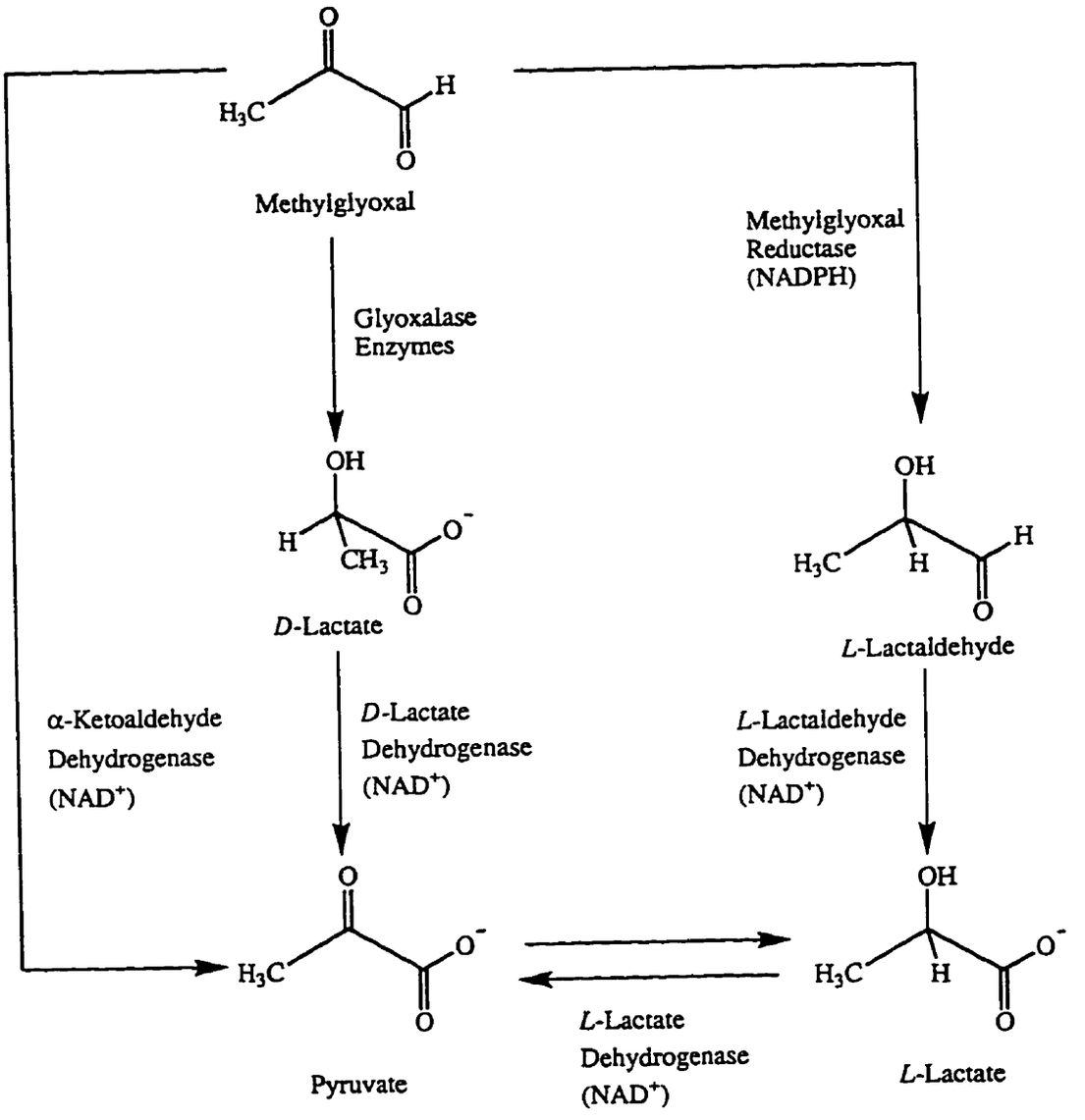

Figure 5. Metabolism of α-ketoaldehydes such as methylglyoxal.

4.3. Lactaldehyde Dehydrogenase

Lactaldehyde dehydrogenase (EC 1.2.1.22) catalyzes the conversion of lactaldehyde to lactic acid. Initial studies on lactaldehyde metabolism were performed in 1953 (Neuberg and Varcellone, 1953) and in 1958 (Sandman and Miller, 1958) but the enzymes involved were not identified until 1985 (Murata *et al.*, 1985b). An NAD^+-utilizing enzyme that metabolizes lactaldehyde has been purified from yeast by Inoue and co-workers (Inoue *et al.*, 1985). Lactaldehyde dehydrogenase has also been purified from *E. coli* (Sridhara and Wu, 1969). The yeast enzyme is specific for *L*-lactaldehyde whereas the *E. coli* enzyme will act upon both *L*-and *D*-lactaldehyde and propionaldehyde. The *E. coli* enzyme has a molecular weight of 100 kDa and is most active above pH 10.5. The yeast enzyme consists of a single polypeptide with a molecular weight of 40 kDa, has a pH optimum of 6.5 and is extremely unstable with an almost complete loss of activity occuring during storage at -20 °C.

4.4. Formaldehyde Dehydrogenase

Formaldehyde dehydrogenase (EC 1.2.1.46) will accept methylglyoxal as a substrate although it will not accept acetaldehyde as a substrate (Schutte *et al.*, 1982). The enzyme has a critical requirement for glutathione (GSH) as a cofactor and is believed to act upon the hemithioacetals of formaldehyde and methylglyoxal. Oxidation by NAD^+

produces S-formylglutathione and S-pyruvoylglutathione, respectively. S-Formylglutathione can be hydrolyzed by S-formylglutathione hydrolase (EC 3.1.2.12) to produce formic acid and regenerate glutathione. However it is believed that S-formylglutathione hydrolase does not hydrolyze S-pyruvoyl-glutathione (Neben, *et al.*, 1980; Uotila and Koivusalo, 1974).

5. Glyoxalase I and Glyoxalase II

It would appear during the course of normal cellular metabolic activity that methylglyoxal is produced endogenously and that methylglyoxal is specifically transformed into S-*D*-lactoylglutathione by glyoxalase I. Glyoxalase I (S-*D*-lactoylglutathione methylglyoxal lyase (isomerising), EC 4.4.1.5), which is believed to be a Zn^{2+}-dependent metalloprotein (Thornalley, 1995), catalyzes the intramolecular disproportionation of nonenzymatically formed hemithioacetals of GSH and α-ketoaldehydes into α-hydroxythioesters.The second enzyme of the system, glyoxalase II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), hydrolyzes thioesters to α-hydroxycarboxylic acids with the consequence that GSH is regenerated. Thus when methylglyoxal is undergoing transformation by the glyoxalase system, it is converted first to S-*D*-lactoylglutathione by glyoxalase I and this compound then undergoes hydrolysis by glyoxalase II to yield GSH and *D*-lactic acid (Figure 6).

The role of *D*-lactate in mammals in unknown. However, the conversion of *D*-lactate to pyruvate by the membrane-bound enzyme *D*-lactate dehydrogenase stimulates transport of several amino acids (Kaback and Milner, 1970; Kaback, 1974) in *E. coli* ML 308-225. The uptake of alanine, aspartic and glutamic acid, glycine, lysine, proline, serine and tryptophan were greatly increased by the presence of *D*-lactic acid in isolated membrane preparations. Histidine-, isoleucine-, leucine-, phenylalanine-, tyrosine- and valine-uptake were also found to be increased in the presence of *D*-lactate but only a two-fold increase in the uptake of cysteine, cystine, methionine and arginine was noted (Kaback and Milner, 1970).

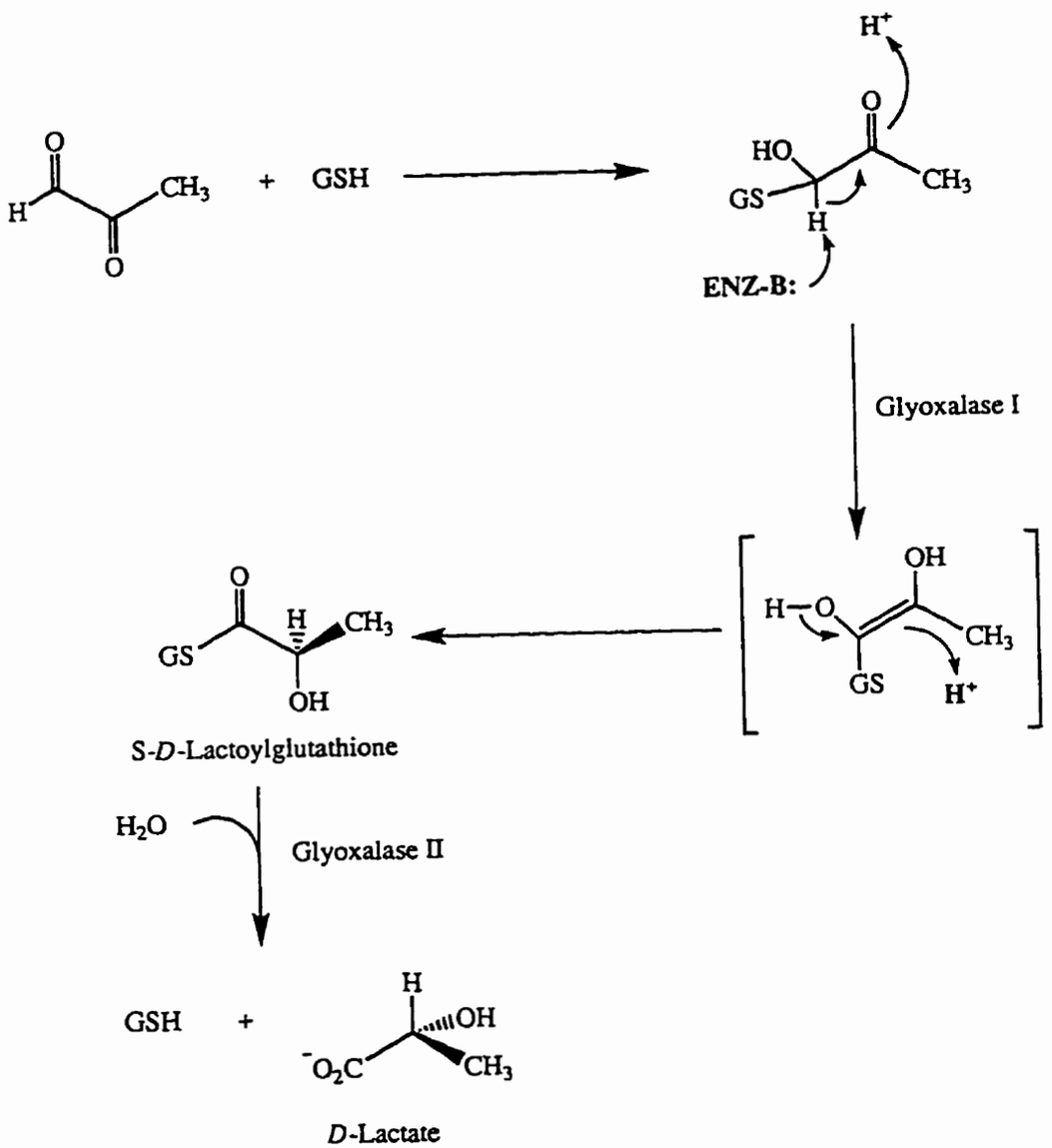

Figure 6. Products of catalysis of glyoxalase I and glyoxalase II.

5.1 Function and Distribution

A number of biological roles for the glyoxalase system have been proposed. It has been postulated that the enzyme system may help control/regulate cell growth and cell division (Stern, 1956), protect mammals from bacteria in the gastrointestinal tract (Aronsson and Mannervik, 1977), and/or may be involved in threonine catabolism (Elliot, 1959; Urata and Granick, 1963; Ray and ray, 1987), heme biosynthesis (Stafforini *et al.*, 1985) and MG detoxification (French and Freedlander, 1958; Riddle and Lorez, 1973).

It is believed that the enzyme is ubiquitous and has been identified and isolated from a number of organisms including bacteria, algae, conifers, yeast, fungi, invertebrates, reptiles, fish, birds and mammals (Widmann, 1929; Still, 1941; Hopkins and Morgan, 1945). Glyoxalase I activity is found to be highest in brain, kidneys and liver but all tissues would appear to possess glyoxalase I (Hopkins and Morgan, 1945; Marmstal *et al.*, 1979). Glyoxalase II has been purified from a number of mammalian sources (Jerzykowski *et al.*, 1978; Ball and Vander Jagt, 1980; Oray and Norton, 1980; Principato *et al.*, 1987) but extensive characterization of this particular enzyme from nonmammalian sources has not yet been attempted (Murat *et al.*, 1986; Talesa *et al.*, 1990; Inoue and Kimura, 1992).

5.2 Isolation and Purification of Glyoxalase I

Glyoxalase I has been purified from a variety of sources including yeast (Marmstal *et al.*, 1979; Vander Jagt and Han, 1973; Murata *et al.*, 1985c; Murata *et al.*, 1988; Inoue *et al.*, 1990; Caccuri *et al.*, 1993), human (Schimandle and Vander Jagt, 1979; Aronsson *et al.*, 1979; Allen *et al.*, 1993; Kim *et al.*, 1993; Ranganathan *et al.*, 1993; Ranganathan and Tew, 1993; Kim *et al.*, 1995), rat (Han *et al.*, 1976; Marmstal and Mannervik, 1979; Mannervik *et al.*, 1981), mouse (Oray and Norton, 1977), sheep (Uotila and Koivusalo, 1975), pig (Mannervik *et al.*, 1972; Aronsson and Mannervik, 1977), rabbit (Elango *et al.*, 1978) and pea (Ramaswany *et al.*, 1983). Vander Jagt *et al.*, have reported only a partial purification of the enzyme from *E. coli* (Vander Jagt, 1975) with little characterization of the partially purified enzyme.

5.2.1 Isolation of Yeast Glyoxalase I

The original purification protocol for glyoxalase I from *Saccharomyces cerevisiae* involved the use of acetone, ethanol and ammonium sulfate precipitation (Marmstal *et al.*, 1979). Glyoxalase I from this source is commercially available from Sigma Chemicals in a variety of grades and it is possible to further purify the commercially available enzyme using S-hexylglutathione-Sepharose 6B affinity column chromatography (Vander Jagt and Han, 1973).

A more recent purification scheme was developed by Caccuri *et al.* Purification was achieved by utilizing ethanol/chloroform and acetone precipitations, DEAE-52, QAE-A50, S-hexylglutathione Sepharose-6B and Sephadex G-100 columns (Caccuri *et al.*, 1993). The specific activity of the enzyme was determined to be approximately 3 500 µmol/min/mg (Caccuri *et al.*, 1993).

While gene cloning of yeast glyoxalase I has yet to be published it is know that there exists a gene which is capable of enhancing yeast glyoxalase I activity (Murata *et al.*, 1988); Inoue *et al.*, 1990). Glyoxalase I activity in yeast cells possessing this gene demonstrated 5-fold increased activity as compared to control cells lacking the gene (Murata *et al.*, 1988). The gene encodes for a large peptide of 106 amino acids (Inoue *et al.*, 1990). How this peptide interacts to activate yeast glyoxalase I is currently unknown.

5.2.2 Isolation of Human Glyoxalase I

The human enzyme has been found to exist in all tissues (Larsen *et al.*, 1975) and purification studies on human erythrocyte glyoxalase I are extensive (Aronsson et al., 1979; Allen *et al.*, 1993). Two alleles for glyoxalase I give rise to three forms of the enzyme (α_2,β_2, and $\alpha\beta$) which appear to be kinetically identical (Bagster and Parr, 1975; Kompf

et al., 1975; Meo *et al.*, 1977).

Isolation of glyoxalase I from human red blood cells has been performed by Allen and coworkers (Allen *et al.*, 1993). Purification was achieved utilizing chloroform/butanol denaturation of cells, acetone and ammonium sulfate precipitation steps and S-hexylglutathione-Sepharose 4B and Sephadex G75 column chromatographies (Allen *et al.*, 1993). The purified enzyme was found to be homodimeric with molecular weight of 46 kDa. The isoelctric pH was determined to be 5.1 and the enzyme obeyed Michaelis-Menten kinetics for MG-GSH hemimercaptal as a substrate in the range 21-817 µM (Allen *et al.*, 1993).

Human cDNA clones encoding for glyoxalase I have been isolated from an U937 cDNA library (Kim *et al.*, 1993). The cDNA fragment encoding for human glyoxalase I was subcloned into pUC19 and the resultant plasmid (pMGLOI) transformed into *E. coli* cells and screened for MG (5 mM) resistance (Kim *et al.*, 1993). The transformants containing pMGLOI acquired MG resistance whereas cells lacking the plasmid were not viable in th epresence of 5 mM MG. Lysates of *E. coli*/pMGLOI showed 110-fold higher activity (2.2 µmol/min/mg) than lysates lacking the plasmid (0.02 µmol/min/mg). The cDNA encoded for a protein of 184 amino acids (molecular weight of 20 719 Da). Research by Ranganathan and coworkers

on cDNA clones encoding for human colon glyoxalase I yielded an identical amino acid sequence to that reported by Kim and coworkers (Ranganathan *et al.*, 1993; Kim *et al.*, 1993).

A recent study concerning cDNA cloning and characterization of human glyoxalase I from fibrosarcoma HT-1080 cells yielded two different cDNA clones (Kim *et al.*, 1995). The two clones are nearly identical except for a substitution at position 111 of the amino acid sequence. The first clone with an alanine at amino acid position 111 is designated pMGLOI-A and the second clone with glutamic acid at amino acid position 111 is designated pMGLOI-E (Kim *et al.*, 1995). Lysates of HT-1080 cells yielded 3 distinct bands containing glyoxalase I activity which is indicative of the presence of 2 homodimers (GLO-A/GLO-A and GLO-E/GLO-E) and one heterodimer (GLO-A/GLO-E) (Kim *et al.*, 1995).

Glyoxalase I purified from human colon cancer cells and normal human colon cells yielded differences in enzyme specific activities. Normal human colon glyoxalase I had a specific activity of 31 µmol/min/mg while the specific activity of human colon cancer glyoxalase I was 54 µmol/min/mg (Ranganathan and Tew, 1993).

5.2.3 Isolation of Rat Glyoxalase I

Purification from rat liver was achieved by using ammonium sulfate and chloroform/ethanol precipitation steps, CM-Sephadex C-50, Sephadex G-100 and DEAE-Sephadex A-50 (Han *et al.*, 1976). Purification of the enzyme from rat erythrocytes was achieved by using chloroform/ethanol fractionation, blue-dextran affinity chromatography and hydroxyapatite chromatography (Marmstal and Mannervik, 1979).

5.2.4 Isolation of *E. coli* Glyoxalase I

Remarkably little is known about glyoxalase I from *E. coli*. Vander Jagt has partially purified the enzyme from *E. coli* K-12 using ammonium sulfate fractionation, CM-Sephadex and Sephadex G-100 chromatography (Vander Jagt, 1975). The enzyme from K-12 was found to be unstable at all stages of purification and very little characterization was performed on this enzyme.

5.2.5 Isolation of *Pseudomonas putida* Glyoxalase I

Bacterial glyoxalase I from *P. putida* was originally purified and studied by Rhee and co-workers (Rhee *et al.*, 1986). The isolation procedure involved DEAE-cellulose, butyltoyopearl 650 M hydrophobic chromatography and S-hexylglutathione agarose affinity chromatography and Sephadex G-150 chromatography.

Molecular cloning of *P. putida* glyoxalase I gene in *E. coli* has been performed by Rhee and coworkers (Rhee *et al.*, 1987). The glyoxalase I gene from P. putida was cloned into the vector pBR322 (BamH I site) as a Sau3A I chromosomal DNA fragment and the plasmid designated as pGI318. This plasmid was then subjected to Hind III restriction enzyme digestion and then ligated to generate the plasmid pGI423. This plasmid was then introduced into *E. coli* C600 cells and screened for resistance to MG (1.2 mM) (Rhee *et al.*, 1987). Approximately 150-fold increae in glyoxalase I activity was observed in *E. coli* C600 cells containing pGI423.

The gene encoding for glyoxalase I from *P. putida* has been cloned into the expression vector pBTacI and the plasmid transformed into *E. coli* JM109 cells (Lu *et al.*, 1994). Induction by isopropylthio-galactoside resulted in 4 000-fold higher expression than JM109 cells lacking the plasmid (Lu *et al.*, 1994). The open reading frame encodes for a protein of 173 amino acids (molecular weight of 19 526 Da). The N-terminal methionine is lost after translation to yield a protein of 19 407 Da. The K_m for *P. putida* glyoxalase I was determined to be 390 μM for the MG-GSH hemimercaptal (50 mM potassium phosphate, pH 7.0) (Lu *et al.*, 1994).

Table 1. Comparison of glyoxalase I from various sources.

Source	MW (kDa)	Specific Activity[a] (μmol/min/mg)	Number of Subunits
Yeast	32-35[b, c]	1 200[b]	1[b]
Human Erythrocyte	44-46[d, e, f]	1 020[e]	2[e]
Rat Erythrocyte	54[g]	950[g]	2[g]
Pig Erythrocyte	48[h]	957[h]	2[h]
Rat Liver Hepatocyte	46[d]	950[g]	2[d]
Sheep Liver Hepatocyte	49.5[i]	4 000[i]	2[i]
P. putida	20[j]	360[j]	1[j]

a. Conversion of hemimercaptal of MG-GSH to S-*D*-lactoylglutathione; b. Marmstal *et al.*, 1979; c. Vander Jagt and Han, 1973; d. Schimandle and Vander Jagt, 1979; e. Aronsson *et al.*, 1979; f. Allen *et al.*, 1993; g. Marmstal and Mannervik, 1979; h. Aronsson and Mannervik, 1977; i. Uotila and Koivuslao, 1975; j. Rhee *et al.*, 1986

5.3 α-Ketoaldehyde Substrate Specificity

Glyoxalase I will act upon hemimercaptals generated from a number of structurally differing α-ketoaldehydes. However α-ketoaldehydes which possess severe steric hinderance tend not be acceptable as substrates. Enzymes such as glutathione S-transferases (Monks *et al.*, 1985; Stevens *et al.*, 1986; Monks and Lau, 1987), cytochrome P-450 (Wiseman and Woods, 1979; Wislocki *et al.*, 1980; Woods and Wiseman, 1980; King *et al.*, 1984; Schuetz and Guzelian, 1984; Azari and Wiseman, 1990) and epoxide hydrolase (Oesch, 1980; Barman, 1987) demonstrate such expansive specificities for substrates. This has led to the hypothesis that the glyoxalase enzymes from various sources serve as detoxification enzymes.

Table 2. α-Ketoaldehydes that can be utilized by glyoxalase I from various sources[a].

Aliphatic α-ketoaldehyde	Aromatic α-ketoaldehyde
Glyoxal	Phenylglyoxal
Methylglyoxal	*p*-Chlorophenylglyoxal
Hydroxypyruvaldehyde	*p*-Hydroxyphenylglyoxal
Phosphohydroxypyruvaldehyde	*p*-Bromophenylglyoxal
4,5-Dioxovalerate	*p*-Methylphenylglyoxal
3-Ethoxy-2-oxobutyraldehyde	*p*-Methoxyphenylglyoxal
t-Butylglyoxal	*m*-Methoxyphenylglyoxal
Fluoromethylglyoxal	*p*-Nitrophenylglyoxal
Glutathiomethylglyoxal	2,4-Dimethylphenylglyoxal
	2,4,6-Trimethylphenylglyoxal

a. References: Hopkins and Morgan, 1948; Racker, 1951; Weaver and Hardy, 1961; Han et al., 1976; Vander Jagt, 1975; Vander Jagt et al., 1974; Vander Jagt *et al.*, 1975; Kozarich *et al.*, 1981; Kozarich *et al.*, 1982.

5.4 Cosubstrate Specificity

Glutathione is the natural endogenous cosubstrate for glyoxalase I (Lohmann, 1932). Other glutathione analogues, regardless of whether they are naturally occuring or produced synthetically, are not as effective as cosubstrates when compared to GSH (Carnegie, 1963).

Table 3. Cosubstrate specificity for glyoxalase I from various sources.

Compound	Effect
Glutathione	Natural cosubstrate[a]
γ-Glutaminylcysteinyl-β-alanine	Active[b]
α-Glutamylcysteinylglycine	Active[c]
β-Aspartylcysteinylglycine	Active[c]
γ-*D*-Glutamylcysteinylglycine	Active[d]
N-*D,L*-AlanylGSH	Active[d]
N-*D,L*-ValylGSH	Active[d]
N-AcetylGSH	Active[e]
Cysteinylglycine	Inactive[c]
γ-Glutamylcysteine	Inactive[f]
Cysteine	Inactive[g]
γ-Glutamyl-*L*-allothiothreonylglycine	Inactive[h]
γ-Glutamyl-*L*-thiothreonylglycine	Inactive[h]
GSH dimethyl ester	Inactive[e]
GSH glycyl methyl ester	Active[i]

References: a. Lohmann, 1932; b. Carnegie, 1963; c. Behrers, 1941; d. Wieland *et al.*, 1956; e. Vander Jagt and Han, 1973; f. Kermack and Mattheson, 1957; g. Woodward and Reinhardt, 1942; h. Xie and Creighton, 1991; i. Hamilton and Creighton, 1992.

Acetylation of the α-amino group of GSH altered K_m whereas blocking of the GSH carboxylates rendered the molecule ineffective as a cosubstrate (Vander Jagt and Han, 1973). Methyl and ethyl esterification of the glycyl carboxyl group generated esters which were moderately active as substrates of yeast glyoxalase I (Hamilton and Creighton, 1992).

5.5. Glyoxalase I: Kinetics and Mechanism of Action

The nonenzymatic reaction between GSH and α-ketoaldehydes generates an equilibrium with two hemithioacetal diastereomers. Given that S-*D*-lactoylglutathione is formed with high optical purity (>95%) (Han *et al.*, 1977; Azari and Wiseman, 1990) and on the basis of NMR studies (Brown *et al.*, 1981) it would be logical to conclude that only one of the diastereomeric pair would be acceptable as a substrate. Isotopic trapping experiments by Griffis *et al.* demonstrated nonstereospecific substrate usage by the enzyme (Griffis *et al.*, 1983). Recent experiments concerning yeast glyoxalase I have indicated that the enzyme utilizes both diastereomers of the methylglyoxal/GSH hemimercaptal indiscriminately but prefered only one diastereomer of the phenylglyoxal/GSH hemimercaptal (Rae *et al.*, 1994). If the enzyme does not discriminate between diastereomeric pairs then a situation similar to the ability of phosphoglucose isomerase to utilize both α-and β-anomers of glucose-6-phosphate comes to mind (Rose, 1975). Kinetic analysis of the turnover rate for glyoxalase I indicates that the rate constant approaches values for a diffusion-controlled reaction. Catalytic efficiencies for various α-ketoaldehyde substrates varies between 10^6-10^7 $M^{-1} \cdot s^{-1}$ (Table 4) indicating that the enzyme is efficient. These values approach those determined for other enzymes such as acetylcholinesterase, crotonase, fumarase, triosephosphate isomerase

($1.6x10^8$, $2.8x10^8$, $1.6x10^8$ and $2.4x10^8$ $M^{-1}\cdot s^{-1}$, respectively) in which k_{cat}/K_m is close to the diffusion controlled association rate (Zubay, 1988).

Table 4. Kinetic parameters for various α-ketoladehydes with yeast glyoxalase I.

α-Ketoaldehyde	K_m (10^{-4} M)[a]	k_{cat}/K_m (10^6 $M^{-1}\cdot s^{-1}$)[b]
Methylglyoxal	3	3.5
Hydroxypyruvaldehyde	8	0.57
t-Butylglyoxal	2	0.0015
Phenylglyoxal	2	4.8
p-Methylphenylglyoxal	0.4	7.2
p-Methoxyphenylglyoxal	0.4	6.3
p-Bromophenylglyoxal	0.3	16.2
m-Methoxyphenylglyoxal	0.6	15.3
p-Nitrophenylglyoxal	0.9	8.3
2,4-Dimethylphenylglyoxal	0.4	4.2
p-Hydroxyphenylglyoxal	0.7	4.7

a. K_m for yeast glyoxalase I, 25 °C, pH 7.0.; references: Vander Jagt *et al.*, 1972 and Vander Jagt *et al.*, 1975; b. reference: Vander Jagt and Han, 1973.

Two proposals for the mechanism of glyoxalase I catalysis have been hypothesized: intramolecular hydride shift versus enediol(ate) formation (Figure 7). The intramolecular hydride shift hypothesis had

been accepted until the mid-1970s. Lack of tritium incorporation into *D*-lactate supported the hydride shift mechanism (Rose, 1957).

If a protected basic amino acid is involved in proton abstraction then lack of solvent exchange may incorrectly favor a hydride shift hypothesis. More recent studies involving NMR experiments indicate that catalysis may occur via an enediol(ate) intermediate (Hall *et al.*, 1976). Isotope exchange studies demonstrated that low level incorporation of deuterium from D_2O into *D*-lactate is temperature-dependent (15% at 22 °C, 22% at 35 °C) (Hall *et al.*, 1976) and these experiments seem to favor the protected base argument. Experiments with flavins also seem to favor the enediol(ate) hypothesis as enediols are more efficiently trapped than are hydrides (Shinkai *et al.*, 1970; Shinkai *et al.*, 1974; Hemmerich *et al.*, 1977). Flavin trapping experiments with 3-methyl-tetra-O-acetylriboflavin on glyoxalase I and the hemithioacetal of phenylglyoxal resulted in the thiol ester of benzoylformate being formed as opposed to the thiol ester of mandelate being formed (Shinkai *et al.*, 1981; Ueda *et al.*, 1984; Douglas *et al.*, 1985).

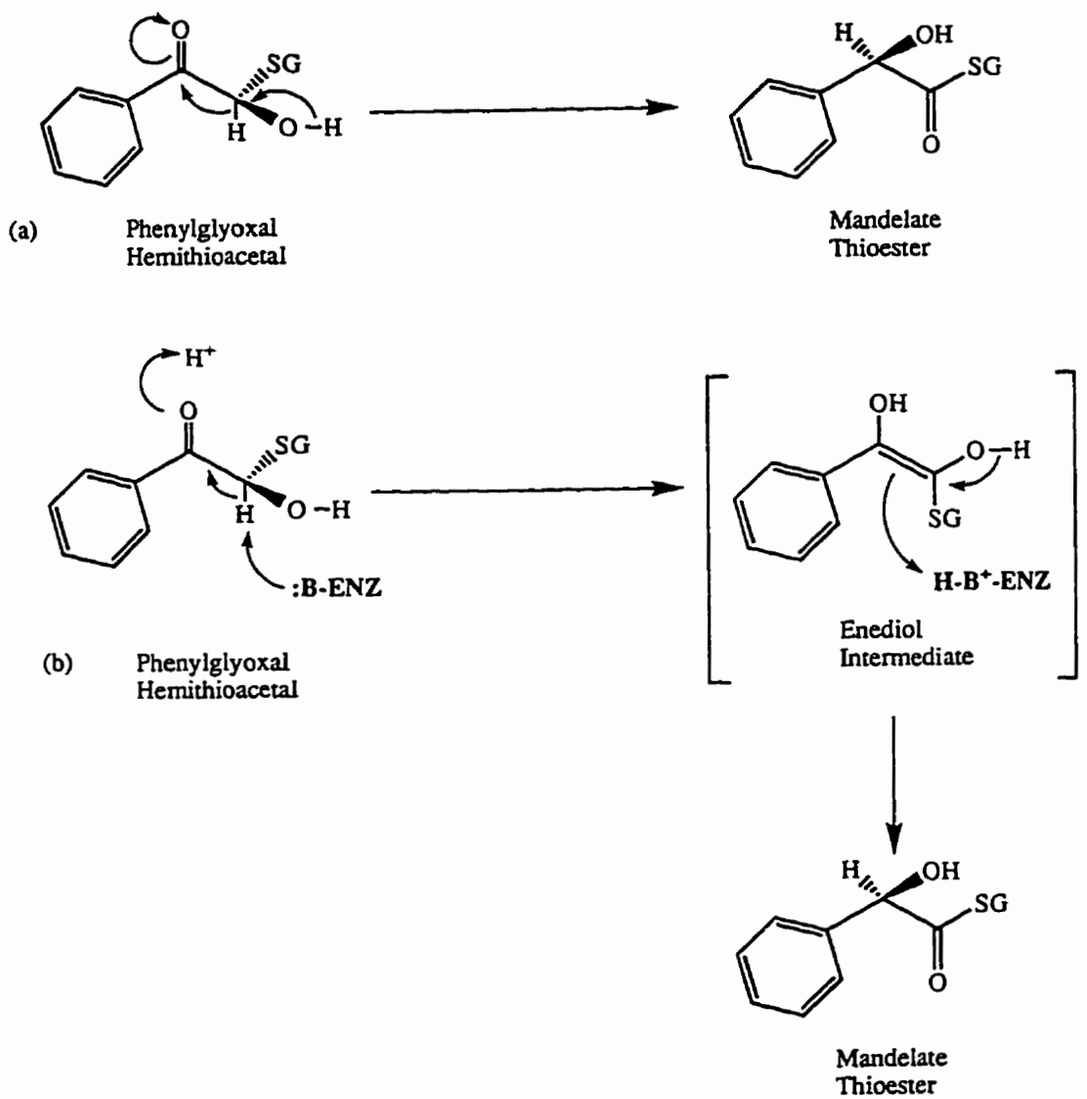

Figure 7. Intramolecular hydride shift (a) versus cis-enediolate mechanism (b) for glyoxalase I catalysis of the hemithioacetal of phenylglyoxal.

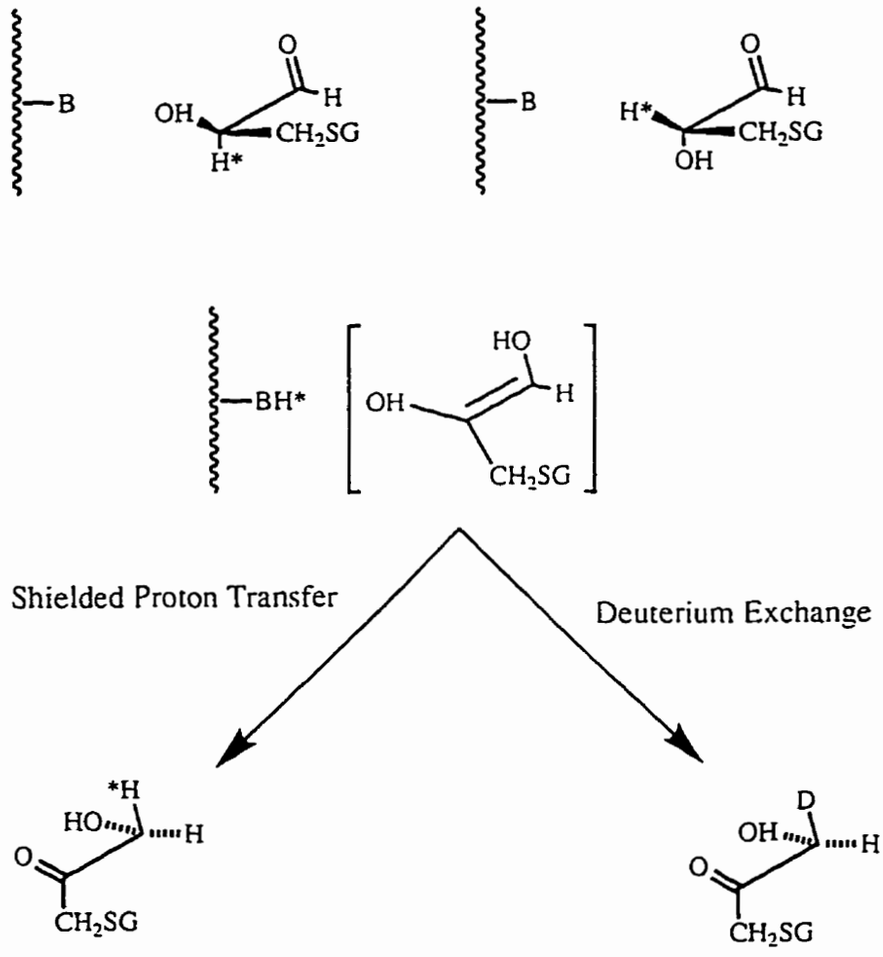

Figure 8. Mechanism of action of human glyoxalase I upon (R)- and (S)-glutathiolactaldehyde.

Landro and coworkers have performed NMR experiments on stereochemically "locked" analogues of the diastereomeric hemimercaptals of MG and GSH (Landro *et al.*, 1992). Both (R)- and (S)-glutathio-lactaldehyde (Figure 8) were capable of being converted to glutathiohydroxyacetone by human glyoxalase I at a rate of 0.8 s^{-1} and 0.4 s^{-1}, respectively (Landro *et al.*, 1992). It was possible to measure the rate of disappearance of the hydrated aldehyde by proton nuclear magnetic resonance (^1H NMR)(resonance measured at 4.85 ppm) and the appearance of glutathiohydroxyacetone (the "expected" product of glyoxalase I catalysis) (resonance measured at 4.39 ppm). When both (R)- and (S)-glutathiolactaldehyde were exposed to glyoxalase I in deuterated phosphate buffer an AB quartet was observed at 4.39 ppm. Also observed was the formation of a single broadened triplet 0.015 ppm upfield from the AB quartet. This triplet was observed regardless if either (R)- or (S)-glutathiolactaldehyde was used as a substrate (Landro *et al.*, 1992). Proton transfer was observed during the isomerization of both analogues to yield deuterium incorporation into the *pro*-S position (Landro *et al.*, 1992). Based upon previous work by Chari and Kozarich, this triplet was assigned to the pro-*R* proton of glutathiohydroxyacetone coupled to deuterium (Chari and Kozarich, 1983). These results would seem to indicate that glyoxalase I nonstereospecifically abstracts a proton from the hemimercaptal of MG and GSH but proton addition is stereospecific to

yield S-*D*-lactoylglutathione.

5.6 Glyoxalase I: Metal Ion Requirement

Exposing glyoxalase I from various sources to ethylenediamine tetraacetic acid (EDTA) often results in the rapid inactivation of the enzyme indicating that a metal cofactor is necessary for catalysis or is necessary for maintaining the structural integrity of the enzyme.

Approximately 30 years ago it was believed that glyoxalase I was a Mg^{2+}-dependent metalloprotein due to its efficient activation by Mg^{2+} (Davis and Williams, 1966). Work by Aronsson *et al.*, in the late 1970's overturned this hypothesis with research that indicated that both human and yeast glyoxalase I were Zn^{2+}-dependent proteins with each subunit containing one Zn^{2+} (Aronsson *et al.*, 1978). Data generated concerning metal replacement studies has been accomplished with the human erythrocyte enzyme but not the yeast enzyme. The human enzyme will regain one-half of its initial velocity upon reconstitution with Zn^{2+} whereas the yeast enzyme cannot be reactivated after metal depletion (Aronsson *et al.*, 1978). Table 5 indicates that the small dissociation constant for Zn^{2+} for human erythrocyte glyoxalase I indicates Zn^{2+} to be the prefered metal ion (Sellin and Mannervik, 1983).

From Table 6 it can be seen that Zn^{2+} and Mg^{2+} are nearly equally as effective at activating the enzyme for the enzymatic conversion of methylglyoxal. Other studies indicate that Cd^{2+}, Ba^{2+}, Fe^{2+}, Ca^{2+} and Hg^{2+} are incapable of activating the enzyme (Uotila and Koisuvalo, 1975). Apoglyoxalase I from human erythrocytes and calf liver is exceptionally versatile and can be reconstituted with Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+} (Davis and Williams, 1966; Uotila and Koisuvalo, 1975; Han *et al.*, 1977; Aronsson *et al.*, 1978; Sellin *et al.*, 1983; Sellin and Mannervik, 1984), V^{2+} (Vander Jagt and Topscott, 1978) and Ga^{3+} (Vander Jagt *et al.*, 1980).

Table 5. Metal ion dissociation constants for human erythrocyte glyoxalase I[a].

Divalent Metal Cation	Dissociation Constant
Zn^{2+}	2.7×10^{-11} M
Co^{2+}	3.06×10^{-10} M
Mn^{2+}	4.9×10^{-9} M
Mg^{2+}	7.0×10^{-6} M

a. Reference: Sellin and Mannervik, 1983.

Fluorescence quenching experiments by Han *et al.*, have indicated that the metal ion resides near the active site (Han *et al.*, 1977). Dansylation of rat erythrocyte apoglyoxalase I followed by reintroduction of Mn^{2+} resulted in fluorescence quenching. Further work with human erythrocyte Zn^{2+}-, Mg^{2+}- and Co^{2+}-glyoxalase I has demonstrated that they had no effevt on k_{cat} for the conversion of methylglyoxal. A pronounced sensitivity in k_{cat} by Mg^{2+}-, Mn^{2+}-, Co^{2+}- and Ni^{2+}-glyoxalase I for phenylglyoxal is apparent (Uotila and Koisuvalo, 1975; Vander Jagt and Topscott; Vander Jagt *et al.*, 1980).

Spectroscopic studies on human erythrocyte glyoxalase I support the hypothesis that the metal ion is far enough removed from the substrate such that direct coordination is not possible. Work on Mn^{2+}-glyoxalase I and electron paramagnetic resonance (EPR) and visible absorption studies on Co^{2+}-glyoxalase I give credence to the idea that coordination is second sphere through one or two water molecules (Sellin *et al.*, 1982a; Sellin *et al.*, 1982b).Extended x-ray absorption fine structure (EXAFS) studies for human Zn^{2+}-glyoxalase I have indicated that enzyme-Zn^{2+} coordination is in a distorted octahedral or hepta-coordination geometry (Garcia-Iniquez *et al.*, 1984).

Table 6. Activation of human glyoxalase I by various metal ions for various substrates[a].

	Relative k_{cat} for M^{n+}-glyoxalase I						
α-Ketoaldehyde	Zn^{2+}	Mg^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	V^{2+}	Ga^{3+}
Methylglyoxal	1.0	1.2	0.6	1.0	0.5	0.3	0.5
Phenylglyoxal	-	0.6	0.04	0.8	1.5	-	-
p-Chloro-phenylglyoxal	-	0.8	0.2	3.2	3.5	-	-
p-Methoxy-phenylglyoxal	-	0.4	0.02	0.4	1.5	-	-

a. k_{cat} values relative to Zn^{2+}-glyoxalase I with methylglyoxal (Uotila and Koisuvalo, 1975; Vander Jagt and Topscott; Vander Jagt *et al.*, 1980).

Even with extensive metal-glyoxalase I studies the role of the metal ion in the process of catalysis has not been elucidated. It is known that k_{cat} values for the yeast enzyme with various α-ketoaldehydes are relatively similar which may indicate that the role of the metal is invariant (Vander Jagt *et al.*, 1972). The role of the metal in yeast glyoxalase I may be to polarize the ketone functionality of the hemithioacetal via an intervening water molecule (Figure 9). Changes in

k_{cat} for various metal-glyoxalases may be due to decreased or altered polarization of various α-ketoaldehydes.

5.7 Glyoxalase I: Active Site Studies

Amino group specific reagents such as 1-fluoro-2,4-dinitrobenzene, 5-dimethylaminonaphthalene-1-sulfonyl chloride and 2,4,6-trinitrobenzenesulfonate are capable of inactivating yeast and porcine erythrocyte glyoxalase I (Mannervik *et al.*, 1975). Protection against inactivation can be afforded by preincubating the enzyme with glutathione derivatives (Han *et al.*, 1977). The yeast enzyme is rendered inactive when exposed to 2,3-butanedione, phenylglyoxal and camphorquinone-10-sulfonic acid which are arginine-modifying reagents (Schasteen and Reed, 1983). Inactivation could be prevented by the presence of glutathione, substrate or S-*D*-lactoylglutathione (Schasteen and Reed, 1983). Loss of activity in the absence of protective molecules would seem to suggest that arginine(s) responsible for anionic binding are being modified. The tyrosine-modifying agent tetranitromethane, has also been found to inactivate yeast glyoxalase I (Carrington and Douglas, 1985). A known glyoxalase I inhibitor, S-(*p*-bromobenzyl)-glutathione, was found to reduce inactivation by tetranitromethane (Carrington and Douglas, 1985). Ethoxyformylation and photo-oxidation of

histidines in yeast glyoxalase I indicate that this amino acid may play a catalytic or structural role (Hall *et al.*, 1976; Jordan *et al.*, 1983).

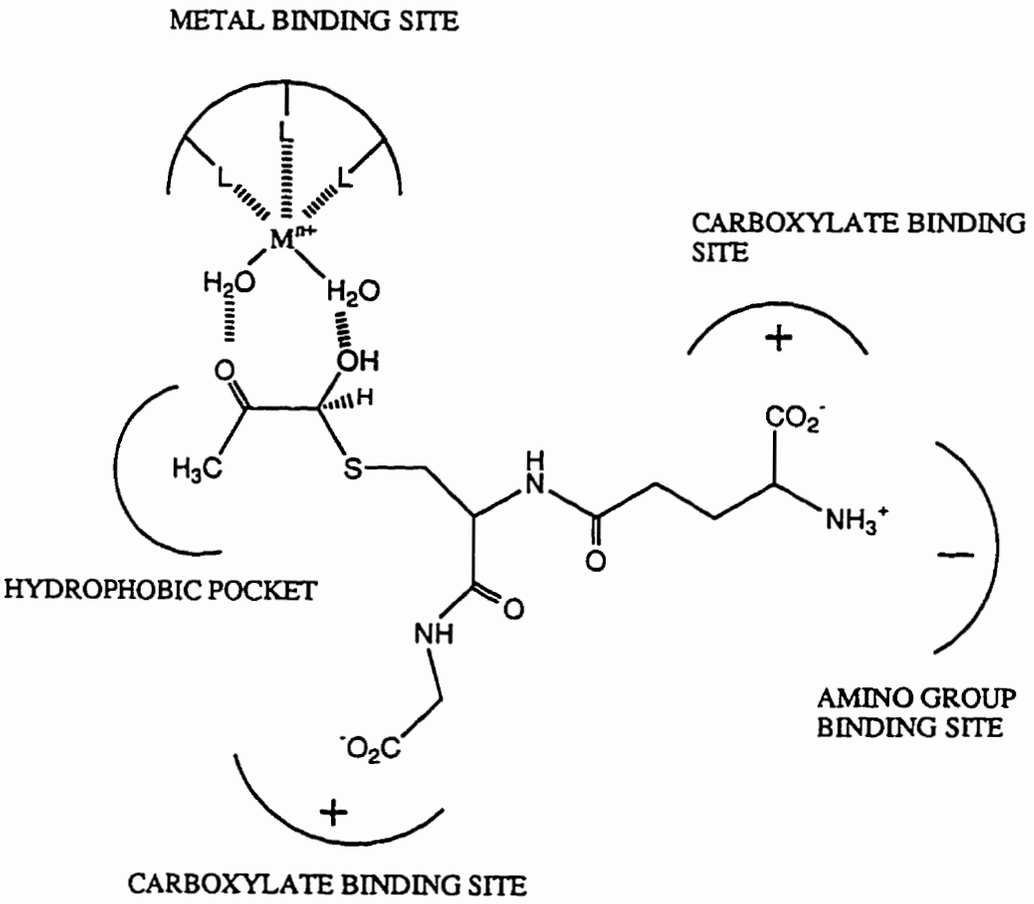

Figure 9. Hypothetical active site for glyoxalase I based on the model proposed by Carrington and Douglas (Carrington and Douglas, 1985).

6. Glyoxalase II: Kinetics and Mechanism of Action

From Table 7 it can be seen that glyoxalase II recognizes and preferentially binds thioesters of glutathione but does not appear to recognize coenzyme A and thioglycolate thioesters nor oxygen esters (Uotila, 1973). The presence of a C-2 hydroxyl is not an absolute requirement for glyoxalase II catalysis to occur perhaps indicating that glyoxalase II is not strictly specific for the products of catalysis of glyoxalase I. Catalytic efficiency of glyoxalase II is on the order of 10^7 $M^{-1} \cdot s^{-1}$ which is approximately an order of magnitude less than that of acetylcholinesterase (Ball and Vander Jagt, 1981; Guha *et al.*, 1988).

Table 7. Kinetic parameters for various substrates for human liver glyoxalase II[a].

Substrate	K_m (μM)	V_{max} (relative)
S-Lactoylglutathione	190	100
S-Glycerylglutathione	109	62
S-Glycolylglutathione	70	39
S-Mandeloylglutathione	16	5
S-Acetoacetylglutathione	295	56
S-Succinylglutathione	153	29
S-Formylglutathione	153	38
S-Acetylglutathione	266	9
S-Propionylglutathione	213	14

a. Reference: Uotila, 1973.

Comparing the pH titration curves of the enzyme in H_2O with that in D_2O led to the conclusion that general acid-general base catalysis was not responsible for the enzyme's mechanism of action (Ball and Vander Jagt, 1981). Incubating diisopropylphosphofluoridate (serine-modifying) (Ball and Vander Jagt, 1981), phenylmethanesulfonyl fluoride (PMSF) (serine-modifying) (Douglas *et al.*, 1984), 5,5'-dithiobis-2-nitrobenzoic acid (cysteine-modifying) (Douglas *et al.*, 1984), 2-hydroxy-5-nitrobenzyl bromide (tryptophan-modifying) (Douglas *et al.*, 1984) and tetranitromethane (tyrosine-modifying) (Douglas *et al.*, 1984) with glyoxalase II did not lead to inactivation of the enzyme, these results appear to indicate that serine, cysteine, tryptophan and tyrosine are not crucial for catalysis whereas amino acid modification studies utilizing methylene blue and diethyl pyrocarbonate resulted in enzyme inactivation indicative of the presence of a crucial histidinyl(s) which may exist within the active site (Figure 10). Recently the overexpression of glyoxalase II by Ridderstrom and co-workers has been reported and the ready availability of substantial quantities of the enzyme for structural studies should eventually lead to increased mechanistic information on this enzyme (Ridderstrom *et al.*, 1996).

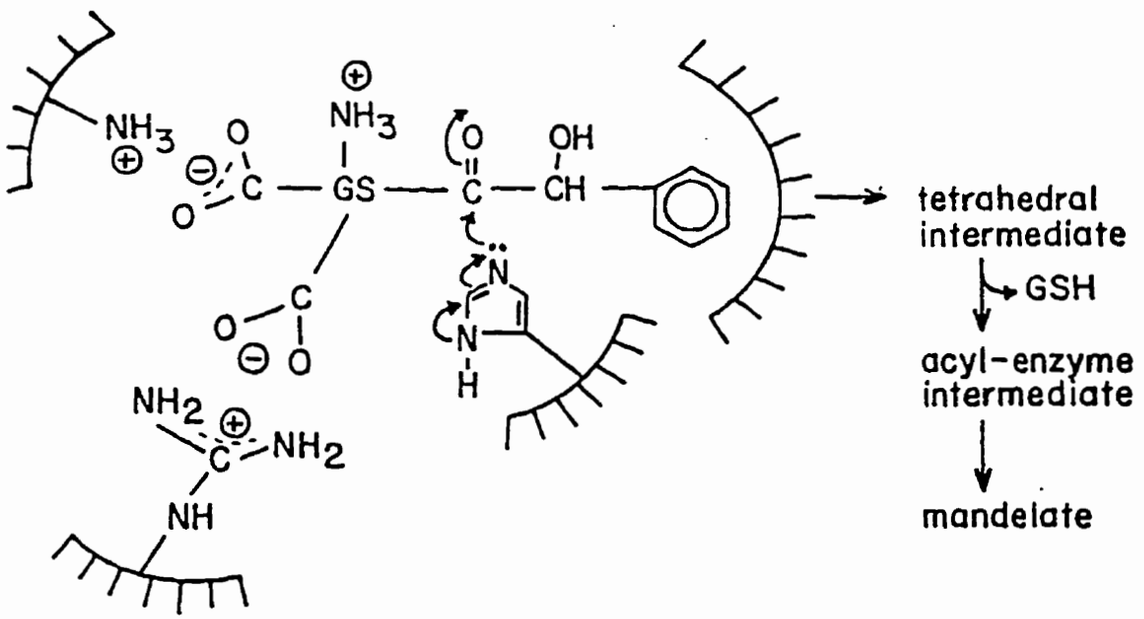

Figure 10. Hypothetical active site for glyoxalase II as proposed by Ball and Vander Jagt (Ball and Vander Jagt, 1981).

7. Glyoxalase III: A Novel Enzyme Capable of Direct Conversion of Methylglyoxal into *D*-Lactate

Recent work by Misra *et al.*, has shown that an enzyme capable of transforming methylglyoxal and phenylglyoxal into their respective α-hydroxycarboxylic acids without the requirement for glutathione exists in wild-type *E. coli* K-12 (Misra *et al.*, 1995). Substrate specificity studies indicated that only methylglyoxal and phenylglyoxal could be enzymatically transformed. Glyoxalase III was found to be an α_2 homodimer with each subunit exhibiting a molecular weight of 44 kDa. Optimal pH experiments did not indicate a sharp pH optima. Below pH 5.0 enzymatic activity decreased rapidly but between pH 6.0-8.0 enzyme activity was constant. Above pH 8.0 studies were performed using boric acid/sodium borate and sodium carbonate/sodium bicarbonate and the results showed a moderate drop in activity of 20-25% at pH 10.0. Glyoxalase III has been found to be sensitive to thiol-modifying reagents and may indicate that a crucial sulfhydryl is required for catalysis. This paper is the only known report on the existence of glyoxalase III from any organism.

8. Inhibition Studies

8.1 Inhibition Studies with Glyoxalase I from *Saccharomyces cerevisiae*

Previous studies from our laboratory and those of other researchers have demonstrated that glutathione-quinone conjugates (Barnard and Honek, 1989; Allen *et al.*, 1993; Barnard and Honek, 1994; Douglas and Keysworth, 1994) and troponoids (derivatives of cycloheptatrienone) (Barnard and Honek, 1994) are good inhibitors of yeast glyoxalase I. In addition, these compounds have also demonstrated good antimalarial activity (Barnard and Honek, 1994). Troponoids which were the most inhibitory to glyoxalase I contained a hydroxyl at the C-2 position of the troponoid ring. Based upon these results, syntheses of hydroxychavicol derivatives (Figure 11) were performed in the laboratory of Dr. Judy Bolton (Queen's University, Kingston, Ontario) and forwarded to us for testing against yeast glyoxalase I. None of the compounds tested were found to be good inhibitors of the enzyme against a GSH-MG hemimercaptal concentration of 1.6 mM (Table 8). Compounds such as catechol and resorcinol have been shown to be poor inhibitors of glyoxalase I (146) while compounds such as 2-glutathionyl-1,4-benzoquinone and 2,3-bis-glutathionyl-1,4-naphthoquinone are relatively good inhibitors of yeast glyoxalase I. Perhaps the positioning of glutathione on hydroxychavicol is disadvantageous for binding of these compounds to the enzyme. Compounds with hydroxyls adjacent to one

another typically tend to form strong intramolecular hydrogen bonds which may hinder interactions with the putative metal binding site of yeast glyoxalase I.

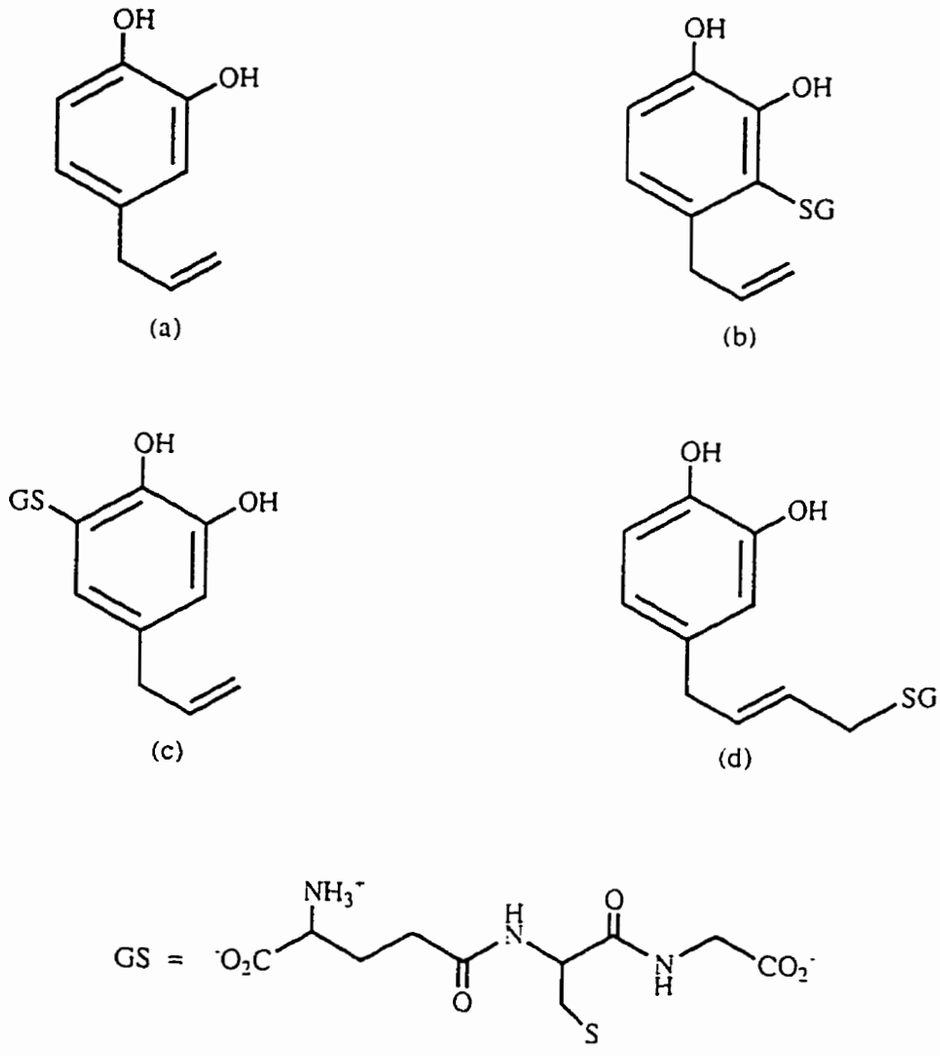

Figure 11. Structures of hydroxychavicol and glutathione adducts of hydroxychavicol; (a) hydroxychavicol; (b) 3-glutathionyl hydroxychavicol; (c) 6-glutathionyl hydroxychavicol; (d) glutathionyl-2-pyrogallyl-*trans*-but-2-ene.

Table 8. Inhibition levels (IC_{50}) for hydroxychavicol and analogues against yeast glyoxalase I[a].

Compound	IC_{50} (mM)
Hydroxychavicol	0.73
3-Glutathionyl hydroxychavicol	0.78
6-Glutathionyl hydroxychavicol	>1
Glutathionyl-2-pyrogallyl-*trans*-but-2-ene	>1

a. Assayed at 25 °C in 50 mM potassium phosphate, pH 6.6. A MG-GSH hemimercaptal concentration of 1.6 mM was used in the assay.

8.2 Inhibition Studies with Glyoxalase II from Bovine Liver

A number of glutathione analogues were previously tested for inhibitory activity against bovine liver glyoxalase II (Figure 12). Work by Hsu and Norton (hsu and Norton, 1983) and Bush and Norton (Bush and Norton, 1985) have shown that S-carbobenzoxy-*L*-glutathione and similar derivatives are good inhibitors of rat and human glyoxalase II. Under our assay conditons S-carbobenzoxy-*L*-glutathione was found to be a moderate inhibitor of the enzyme from bovine liver (Barnard and Honek, 1994). In order to examine electronic effects on the inhibition of glyoxalase II by S-carbobenzoxy-*L*-glutathione, several heteroatom substitutions (Figure 12) were tested and evaluated as potential inhibitors of glyoxalase II. All heteroatom substitutions resulted in

poor inhibitors of the enzyme (Table 9). The molecule S-(N-benzyl-carbamoyl)-*L*-glutathione caused only 20% inhibition at 1.17 mM concentration. Transitions from dithiocarbamoyl to carbamoyl to carbobenzoxy resulted in decreased double bond character from R-NH-CS-R' to R-NH-CO-R' to R-O-CO-R' (Sundstrom, 1967). A decrease in double bond character will result in increased rotational freedom which may allow S-carbobenzoxy-*L*-glutathione to "fit" better into the enzyme active site and may explain its inhibitory activity when compared to benzylthiocarbamoyl and benzylcarbamoyl analogues.

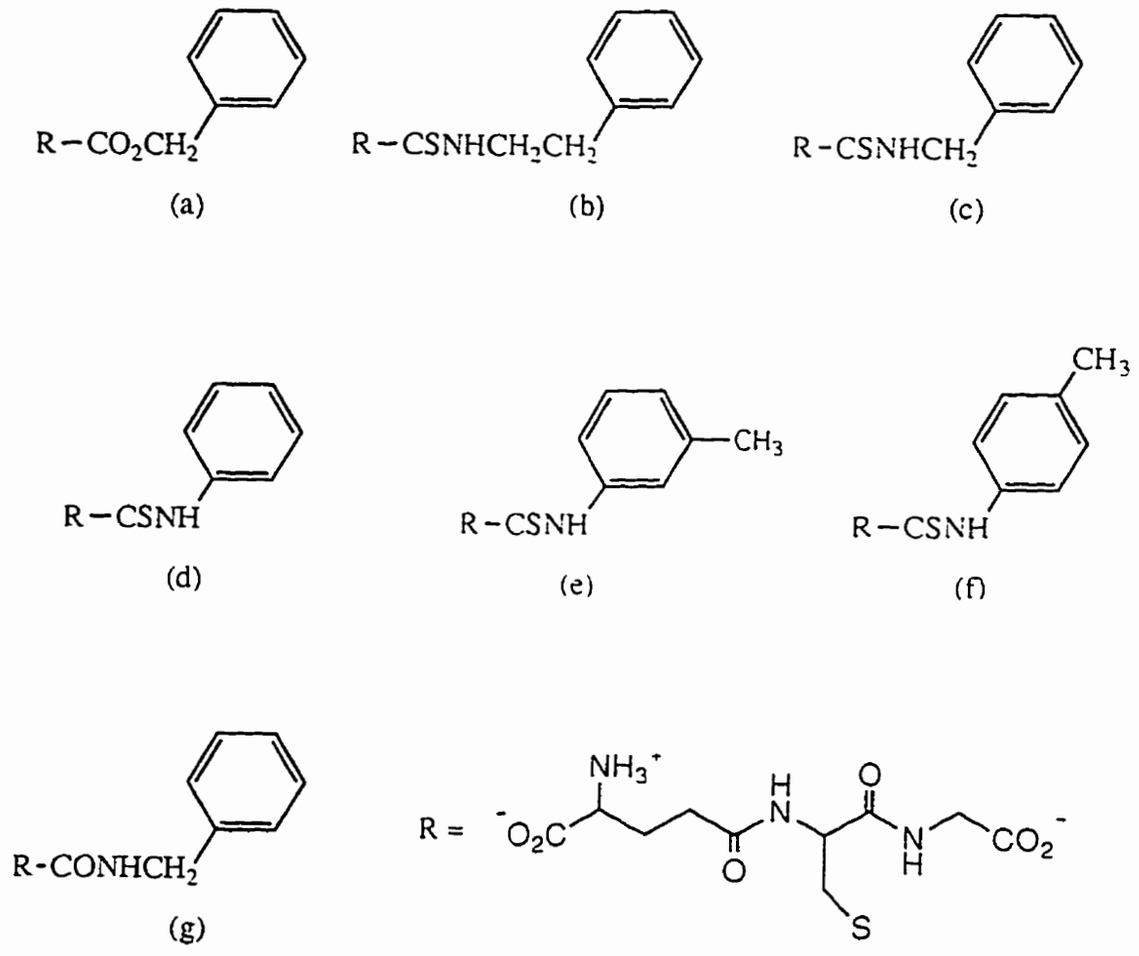

Figure 12. Analogues of S-carbobenzoxy-*L*-glutathione tested against bovine liver glyoxalase II (Barnard and Honek, 1994); (a) S-carbobenzoxy -*L*-glutathione; (b) S-(N-phenethylthiocarbamoyl)-*L*-glutathione; (c) S-(N-benzylthiocarbamoyl)-*L*-glutathione; (d) S-(N-phenylthiocarbamoyl)-*L*-glutathione; (e) S-(N-*m*-tolylthiocarbamoyl)-*L*-glutathione; (f) S-(N-*p*-tolylthiocarbamoyl)-*L*-glutathione; (g) S-(N-benzylcarbamoyl)-*L*-glutathione.

Table 9. Glutathione analogues as inhibitors of bovine glyoxalase II (Barnard and Honek, 1994).

Compound	IC_{50} (μM)[a]
S-Carbobenzoxy-*L*-glutathione	180
S-(N-Phenethylthiocarbamoyl)-*L*-glutathione	NI[b]/1.59 mM
S-(N-Benzylthiocarbamoyl)-*L*-glutathione	NI/1.64 mM
S-(N-Phenylthiocarbamoyl)-*L*-glutathione	NI/1.70 mM
S-(N-*m*-Tolylthiocarbamoyl)-*L*-glutathione	NI/1.64 mM
S-(N-*p*-Tolylthiocarbamoyl)-*L*-glutathione	NI/1.64 mM
S-(N-Benzylcarbamoyl)-*L*-glutathione	IC_{20}/1.17 mM

a. Standard errors are ± 10% of values shown; b. NI - not inhibitory.

9. Research Goals

Work on the mechanistic and structural aspects of the glyoxalase enzyme system can at best be considered minimal especially when compared to work performed on human and yeast glyoxalase I. To achieve a better understanding of *E. coli* glyoxalase I as well as the glyoxalase I mechanism in general, we proposed to isolate and characterize the enzyme in terms of mechanistic and structural features. There exist a number of inhibitors of yeast glyoxalase I which would be interesting to evaluate against *E. coli* glyoxalase I to determine if common trends exist between the enzyme from the two sources. Site-directed mutagenesis studies would allow for the generation of mutants whose properties would be interesting to compare with the wild-type enzyme and these mutant proteins may aid in the elucidation of crucial residues in *E. coli* glyoxalase I catalysis. An overexpression system making available sufficient quantities of glyoxalase I for crystallographic studies was also a consideration.

CHAPTER 2

EXPERIMENTAL

Materials

Acetic acid (BDH)

Acrylamide (Bio-Rad)

Ammonium Persulfate (Bio-Rad)

Ammonium sulfate (Enzyme grade, Schwarz-Mann Biotech)

Ampicillin (Sigma)

Azocoll™ (Calbiochem)

BioLyte 3-10 (Bio-Rad)

Boric acid (Sigma)

Bovine Serum Albumin (Sigma)

Bromophenol blue (Sigma)

Calcium chloride dihydrate (Fisher Scientific)

Carbenicillin (Sigma)

60 U/mg α-Chymotrypsin Type II from Bovine Pancreas (Sigma)

Cobalt (II) chloride hexahydrate (Fisher Scientific)

Coomassie Brilliant Blue G-250(Sigma)

Coomassie Brilliant Blue R-250(Sigma)

3-(Cyclohexylamino)-1-propane sulfonic acid (ICN Biochemicals)

Diethylpyrocarbonate (Sigma)

Dimethyl sulfoxide (American Chemical Ltd)

Ellman's reagent (Boehringer Mannheim)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma)

Ethylenediamine tetraacetic acid (Sigma)

Ethyleneglycol-bis-(2-aminoethyl ether)-N,N'-tetraacetic acid (Boehringer Mannheim)

Formaldehyde (Sigma)

Formic acid (American Chemicals Ltd.)

Glutaraldehyde, 25% solution (Spectrum Chemicals; Sigma)

Glutathione, reduced (Sigma)

Glycerol (BDH)

Glycine, ultra pure (ICN Biochemicals)

Glyoxalase I, Grade X from yeast (Sigma)

Glyoxalase II, Bovine liver (Sigma)

S-Hexylglutathione agarose(Sigma)

Histidine, free base (Sigma)

HTP Econo-Pac CHT-II (Bio-Rad)

N,N-bis(2-Hydroxyethyl)glycine (Sigma)

Imidazole (ICN Biochemicals)

Iodoacetamide, crystalline (Sigma)

S-*D*-Lactoylglutathione (Sigma)

Lysozyme from bacteriophage λ (courtesy Henry Duewel)

Magnesium chloride hexahydrate (BDH)

Manganese (II) chloride tetrahydrate (Fisher Scientific)

α-Mercaptoethanol (Sigma)

Mercury (II) chloride (Sigma)

N,N'-Methylene-bis-acrylamide (Bio-Rad)

Methylglyoxal (Sigma)

Molecular weight markers (Pharmacia)

Mono P HR 5/20 (Pharmacia)

Mono Q HR 5/5 (Pharmacia)

2-(N-Morpholino)ethanesulfonic acid (Sigma)

3-(N-Morpholino)propanesulfonic acid (Sigma)

Nickel (II) chloride hexahydrate (Fisher Scientific)

Nicotinamide adenine dinucleotide, oxidized (Sigma)

Nicotinamide adenine dinucleotide phosphate, reduced (Sigma)

S-Octylglutathione agarose (Sigma)

Phast-Gel, buffer strips (Pharmacia)

Phast-Gels, Homogenous 20, High Density (Pharmacia)

Phenylmethanesulfonyl fluoride (Boehringer Mannheim)

Phenyl-Superose HR 10/10 (Pharmacia)

Phosphoric acid (J.T. Baker Chemicals)

PM-10 membranes (Amicon)

Polybuffer 74 (Pharmacia)

Polyvinylidene difluoride membranes (Pharmacia)

Potassium bromide (J.T. Baker Chemicals)

Potassium chloride (J.T. Baker Chemicals)

Potassium phosphate, monobasic (J.T. Baker Chemicals)

Q-Sepharose Fast Flow (Pharmacia)

Silver nitrate (J.T. Baker Chemicals)

Sodium acetate (BDH)

Sodium bromide (J.T. Baker Chemicals)

Sodium carbonate (BDH)

Sodium chloride (BDH)

Sodium dodecyl phosphate (Bio-Rad)

Spectra/Por® dialysis membranes, 12-14 kDa MWCO (Spectra/Por)

Sodium thiosulfate (American Chemicals Ltd.)

Streptomycin sulfate

Superdex 75 HR 10/30 (Pharmacia)

Tris(hydroxymethyl)aminomethane (ICN Biochemicals)

N,N,N′,N′-Tetramethylethylenediamine (Bio-Rad)

Thiamine (ICN Biochemicals)

Trichloroacetic acid (American Chemicals, Ltd.)

Trypsin (Sigma)

Tryptone (Difco)

Uracil (Kodak)

Yeast Extract (Difco)

Zinc chloride (J.T. Baker)

Zinc sulfate (J.T. Baker)

Equipment

Amicon Ultrafiltration unit (Amicon)

Beckmann model J2-21 centrifuge (Beckmann)

Beckmann JA-14, JA-20 rotors (Beckmann)

Cary13 UV-VIS spectrometer (Varian)

Centricon concentrators, 10 kDa cut-off (Amicon)

Fast Protein Liquid Chromatography unit (Pharmacia)

French Press (Carver Laboratory Press)

IEC Centra-7R Centrifuge (IEC)

Mini-Trans-blot Electrophoretic Transfer Cell (Bio-Rad)

Mini Protean II Unit (Bio-Rad)

Phast System Separation and Development Unit (Pharmacia)

Porton Micro-sequencer model 2090 (Porton)

Preparative Isoelectric Focusing Unit (Rotofor) (Bio-Rad)

Sonicator W-225 (Heat Systems-Ultasonics Inc.)

Triple quadrupole mass spectrometer (Fisons VG Quatro II)

1. Enzyme and Substrate Assays and Analyses

1.1 Methylglyoxal Dehydrogenase

The assay mixture (1.0 mL total volume) for methylglyoxal dehydrogenase consisted of 20 mM methylglyoxal, 5.0 mM NAD^+, 100 mM potassium phosphate (pH 8.0) and enzyme. Activity was determined at 25 °C by measuring the increase at 340 nm due to reduction of NAD^+ to NADH (Rhee *et al.*, 1987a).

1.2 Methylglyoxal Reductase

The assay mixture (1.0 mL total volume) for methylglyoxal reductase consisted of 20 mM methylglyoxal, 0.15 mM NADPH, 100 mM sodium acetate (pH 6.6) and enzyme. Activity was determined at 25 °C by measuring the decrease in 340 nm due to oxidation of NADPH to $NADP^+$ (Rhee *et al.*, 1987a).

1.3 Glyoxalase I

The assay mixture (total volume of 3.0 mL) for glyoxalase I consisted of 27 mM methylglyoxal, 1.8 mM glutathione, 50 mM degassed potassium phosphate buffer (pH 6.6) and enzyme. Activity was determined at 25 °C by measuring the increase at 240 nm due to S-*D*-lactoyl-glutathione formation (Uotila, 1973; Barnard and Honek, 1989).

1.4 *Saccharomyces cerevisiae* Glyoxalase I Inhibitor Assays

The uv spectrum of S-*D*-lactoylglutathione was measured in the presence and absence of various potential inhibitors. Spectrophotometric studies were performed at 1.6 mM hemimercaptal (with or without inhibitor as necessary; GSH and MG were allowed to equilibrate for 15 minutes before the addition of enzyme) (Barnard and Honek, 1989; Barnard and Honek, 1994). Enzymatic reactions were initiated by the addition of 0.23 U/mL of yeast glyoxalase I. Reactions were quenched with the addition of trichloroacetic acid (TCA) (220 mM final concentration) and solutions diluted 10-fold with 50 mM potassium phosphate buffer (pH 6.6) and absorbance at 240 nm measured. All measurements were performed at 25 °C in triplicate. Controls to confirm that TCA did not degrade S-*D*-lactoylglutathione under the above conditions were performed.

1.5 Glyoxalase II Inhibitor Assays

Glyoxalase II assays were based upon a modification of the method of Principato *et al.* (Principato *et al.*, 1987b). The assay mixture contained inhibitor (1 µL), 0.85 mL 100 mM potassium phosphate buffer (pH 6.6) containing 0.2 mM Ellman's reagent, 0.1 mL S-*D*-lactoyl-glutathione and the reaction was initiated by 0.05 mL of glyoxalase. GSH formation was monitored at 412 nm by following the rate of production of thionitrobenzoate. The initial concentration of S-*D*-lactoylglutathione

was 1.6 mM. It was necessary to perform inhibition studies at pH 6.6 to minimize hydrolysis of thiocarbamoyl and carbamoyl compounds. Compound hydrolysis under these conditions amounted to less than 0.1% of the initial concentration over the course of 15 minutes. Compounds tested were dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentration with buffer. DMSO accounted for less than 5% vol/vol and did not affect enzyme activity.

1.6 K_m and V_{max} Studies

The following equation was used to determine hemimercaptal concentrations (Vince *et al.*, 1971; Vander Jagt et al., 1972):

$$K_d = 3.1 \text{ mM} = ([MG - x][GSH - x])/[x]$$

where [x] = hemimercaptal concentration

Assay mixtures (1.0 mL total volume) contained either 0.1 mM (2.43 mM MG and 0.229 mM GSH), 0.15 mM (3.66 mM MG and 0.28 mM GSH), 0.2 mM (4.85 mM MG and 0.33 mM GSH), 0.3 mM (8.11 mM MG and 0.42 mM GSH), 0.545 mM (12.17 mM MG and 0.61 mM GSH), 1.12 mM (24.27 mM MG and 1.27 mM GSH) or 2.85 mM (60.85 mM MG and 3.0 mM GSH) hemimercaptal in 50 mM potassium phosphate (pH 6.6). Glutathione and methylglyoxal were allowed to equilibrate for 15 minutes before the addition of glyoxalase I.

Reactions were initiated by the addition of *E. coli* glyoxalase I (1 µL). Prior to initiation of enzyme reaction, *E. coli* glyoxalase I was incubated with $NiCl_2$ (final concentration of 20 mM) for 20 minutes at 4 °C. The presence of $NiCl_2$ was also tested and found not to chemically alter the product formation or degradation in the absence of enzyme.

1.7 Methylglyoxal Purification and Analyses

Commercially available 40% aqueous solutions of methylglyoxal were distilled under atmospheric pressure and the fractions which distilled between 92 °C-96 °C were collected and analyzed for methylglyoxal content. Analyses were performed with modification of the method of Gawehn (Gawehn, 1985). The reference cuvette contained 3.02 mL of 50 mM potassium phosphate buffer (pH 6.6), 50 µL of 30 mM GSH and 20 µL of 50-fold diluted stock methylglyoxal. The reaction cuvette contained 3.00 mL of buffer, 50 µL of GSH and 20 µL of Grade X yeast glyoxalase I (0.66 mg/mL). The diluted methylglyoxal(20 µL) was added to the reaction cuvette and formation of product was monitored at 240 nm. All measurements were performed at 25 °C.

1.8 Glutathione Assays

Reduced glutathione solutions were prepared by dissolving 50 mg of GSH per mL of degassed 50 mM potassium phosphate buffer, pH 7.4. A 3 600-fold diluted aliquot of the stock solution was titrated with 5 µL of 323 mM Ellman's reagent (Ellman, 1959). The formation of 2-nitrothiobenzoate at 412 nm (ε_M = 14 150 $M^{-1} \cdot cm^{-1}$) (Collier, 1973) was monitored until no further increase was noted with successive additions of Ellman's reagent and the absorbance readings were recorded. All measurements were performed at 25 °C.

1.8 Azocoll™ Analyses for the Presence of Proteases

Azocoll™ is an insoluble protein-dye conjugate which can be hydrolyzed by various proteases to yield soluble, colored peptide fragments with a characteristic absorbance at 520 nm (Chavira *et al.*, 1984). Azocoll™ (50 mg) was suspended in 50 mM potassium phosphate buffer, pH 7.0 (Chavira *et al.*, 1984). For the blank, azocoll was suspended in 5.0 mL of buffer. For PMSF-treated samples, 4.0 mL of buffer was used to suspend the azocoll™ and 1.0 mL of sample was added. Similarly, protein sample which had not been exposed to PMSF was added to an azocoll™ suspension. As a positive control, 200 µg of chymotrypsin (type II, bovine pancreas; 12U) was added to 4.0 mL of an

azocoll™ suspension. All samples were incubated with shaking at 37 °C for 30 minutes. At the end of 30 minutes samples were filtered through pasteur pipettes which had been plugged with glass wool. Absorbance readings at 520 nm were performed on the collected filtrates.

2. Organisms and Growth Conditions

2.1 Wild-type *E. coli* MG1655

A single colony of *E. coli* MG1655 (λ-, F-) (Jensen, 1995) was preinoculated in 10 mL of autoclaved sterilized Luria-Bertani (LB) growth medium (10 g tryptone, 10 g NaCl and 5 g yeast extract per litre) at 37 °C overnight. The overnight culture was then diluted 1:100 into fresh medium (990 mL) and incubated at 37 °C with shaking for approximately 7 hours.

2.2 DH5α containing pDM1

DH5α (*supE*44, Δ*lac*U169(φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyr*A96, *thi*-1, *rel*A1) containing the pDM1 plasmid was treated in a similar manner as *E. coli* MG1655 organism with the exception that 50 mg of ampicillin was added per litre of growth medium.

2.3 DF502 containing pDM7

DF502 (*galK*35, λ-, *edd*-1, *his*-68, *rpsL*125, Δ(*rhaD-tpiA*)269, *purD*34, *pfkB*1) (Strauss and Gilbert, 1985) containing pDM7 was grown in medium consisting of LB media (900 mL) supplemented with 25 mL of 1.6 mg/mL histidine, 25 mL of 0.04 mg/mL thiamine, 25 mL of 0.064 mg/mL $ZnSO_4$, 25 mL of 8% glycerol, 40 mg uracil and 50 mg carbenicillin (Babul, 1978; Pahel *et al.*, 1979; Alber and Kowalski, 1982).

A single colony was preinoculated into 10 mL of the supplemented LB media at 37 °C overnight with shaking. The overnight culture was diluted 1:100 into fresh medium (990 mL) and was incubated at 37 °C with shaking for approximately 9 hours.

3. Cell Harvesting and Storage

Cells were harvested by centrifugation at 15 300 x g (Beckmann JA-14 rotor), 4 °C for approximately 10 minutes. After the growth media had been decanted from the cells, the cells were resuspended in distilled-deionized water and centrifuged again under the aforementioned conditions. Cells which where to be stored were rapidly frozen in liquid nitrogen and stored at -80 °C.

4. Cell Breakage

4.1 Sonication

Cells were disrupted by sonication using a Sonicator W-225 (Heat Systems-Ultrasonics, Inc.). Cells were resuspended in 20 mM tris(hydroxymethyl)aminomethane (TRIS), pH 7.0 (1.0 g of cells per 10 mL TRIS buffer). In order to control temperature during homogenizations, the beaker containing the resuspended cells was maintained in an ice-bath. Cells were pulse sonicated for one minute (70% duty cycle). After the initiation of sonication, sufficient phenylmethanesulfonyl fluoride was added such that the final concentration was 1.0 mM. To remove cell debris the lysate was centrifuged at 12 100 x g (Beckmann JA-20 rotor), 4 °C for 10 minutes.

Depending upon the purification protocol, the supernatant had sufficient glycerol added such that the final glycerol concentration was 30% vol/vol.

4.2 French Press

Cells which were to be disrupted by French Press were resuspended in cooled 50 mM potassium phosphate, pH 6.6 (1.0 g of cells per 10 mL of phosphate buffer). Cells were disrupted at a pressure of 10 000 pounds per square inch. After the lysate had been collected with cooling in an

ice bath, PMSF dissolved in 1 mL of acetone was added to a final concentration of 1.0 mM. To remove cell debris, the lysate was centrifuged at 12 100 x g (Beckmann JA-20 rotor), 4 °C for 10 minutes.

5. Removal of Nucleic Acids

5.1 $MnCl_2$ Precipitation

Precipitation of nucleic acids was achieved by mixing 40 mL of 0.5 M $MnCl_2$ per 9 400 mg of total protein (Chiu and Fengold, 1969). The mixture was allowed to stir gently overnight at 4 °C. The precipitate was removed by centrifugation at 12 100 x g (Beckmann JA-20 rotor), 4 °C for 15 minutes and the supernatant kept for further purification.

5.2 Streptomycin Sulfate Precipitation

Freshly prepared 10% wt/vol streptomycin sulfate was added with stirring to the supernatant (30 µL of streptomycin per mL supernatant). This amount yielded a final streptomycin concentration of 0.3% wt/vol. The suspension is allowed to stir at 4 °C for 10 minutes and then centrifuged at 12 100 x g (Beckmann JA-20 rotor), 4 °C for 30 minutes (Kaplan, 1971).

5.3 Protamine Sulfate Precipitation

Approximately 5 mg of protamine sulfate was added per gram of protein in solution and left to stir overnight at 4 °C (Scopes, 1987). Precipitate was removed by centrifugation at 12 100 x g (Beckmann JA-20 rotor), 4 °C for 10 minutes.

6. Ammonium Sulfate Precipitation of Proteins

The quantities of ammonium sulfate required to precipitate proteins were based upon information derived from Scopes (Scopes, 1987). For a 0%-30% fraction approximately 16.6 g is required per 100 mL of protein solution. For a 30%-60% fraction an additional 18.4 g is required per 100 mL of 30% ammonium sulfate protein solution. The ammonium sulfate was added to protein solutions (kept at 4 °C on ice) with gentle stirring over the course of 45 minutes. After all the solid ammonium sulfate was added, a further stirring time of 30 minutes was allowed. Pellets were isolated by centrifugation (22 100 x g; Beckmann JA-20 rotor), 4 °C for 15 minutes and stored at -20 °C.

7. Column Chromatography and Purification Protocols

7.1 Q-Sepharose Fast Flow Column

Loading of the Q-Sepharose fast Flow Column (1 cm x 30 cm) was performed at a flow rate of 1.0 mL/min at room temperature. Volume of application was dependent upon the quantity of cells disrupted but typically varied between 30-50 mL.

The fast performance liquid chromatography (FPLC) system (Pharmacia) utilized was a dual pump system (Pump A and Pump B). In this purification step Pump A contained 20 mM TRIS, 30% glycerol, pH 7.0 and Pump B contained 20 mM TRIS, 1 M KCl, 30% glycerol, pH 7.0. After the contents of the Superloop loading column had been applied to the column utilizing the buffer in pump A, washing was continued until a decrease in 280 nm absorbance had occurred and a stable baseline had been achieved. The gradient was applied at a rate such that the salt concentration gradient increased at a rate of 10 mM/min. This ensured that proteins were not shocked from the column. It was necessary to maintain a wash of 1 M KCl for 60 minutes to elute tightly bound proteins (Table 10). The protein fractions were measured for activity and were pooled.

Table 10. FPLC protocol settings for the isolation of proteins from the Q-Sepharose Fast Flow chromatographic column.

Time (min)	Status	Value/Flow rate
0.0	CONC %B	0.0
0.0	ML/MIN	1.00
0.0	CM/MIN	0.2
0.0	VALVE POS	1.1
5.0	CONC %B	0.0
105	CONC %B	100
165	CONC %B	100
180	CONC %B	0.0

7.2 Mono Q Ion-Exchange Column

Prior to column loading, samples were dialyzed using Spectra/Por® 12-14 kDa molecular weight cutoff dialysis tubing (2 x 1 L, 12 hr, 4 °C) against 20 mM TRIS, pH 7.0. Dialysis tubing was prepared by boiling fresh dialysis tubing in 250 mL of Milli-Q water for 15 minutes. Dialyzed protein samples were applied to an anion exchange Mono Q HR 5/5 (0.5 cm x 5 cm) column that had been equilibrated with 20 mM TRIS, pH 7.0 (Buffer A). Proteins were eluted with an increasing salt concentration gradient (Buffer A + 1 M KCl; Buffer B; buffers maintained at 4 °C on ice) and assayed for glyoxalase I activity. Fractions were collected at room temperature but stored immediately at 4 °C.

Table 11. FPLC protocol settings for isolation of proteins from the Mono Q column.

Time	Status	Value/Flow Rate
0.0	CONC %B	0.0
0.0	ML/MIN	0.50
0.0	CM/MIN	0.20
0.0	VALVE.POS	1.2
5.0	VALVE.POS	1.1
10.0	CONC %B	0.0
90.0	CONC %B	40.0
90.0	CONC %B	100.0
100.0	CONC %B	100.0
105.0	CONC %B	0.0

7.3 Mono P Column Chromatography

7.3.1 Mono P used as an Isoelectric Focusing Column

Samples which were to undergo chromatofocusing were dialyzed using Spectra/Por® 12-14 kDa molecular weight cutoff dialysis tubing (2 x 1 L, 12 hrs, 4 °C) against 25 mM histidine (pH 6.4) buffer. Dialysis tubing was prepared by boiling fresh dialysis tubing in 250 mL of Milli-Q water for 15 minutes. Dialyzed samples were then applied to a Mono P HR 5/20 (0.5 cm x 20 cm) column which had been previously equilibrated with the 25 mM histidine buffer (pH 6.4). Protein elution was achieved by the generation of a linear gradient which was due to the application of 10-

fold diluted Polybuffer 74 (pH 4.0). A specific program was not used since only one pump of the FPLC was required. However, the flow rate was maintained at 0.5 mL/min and the chart speed was 0.2 cm/min. Buffers were maintained at 4 °C on ice. Fractions were collected at room temperature but stored immediately at 4 °C.

7.3.2 Mono P used as an Ion-Exchange Column

Samples which were to undergo ion-exchange were dialyzed using Spectra/Por® 12-14 kDa molecular weight cutoff dialysis tubing (2 x 1 L, 12 hr, 4 °C) against 20 mM TRIS, 15% vol/vol glycerol, pH 7.0. Dialysis tubing was prepared by boiling fresh dialysis tubing in 250 mL of Milli-Q water for 15 minutes. Protein samples were applied to a Mono P HR 5/20 (0.5 cm x 20 cm) column which had been previously equilibrated with 20 mM TRIS, 15% vol/vol glycerol, pH 7.0. The eluting buffer consisted of 20 mM TRIS, 1 M KCl, 15% vol/vol glycerol, pH 7.0. The rate of increase in the salt gradient was 10 mM/min (Table 12). Buffers were maintained at 4 °C on ice. Fractions were collected at room temperature but stored immediately at 4 °C.

Table 12. FPLC protocol settings for the isolation of proteins from a Mono P chromatography column when used as an ion-exchanger.

Time (min.)	Status	Value/Flow Rate
0.0	CONC %B	0.0
0.0	ML/MIN	0.5
0.0	CM/MIN	0.2
0.0	VALVE POS	1.1
100	CONC %B	100
130	CONC %B	100
145	CONC %B	0.0

7.4 Superdex 75 Molecular Sieving Column

Samples (200 µL maximum volume) were loaded onto a Superdex 75 HR 10/30 (1 cm x 10 cm) column and were eluted with 150 mM potassium phosphate, pH 6.6, 15% vol/vol glycerol. The following program was used to elute proteins from the column. Buffers were maintained at 4 °C on ice. Fractions were collected at room temperature but stored immediately at 4 °C.

Table 13. FPLC protocol settings for eluting proteins from a Superdex 75 HR 10/30 column.

Time	Status	Value/Flow Rate
0.0	CONC %B	0.0
0.0	ML/MIN	0.50
0.0	CM/MIN	0.20
0.0	VALVE.POS	1.2
5.0	VALVE.POS	1.1
60.0	CONC %B	0.0

7.5 Phenyl-Superose Column

Solid ammonium sulfate was added to protein samples (50 mM potassium phosphate, pH 6.6) to give a final concentration of 2 M. Samples were applied to a Phenyl-Superose HR 10/10 column (1 cm x 10 cm) which had been previously equilibrated with 50 mM potassium phosphate, pH 6.6 and 2 M ammonium sulfate (Buffer A). Proteins were eluted from the column with a decreasing salt gradient (2 M to 0 M) and Milli-Q water was the final eluant (Buffer B). The following program was used to elute proteins from the column:

Table 14. FPLC protocol settings for protein elution from the Phenyl-Superose HR 10/10 column.

Time	Status	Value/Flow rate
0.0	CONC %B	0.0
0.0	ML/MIN	0.5
0.0	CM/MIN	0.2
0.0	VALVE.POS	1.2
5.0	VALVE.POS	1.1
10.0	CONC %B	0.0
70.0	CONC %B	100.0
90.0	CONC %B	100.0

7.6 Hydroxyapatite Column

Protein samples (1 mM potassium phosphate, pH 6.8; Buffer A) were loaded onto a HTP Econo-Pac CHT-II (Bio-Rad) (5 mL) column and eluted with increasing potassium phosphate concentrations (400 mM potassium phosphate, pH 6.8; Buffer B). The following program was used to elute proteins from the column:

Table 15. FPLC protocol settings for elution of proteins from the HTP Econo-Pac column.

Time	Status	Value/Flow Rate
0.0	CONC %B	0.0
0.0	ML/MIN	0.5
0.0	CM/MIN	0.2
0.0	VALVE.POS	1.2
2.0	VALVE.POS	1.1
10.0	CONC %B	0.0
25.0	CONC %B	75.0
28.0	CONC %B	75.0
33.0	CONC %B	0.0

7.7 S-Hexylglutathione and S-Octylglutathione Affinity Chromatography

Approximately 1 mL of swollen S-hexylglutathione agarose or S-octylglutathione agarose was added to a disposable Pasteur pipette which had been plugged with glass wool. Gels were equilibrated with 50 mL of 10 mM potassium phosphate, pH 6.6. Protein samples (50 µL) were applied to the column and washed with 20 mL of 10 mM potassium phosphate containing 0.2 M KCl, pH 6.6. Attempts to elute glyoxalase I first with buffer containing 5.0 mM GSH and then with 50.0 mM GSH proved ineffective.

8. Concentration of Protein Samples

For volumes greater than 10 mL, proteins were concentrated by ultrafiltration (Amicon unit, PM-10 membrane). For volumes of protein solutions less than 10 mL, Centricon™ concentrators (Amicon) with a 10-kDa MW cutoff were centrifuged (IEC Centra-7R) at 10 000 rpm to concentrate protein.

9. Protein Blotting to PVDF Membrane for N-Terminal Sequencing and Amino Acid Analysis.

The separating gel (20%) consisted of 1.025 mL Milli-Q water, 3.75 mL of 1.5 M TRIS (pH 8.8), 150 µL of 10% wt/vol sodium dodecyl sulfate (SDS), 10 mL of 30% wt/vol acrylamide and 75 µL N,N,N′,N′-tetramethylethylenediamine (TEMED). The polymerization reaction was initiated by 75 µL of 10% wt/vol ammonium persulfate. The gel was cast and allowed to polymerize overnight. The stacking gel (4%) consisted of 6.1 mL Milli-Q water, 2.5 mL of 0.5 M TRIS (pH 6.8), 100 µL of 10% wt/vol SDS, 1.3 mL of 30% wt/vol acrylamide and 10 µL TEMED. The polymerization reaction was initiated by 75 µL of 10% wt/vol ammonium persulfate and the stacking gel cast on top of the separating gel and allowed to polymerize.

Blotting of protein bands present in SDS-polyacrylamide gels onto polyvinylidene difluoride (PVDF) membranes were conducted with a modification of the procedure described by LeGendre and Matsudaira (LeGendre and Matsudaira, 1989) with the aid of a mini Trans-blot electophoretic transfer cell (Bio-Rad). Before use, the PVDF membrane (Pharmacia) was rinsed with absolute methanol and then rinsed with 10% vol/vol methanol in 10 mM CAPS, pH 10. The membrane and SDS-polyacrylamide gel were layered between two 3 mm Whatman papers. Transfer and blotting was conducted in a Mini Protean II unit (Bio-Rad) with 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), pH 10 containing 10% vol/vol absolute methanol as the buffer. A constant current of 250 mA was used for the entire duration of the 60 minute run.

The PVDF membrane was transferred into a solution of 50% vol/vol methanol/Milli-Q water containing 0.1% Coomassie Blue R-250 for 5-10 minutes. The membrane was then rinsed several times in 50% vol/vol methanol/Milli-Q water to remove excess dye. The membrane was rinsed twice with Milli-Q water and was allowed to air dry.

The protein band of interest was excised from the PVDF membrane and sent to the Hospital for Sick Children-Pharmacia Biotechnology Centre, Toronto. N-Terminal protein sequencing was performed with on-

line PTH analysis with a Porton gas-phase Micro-sequencer, model 2090.

10. Characterization of *E. coli* Glyoxalase I

10.1 pH Stability

The effect of pH on *E. coli* glyoxalase I was examined using various buffers over the pH range of 2.7 to 10.8. Formate (50 mM; pH 2.7-4.5), acetate (50 mM; pH 3.5-5.7), 2-(N-morpholino)ethanesulfonic acid (MES) (50 mM; pH 5.4-6.6), 3-(N-morpholino)propanesulfonic acid (MOPS) (50 mM; pH 6.4-7.9), N,N-bis(2-hydroxyethyl)glycine (BICINE) (50 mM; pH 7.8-8.8), glycine (50 mM; pH 8.7-10.8) and boric acid (50 mM; pH 8.0-10.1) were used in this study. Approximately 300 µg of glyoxalase I was incubated with the aforementioned buffers over the indicated pH ranges for 15 minutes. Aliquots (10 µL) were removed at the end of the 15 minutes and assayed for formation of S-*D*-lactoylglutathione (1.6 mM hemimercaptal; final volume of 3.0 mL) in 50 mM potassium phosphate, pH 6.6.

10.2 Estimation of Isoelectric Point

10.2.1 Preparative Isoelectric Focusing

The 30%-60% ammonium sulfate pellet was dialyzed overnight against 2 x 1 L of distilled-deionized water containing 20% vol/vol glycerol. The volume of the dialysate was brought to approximately 50 mL (with 20% vol/vol glycerol/water). BioLyte 3-10 (1.37 mL) was also added to the sample. The sample was then focused in the preparative isolectric focusing chamber (Rotofor, Bio-Rad) at constant power (12 W) for approximately 4-6 hours. Samples were harvested and the pH of the sample fractions determined. Fractions were dialyzed against 2 x 1 L (12 hr, 4 °C) of 50 mM potassium phosphate, pH 6.6. After dialysis samples were analyzed for glyoxalase I activity.

10.2.2 Chromatofocusing over Mono P Column

Glyoxalase I was dialyzed overnight against 2 x 1 L (12 hr, 4 °C) of 25 mM histidine, pH 6.2. The dialysate was applied to a Mono P HR 5/20 column (0.5 cm x 20 cm) that had previously been equilibrated with the aforementioned buffer. Elution of the bound enzyme was achieved by a linear gradient of the initial buffer and a 10-fold dilution of Polybuffer 74 (pH 4.0). Fractions were collected and analyzed for glyoxalase I activity.

10.3 Effect of Buffers

Approximately 1.0 g of cells were suspended in 10 mL of 20 mM TRIS, pH 7.0 and the cells were sonicated and cell debris removed as described in **4.1** of this chapter. Aliquots of the protein supernatant (300 µL) were diluted with 1.2 mL of the following buffers:

1. 25 mM histidine, pH 6.6,
2. 25 mM imidazole, pH 7.0,
3. 25 mM TRIS, pH 7.0 and
4. 150 mM potassium phosphate, pH 6.6.

As a result of having sonicated the cells in TRIS buffer, buffers 1,2 and 4 contained a 4 mM TRIS component. Over the course of two days the protein samples, stored at 4 °C, had 50 µL aliquots removed and assayed for residual glyoxalase I activity (1.6 mM hemimercaptal; 3.0 mL assay volume).

10.4 Effect of Glycerol on *E. coli* Glyoxalase I Stability

Approximately 1.7 g of cells were suspended in 10 mL of 20 mM TRIS, pH 7.0 and sonicated and cell debris removed as described in **4.1** of this chapter. Protease inhibitor (PMSF in 1 mL of acetone, 1 mM final concentration) was added to the supernatant and 100 µL aliquots were diluted to 1.0 mL with various glycerol concentrations. Glycerol

concentrations of 0%, 5%, 15%, 25% and 50% were examined for effects on glyoxalase I. The effect of glycerol on stability was examined over the course of several days with storage at 4 °C.

10.5 Chemical Modification Studies

10.5.1 Aspartate and Glutamate Residues

Carboxyl group modification was performed with modifications to the protocol described by Clarke and Yaguchi (Clarke and Yaguchi, 1985). Approximately 155 µg of glyoxalase I was incubated with and without 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (final concentration of 50 mM) in a final volume of 1.02 mL containing 100 mM MES, pH 6.0. All incubations were performed at 25 °C. Residual enzyme activity was determined by removing a 10 µL aliquot at various time intervals and measuring the rate of S-*D*-lactoylglutathione formation (1.6 mM hemimercaptal; final assay volume of 3.0 mL).

10.5.2 Cysteine

Approximately 155 µg of glyoxalase I was treated with or without 20 mM iodoacetamide in a final volume of 1.02 mL containing 100 mM MES, pH 6.0. All incubations were performed in the dark at 25 °C. Residual glyoxalase I activity was determined by removing a 10 µL aliquot at

various time intervals and assaying for product formation (1.6 mM hemimercaptal; final volume of 3.0 mL).

10.5.3 Histidine

Approximately 155 µg of glyoxalase I was treated with diethyl pyrocarbonate (DEPC; in ethanol; final concentration 0.6 mM) and 100 mM MES, pH 6.0 in a final volume of 1.02 mL. All incubations were performed at 25 °C. Residual glyoxalase I activity was determined by removing 10 µL aliquots at various time intervals and assaying for product formation (1.6 mM hemimercaptal; final volume of 3.0 ml).

10.6 Effect of Metal Salts

The effect of metal salts including transition metals was examined. NaCl, NaBr, KCl and KBr were examined at 200 mM (25 mM MOPS, pH 7.6). $CaCl_2$ and $MgCl_2$ were examined at a concentration of 20 mM (25 mM MOPS, pH 7.6). $ZnCl_2$, $HgCl_2$, $MnCl_2$ and $CoCl_2$ were all examined at a concentration of 20 mM (100 mM MES, pH 6.0). $NiCl_2$ was examined at 200 µM, 2 mM and 20 mM in 100 mM MES, pH 6.0. Approximately 155 µg of glyoxalase I was incubated with the metals for either 5 or 15 minutes at 4 °C. Aliquots (10 µL) were removed and assayed for glyoxalase I activity (1.6 mM hemimercaptal; final volume of 3.0 mL). Control curves

to determine if the various metals could cause an increase in A240 were generated by incubating the various metals under the aforementioned conditions but without the presence of glyoxalase I.

11. Pharmacia Phast Gel Conditions

11.1 Loading Buffer

The amount of reagent indicated below is for the preparation of 100 mL of loading buffer.

SDS	1.0 g
α-Mercaptoethanol	200 µL
Bromophenol Blue	20 mg
Glycerol	40 mL
0.5 M TRIS, pH 6.8	20 mL
Milli-Q Water	60 mL

Protein samples were mixed 1:1 with loading buffer and boiled for at least 5 minutes at 100 °C prior to the electrophoretic run.

12. Protein Visualization for Protein Content Determinations

12.1 Coomassie Brilliant Blue Solution

Quantitation of protein was performed using the commercial Dye Reagent Concentrate (Bio-Rad Protein Kit) or by prepared dye reagent:

1. Coomassie Brilliant Blue G-250	100 mg
2. 95% Ethanol	50 mL
3. 85% Phosphoric acid	100 mL
4. Distilled-deionized water	850 mL

Coomassie Brilliant Blue G-250 was dissolved in ethanol and under stirring the phosphoric acid and water were added. Prior to use, the dye reagent was filtered through Whatman no. 1 paper. Regular or microprotein assays were conducted by the method of Bradford (Bradford, 1976).

12.2 Silver Staining of Proteins in Polyacrylamide Gels

Reagents and conditions with minor modifications were based upon the procedure of Heukeshoven and Dernick (Heukeshoven and Dernick, 1988).

1. 20% Methanol/10% acetic acid/ 70% Milli-Q water (30 min)
2. 30% Ethanol/10% acetic acid/70% Milli-Q water (15 min)
3. 30% Ethanol/10% acetic acid/70% Milli-Q water (15 min)
4. 0.5% Glutaraldehyde and 0.1% sodium thiosulfate in 30% ethanol/water/ 0.4% sodium acetate, pH 6.0 (15 min)

5-9. Milli-Q water (2 min)

10. 20 mg $AgNO_3$ plus 50 µL formaldehyde in 200 mL Milli-Q water (20 min)
11. Milli-Q water (2 min)
12. 5 g Na_2CO_3 plus 80 mL formaldehyde in 200 mL Milli-Q water
13. 10% Acetic acid/water (1 min)
14. 10% Acetic acid/10% glycerol/80% Milli-Q water

All steps involving liquids are expressed as per cent volume/volume. Steps involving solids are expressed as the number of grams used or are expressed in per cent weight/volume.

CHAPTER 3

RESULTS and DISCUSSION

1. Theory behind Chromatograhic Methods Utilized to Purify *E. coli* Glyoxalase I.

1.1 Q-Sepharose Fast Flow

Q-Sepharose Fast Flow resin can be used in a preparative ion-exchange column due to its high binding capacity for anionic proteins. However, the binding capacity advantage is somewhat offset by variable bead sizes which results in decreased resolution during purification (Pharmacia FPLC manual).

1.2 Mono P

Mono P contains both tertiary and quarternary amines which can interact with a "Polybuffer" to generate a linear pH gradient. Proteins are eluted from the column once they experience a pH equal to their isoelectric point. By virtue that the Mono P beads possess tertiary amines, it can also be used as an ion-exchange column.

1.3 Superdex 75

Gel filtration is a technique that has been in existence since 1957 (Porath and Flodin, 1957). Gels consist of a cross-linked three-dimensional network in bead form. The pores within the beads are of varying sizes such that larger molecules are excluded but molecules smaller than the pore size may enter the pores. Superdex 75 HR 10/30 (1 cm x 10 cm) has a useful molecular range of 3 to 70 kDa.

1.4 Phenyl-Superose

Hydrophobic interactions increase with increasing salt concentration and salts which aid in protein precipitation, ie. ammonium sulfate, tend to enhance hydrophobic interactions to the greatest extent (Scopes, 1987).

1.5 Hydroxyapatite

Hydroxyapatite is an inorganic crystalline matrix ($Ca_{10}(PO_4)_6(OH)_2$) (Scopes, 1987). Interactions are limited to surface-surface ionic interactions. Research by Bernardi has demonstrated that proteins are adsorped to hydroxyapatite at low K^+ concentrations and require increasing K^+ concentrations in order to be eluted. Basic proteins may be eluted by high K^+, Cl^- and low $CaCl_2$ concentrations (Bernardi *et al.*, 1972). Acidic proteins are not eluted by KCl, NaCl nor $CaCl_2$ (Bernardi

et al., 1972).

2.Expression, Isolation and Purification of Glyoxalase I from *E. coli*

2.1. Purification of *E.coli* Glyoxalase I from MG1655

Table 16 summarizes several different attempts to purify glyoxalase I from *E. coli* MG1655, a strain that did not contain glyoxalase I on a multicopy plasmid. This "optimized" protocol was used to determine the amount of glyoxalase I that could be readily obtained from *E. coli* based on constitutive chromosomal expression. Based upon this purification protocol, the specific activity of *E. coli* glyoxalase I is 0.012 µmol/min/mg and was obtained in a yield of approximately 7% at step 5 of the purification protocol. Based upon silver-staining of a Phast-Gel Homogenous 20 mini-gel *E.coli* glyoxalase I had not been purified to apparent homogeneity. However, the main problem of purification of *E.coli* glyoxalase I is the small quantity of glyoxalase I present in the wild-type bacteria (step 6). The low level of apparent purification may indicate that *E. coli* glyoxalase I is unstable at all stages of purification as has been described in a preliminary report by Vander Jagt for *E. coli* K-12 glyoxalase I purification (Vander Jagt, 1975). Based upon these initial findings it was apparent that in order to study *E. coli* glyoxalase I more thoroughly it would be necessary to generate a system capable of overexpressing *E. coli* glyoxalase I.

Table 16. Purification of *E. coli* glyoxalase I from MG1655.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification	Yield
1. Supernatant[a]	4 098	40	10.6	2.6×10^{-3}	1.00	100
2. $MnCl_2$ DNA precipitation	4 056	57	10.1	2.5×10^{-3}	0.96	95
3. 30%-60% ammonium sulfate	1 590	60	9.1	5.7×10^{-3}	2.2	86
4. Isoelectric Focusing (Rotofor)	0.144	4	0.011	7.6×10^{-2}	29.2	0.1
5. Mono Q	approx. 1 µg	2	0.003 7	3.7	1 423	0.035
6. Superdex 75	< 1 µg	1	ND[b]	ND	ND	ND

a. Cells lysed by French press and clarified as described in the **Experimental** section; b. ND - not determined.

2.2 Attempts to Overexpress *E. coli* Glyoxalase I

2.2.1 pDM1 in DH5α

Due to the low enzyme production from wild-type *E. coli* it was necessary to generate a microbial system which would be capable of enhanced glyoxalase I production. Dr. Elisabeth Daub spent considerable time and effort in attempts to generate plasmids containing the glyoxalase I gene. A brief description of the protocol used to generate methylglyoxal-resistant DH5α is therefore presented. *E. coli* chromosomal DNA was partially digested by the restriction enzyme Sau3A I to generate DNA fragments of various sizes. These fragments were fractionated on an agarose gel and fragments in the range of 2-6 kB were isolated and further purified using an Elutip-d column. The DNA fragments were ligated into the vector pBR322 (the vector was linearized with BamH I and dephosphorylated with calf intestinal phosphatase) using T4 DNA ligase and the plasmids transformed into competent DH5α *E. coli* cells. The library was plated on LB agar plates containing 100 μg/mL ampicillin and 3 mM methylgyoxal. Surviving colonies were restreaked on a series of LB agar plates containing increasing concentrations of methylglyoxal (0-5 mM). One plasmid that was isolated and which conveyed methylglyoxal-resistance (MG^R) at 3 mM was called pDM1 (Figure 13).

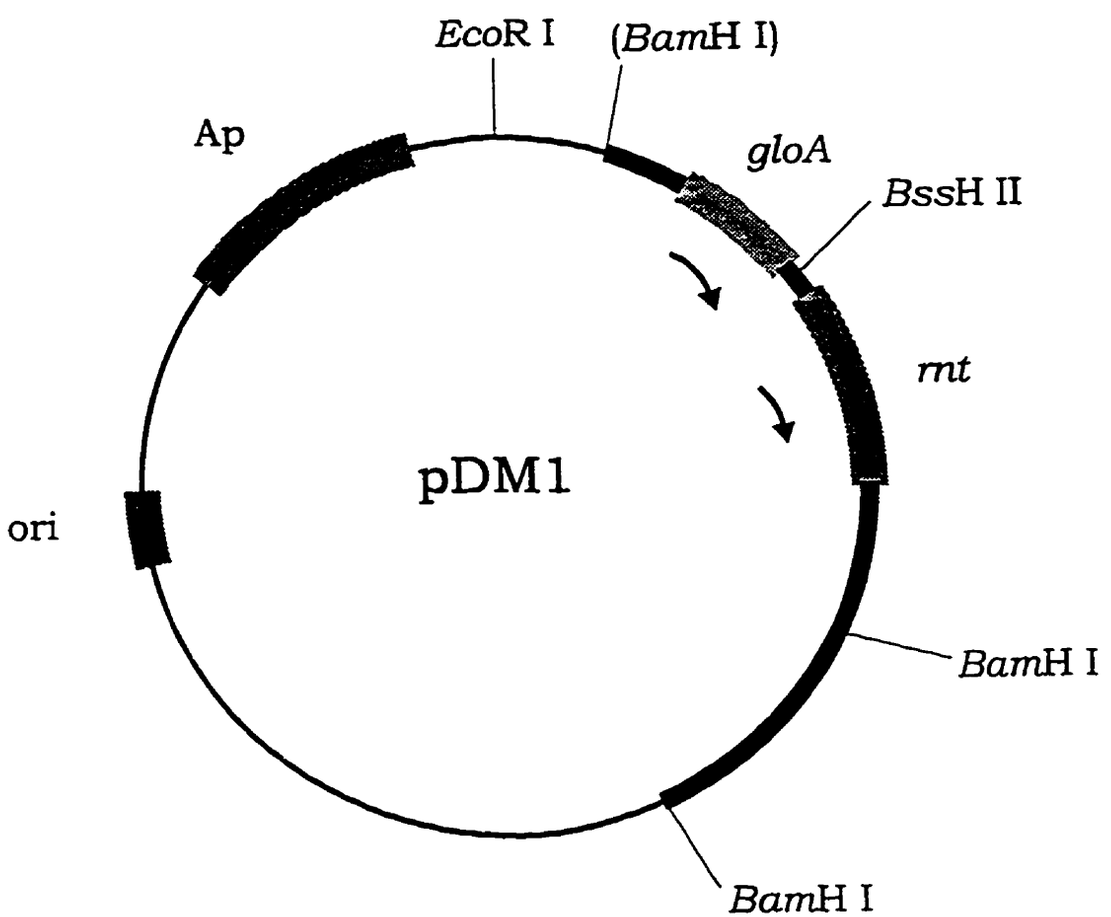

Figure 13. pDM1 plasmid construction; gloA = glyoxalase I and rnt = RNase T. The pBR322 vector is delineated by the thin black line and the insert is delineated by the thickened black line.

Since there are at least 3 known enzymes capable of metabolizing methylglyoxal it was necessary to determine whether glyoxalase I, methylglyoxal reductase or α-ketoaldehyde dehydrogenase was responsible for MG^R in the cells. DH5α cells containing pDM1 were grown in LB medium (100 mL) containing 25 μg/mL ampicillin, harvested and resuspended in 50 mM potassium phosphate, pH 6.6, disrupted by passage through a French Press (10 000 psi) and clarified as described in the **Experimental** section. The crude lysate was treated with streptomycin sulfate, the precipitate removed by centrifugations and a 30%-60% ammonium sulfate fractionation performed. The precipitate was isolated by centrifugation and the pellet resuspended in 50 mM potassium phosphate, pH 6.6. Assays for glyoxalase I, methylglyoxal reductase and α-ketoaldehyde dehydrogenase were performed as described in the **Experimental** section. DH5α cells containing only pBR322 served as a negative control. The plasmid pKR3 was generated in a similar manner to pDM1 except that the library was generated from *Salmonella typhimurium*. Glyoxalase I activity was increased substantially compared to the enzyme from wild-type *E. coli* (Table 17; 1.0 μmol/min/mg versus 6.67×10^{-3} μmol/min/mg; 150-fold increase over the wild-type). Both methylglyoxal reductase and α-keto-aldehyde dehydrogenase levels were similar to wild-type levels. It was therefore assumed based upon these results that the glyoxalase I gene

was being overexpressed by the presence of a multicopy plasmid containing the glyoxalase I gene.

Table 17. Glyoxalase I, methylglyoxal reductase and α-ketoaldehyde dehydrogenase activities in pBR322, pDM1 and pKR3.

	Specific Activity (μmol/min/mg)[a]		
Plasmid	Glyoxalase I	Methylglyoxal Reductase	α-ketoaldehyde Dehydrogenase
pBR322	0.0067	0.0916	0.00174
pDM1	1.0	0.0102	0.00381
pRK3	1.11	0.011	0.00048

a. All values are ±10%; all plasmids in DH5α.

Several purification schemes were used to purify *E. coli* glyoxalase I from DH5α containing pDM1. Purification schemes I-III (Tables 18-20) are essentially identical with the exception of the materials used to precipitate nucleic acids.

Table 18. Purification scheme I for *E. coli* glyoxalase I from DH5α/pDM1.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	739	45	451	0.61	1.00	100
2. $MnCl_2$ DNA precipitation	723	48	448	0.62	1.01	99
3. 30%-60% ammonium sulfate	265	50	318	1.2	2.0	70
4. Isoelectric Focusing (Rotofor)	23	9.4	43.7	1.9	3.1	9.7
5. Mono Q	2.6	3	28.0	10.8	17.7	6.2
6. Superdex 75	0.056	2	0.88	15.8	25.9	0.2
7. Concentrated solution	0.05	0.05	0.90	18.0[b]	29.5	0.2

a. Cells lysed by French Press and clarified as described in the **Experimental** section; b. Specific activity determined in 50 mM potassium phosphate, pH 6.6.

Table 19. Purification scheme II for *E. coli* glyoxalase I from DH5α/pDM1.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	673	50	424	0.63	1.00	100
2. Protamine sulfate	661	50	344	0.52	0.82	81
3. 30%-60% ammonium sulfate	254	50	236	0.93	1.5	56
4. Isoelectric Focusing (Rotofor)	19	9	19	1.0	1.6	4.5
5. Mono Q	2.4	2	6.2	2.6	4.1	1.5
6. Superdex 75	0.016	2	0.05	3.4	5.4	0.01
7. Concentrated solution	0.013	0.050	0.049	3.8[b]	6.0	0.01

a. Cells lysed by French Press and clarified as described in the **Experimental** section; b. Specific activity determined in 50 mM potassium phosphate, pH 6.6.

Table 20. Purification scheme III for *E. coli* glyoxalase I from DH5α/pDM1.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	633	50	380	0.60	1.00	100
2. Streptomycin sulfate	626	50	363	0.58	0.97	95
3. 30%-60% ammonium sulfate	233	50	256	1.1	1.8	67
4. Isoelectric Focusing (Rotofor)	18	6	23	1.3	2.2	6.0
5. Mono Q	2.1	2	9.0	4.3	7.2	2.4
6. Superdex 75	0.028	2	0.14	5.1	8.5	0.04
7. Concentrated solution	0.023	0.050	0.14	6.3[b]	10.5	0.04

a. Cells lysed by French Press and clarified as described in the **Experimental** section; b. Specific activity determined in 50 mM potassium phosphate, pH 6.6.

Table 21. Purification scheme IV for *E. coli* glyoxalase I from DH5α/pDM1.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	658	50	368	0.56	1.00	100
2. Q-Sepharose Fast Flow	86	17	63.6	0.74	1.32	17.3
3. Mono P (chromatofocusing)	6.9	5	5.4	0.78	1.39	1.47
4. Superdex 75	0.053	3	0.16	3.03	5.4	0.04
5. Concentrated solution	0.042	0.05	0.17	4.0[b]	10.6	0.046

a. Cells lysed by sonication and clarified as described in the **Experimental** section; b. Specific activity determined in 50 mM potassium phosphate, pH 6.6.

The quantity of protein isolated depended upon the purification protocol utilized but typically varied between 10 µg to 50 µg of *E. coli* glyoxalase I could be isolated per 6 grams of DH5α/pDM1 cells (1 L of cell growth) to near-homogeneity. A comparison of schemes I and II (Tables 19 and 20) would seem to implicate protamine sulfate as a substance which may be capable of binding *E. coli* glyoxalase I. It is known that *E. coli* α-ketoaldehyde dehydrogenase and yeast phosphofructokinase are adsorbed to protamine sulfate-nucleic acid precipitates (Scopes, 1987). The specific activity (step 7) of purification scheme I (Table 18) is 3-to 6-fold greater than in Tables 19 and 20. This may be due to the presence of Mn^{2+} which we found later to activate *E. coli* glyoxalase I (Table 26). The average length of time between cell growth to step 7 for schemes I-III (Tables 18-20) was approximately 7 days. In order to facilitate a more rapid purification of *E. coli* glyoxalase I scheme IV (Table 21) was implemented. A reduction in purification time (3 days) was found using this protocol. Q-Sepharose Fast Flow has sufficient binding capacity to handle anionic proteins and nucleic acids thus eliminating an overnight nucleic acid precipitation step and ammonium sulfate step. Use of the Q-Sepharose Fast Flow resulted in the elimination of considerable quantities of undesired protein. Similarly, a Mono P chromatographic step is capable

of handling greater quantities of protein than the Mono Q column (maximum of 25 mg of protein). Superdex 75 was used to eliminate proteins of molecular weight less than or greater than 30 kDa. Glyoxalase I from *E. coli* was purified to near-homogeneity (>95% by Phast-Gel mini-gel). However, the total protein typically recovered was only approximately 25 µg per 6 g of DH5α/pDM1 cells lysed (1 L of cell growth).

After step 5 of purification scheme IV (Table 21), S-octyl-glutathione agarose, S-hexylglutathione agarose, hydroxyapatite (HTP Econo-Pac) and Phenyl-Superose columns were utilized to determine if they could be used to enhance enzyme purity. S-Octylglutathione Sepharose 4B has been successfully used as a purification step of mouse liver glyoxalase I (Oray and Norton, 1977) while S-hexylglutathione Sepharose 4B has been used successfully during the purification of rat liver (Marmstal and Mannervik, 1979), human erythrocyte (Aronsson *et al.*, 1979), pig erythrocyte (Aronsson and Mannervik, 1977) and yeast (Marmstal *et al.*, 1979) glyoxalase I. S-Hexylglutathione Sepharose 6B has been used during the purification of rabbit liver glyoxalase I (Elango *et al.*, 1978) while S-hexylglutathione agarose has been used during the purification of *P. putida* glyoxalase I (Rhee *et al.*, 1986). Attempts to use S-hexylglutathione agarose and S-octylglutathione

agarose to further purify *E. coli* glyoxalase I were unsuccessful. No enzyme activity was recovered when 5 mM or 50 mM GSH in 50 mM potassium phosphate, pH 6.6 were used as eluants (>150 mL of eluant was used). It is possible that *E. coli* glyoxalase I forms sufficiently strong interactions with S-hexylglutathione and S-octylgutathione such that it can not be eluted from the columns using GSH. Alternatively, lack of activity may be due to denaturation of the enzyme on the columns.

Neither hydroxyapatite nor Phenyl-Superose were found to be acceptable as steps in the purification of *E. coli* glyoxalase I under a variety of conditions. In order to release *E. coli* glyoxalase I from these columns protracted washing was required (typical elution volumes were 10 mL or more). However, hydroxyapatite has been used succesfully in the purification of sheep liver glyoxalase I (Uotila and Koisuvalo, 1975). Unfortunately, the similarity of *E. coli* glyoxalase I to other glyoxalase enzymes appears not to extend to the success of previous literature purification protocols for other glyoxalase enzymes.

2.2.1.a Amino Acid Sequence and Gene Sequence of *E. coli* Glyoxalase I

A nearly homogeneous sample of *E. coli* glyoxalase I which had been isolated from DH5α/pDM1 was subject to electrophoresis and then electroblotted onto a PVDF membrane. The band of interest was excised from the membrane and underwent N-terminal sequencing at the Pharmacia Biotechnology center in Toronto, Ontario. The first 11 amino acids were determined to be:

^{1}MET-ARG-LEU-LEU-HIS-THR-MET-LEU-ARG-VAL-GLY^{11}.

Sequencing work was peformed by Dr. Elisabeth Daub on the pDM1 plasmid and was initiated from the former BamH I site in pBR322. As a result the following DNA sequence was determined (Figure 14). With the elucidation of the DNA sequence it was possible to predict the amino acid sequence of *E. coli* glyoxalase I (Figure 15). A comparison of the molecular weight for *E. coli* glyoxalase I predicted from the cDNA sequence compared to the molecular weight determined by electrospray mass spectrometry (ESMS) revealed that the molecular weight of *E. coli* glyoxalase I predicted from the cDNA sequence was 12 Da less than that determined experimentally. A re-examination of the sequencing gels revealed that bases coding for 60Asn had been misread as 60Thr. The difference between these two amino acids is approximately 12 Da.

1 ATG CGT CTT CTT CAT ACC ATG CTG CGC GTT GGC GAT TTG CAA CGC

46 TCC ATC GAT TTT TAT ACC AAA GTG CTG GGC ATG AAA CTG CTG CGT

91 ACC AGC GAA AAC CCG GAA TAC AAA TAC TCA CTG GCG TTT GTT CGT

136 TAC GGC CCG GAA ACC GAA GAA GCG GTG ATT GAA CTG ACC TAC AAC

181 TGG GGC GTG GAT AAA TAC GAA CTC GGC ACT GCT TAT GGT CAC ATC

226 GCG CTT AGC GTA GAT AAC GCC GCT GAA GCG TGC GAA AAA ATC CGT

271 CAA AAC GGG GGT AAC GTG ACC CGT GAA GCG GGT CCG GTA AAA GGC

316 GGT ACT ACG GTT ATC GCG TTT GTG GAA GAT CCG GAC GGT TAC AAA

361 ATT GAG TTA ATC GAA GAG AAA GAC GCC GGT CGC GGT CTG GGC AAC

406 TAA

Figure 14. *E. coli* glyoxalase I DNA sequence. Total number of bases is 408 with composition 110 A, 95 C, 115 G and 88 T (Genbank accession number U57363).

1	Met Arg Leu Leu His Thr Met Leu Arg Val Gly Asp Leu Gln Arg
16	Ser Ile Asp Phe Tyr Thr Lys Val Leu Gly Met Lys Leu Leu Arg
31	Thr Ser Glu Asn Phe Glu Tyr Lys Tyr Ser Leu Ala Phe Val Gly
46	Tyr Gly Pro Glu Thr Glu Glu Ala Val Ile Glu Leu Thr Tyr Asn
61	Trp Gly Val Asp Lys Tyr Glu Leu Gly Thr Ala Tyr Gly His Ile
76	Ala Leu Ser Val Asp Asn Ala Ala Glu Ala Cys Glu Lys Ile Arg
91	Gln Asn Gly Gly Asn Val Thr Arg Glu Ala Gly Pro Val Lys Gly
106	Gly Thr Thr Val Ile Ala Phe Val Glu Asp Pro Asp Gly Tyr Lys
121	Ile Glu Leu Ile Glu Glu Lys Asp Ala Gly Arg Gly Leu Gly Asn

Figure 15. Predicted *E. coli* glyoxalase I amino acid sequence from DNA sequence.

Amino acid composition was performed by Pharmacia Biotechnology center, Toronto, Ontario on a sample of glyoxalase I isolated from DH5α/pDM1 that had been electroblotted onto a PVDF membrane. A comparison of some of the amino acids that would not undergo degradation during hydrolysis between the predicted number of residues versus amino acid analysis yielded nearly identical results (Table 22).

Table 22. Amino acid analysis results for *E. coli* glyoxalase I for various residues.

Amino Acid	Residues Predicted	Amino Acid Analysis
HIS	2	1.8 (2)[a]
ALA	10	11.4 (11)
PRO	4	5.1 (5)
VAL	10	10.6 (11)
ILE	7	6.1 (6)
LEU	13	13.3 (13)
PHE	3	2.6 (3)

a. Values in parentheses are rounded to the nearest integer.

2.2.1.b Amino acid Sequence Alignments for Glyoxalase I from *E. coli*, *H. sapiens* and *P. putida*

The sequence alignment for *E. coli*, *Homo sapiens* (*H. sapiens*) and *P. putida* is found in Figure 16. Comparisons and alignments of the sequences were produced by PC/Gene 6.85 software.

The glyoxalase genes from *P. putida* and H. *sapiens* have been isolated and the protein sequences reported (Lu *et al.*, 1994). Comparison of the amino acid sequences for glyoxalase I from *E. coli* with the amino acid sequences from *P. putida* and *H. sapiens* revealed 4 regions possessing considerable homolgy: regions 3-33, 53-64, 72-91 and 104-125 (numbering sequence based upon *E. coli* sequence). Strictly conserved residues (bold) comprise approximately 22.2% (42 amino acids) of the sequence and well-conserved residues (starred) comprise approximately 24.9% (47 amino acids) of the sequence.

```
1.    MR-----------------------------LLHTMLRVGDLQRSIDFY 20
2.    MS--------LNDLNTLPGVTAQADPATAQFVFNHTMLRVKDIEKSLDFY 42
3     MAEPQPPSGGLTDEAALS-CCSDADPSTKDFLLQQTMLRVKDPKKSLDFY 49
                                          * *        ** *

1.    TKVLGMKLLRTSENPEYKYSLAFVGYGPETE---------------EAVI 55
2.    TRVLGFKLVDKRDFVEAKFSLYFLALVDPATIPADDDARHQWMKSIPGVL 92
3.    TRVLGMTLIQKCDFPIMKFSLYFLAYEDKNDIPKEKDEKIAWALSRKATL 99
       *   ** * * *        *    **    **                ***

1.    ELTYNWGVDK-----YELGTA----YGHIALSVDNAAEACEKIRQNGGNV 96
2.    ELTHNHGTERDADFAYHHGNTDPRGFGHICVSVPDVVAACERFEALQVPF 142
3.    ELTHNWGTEDDATQSYHNGNSDPRGFGHIGIAVPDVYSACKRFEELGVKF 149
         *   **       * **     *    ** *** * *** *

1.    TREAGPVKGGTTVIAFVEDPDGYKIELIEEKDAGRGLGN   135
2.    QKRLS--DGRMNHLAFIKDPDGYWVEVIQP----TPL--   173
3.    VKKPD--DGKMKGLAFIQDPDGYWIEILNPNKMATLM--   184
      *  *  *   * *  **      * ***       *
```

Figure 16. Comparison of the amino acid sequences of 1. *E. coli*, 2. *P. putida* and 3. *H. sapiens* glyoxalase I (identical amino acids are in bold and well-conserved amino acids are marked by "*").

2.2.2 Isolation of *E. coli* Glyoxalase I from DF502/pDM7

The rationale behind using *E. coli* strain DF502 was based on the fact that DF502 is deficient in triosephosphate isomerase (TIM) due to a deletion in its chromosomal DNA (Strauss and Gilbert, 1985). It is known that the product of glyoxalase I catalysis of the hemithioacetal of MG and GSH, S-*D*-lactoylglutathione, can affect cell division (Gillespie, 1975). We hypothesized that the presence of MG (produced from TIM) coupled with attempts to overexpress *E. coli* glyoxalase I in bacterial cells could result in increased S-*D*-lactoylglutathione levels and that this increase in S-*D*-lactoylglutathione may adversely affect bacterial cell viability in some fashion and affect our attempts to overexpress *E. coli* glyoxalase I.

According to Dr. Elisabeth Daub's observations, the pDM7 plasmid was extremely unstable in *E. coli* MG1655. Since pDM7 is a high copy number plasmid (pDM7 is pUC18-based) it was possible that overexpression of *E. coli* glyoxalase I coupled with MG production from TIM was depleting cells of GSH resulting in pDM7 instability in MG1655. Therefore if pDM7 were to be transformed into a bacterial strain deficient in TIM a reduction in MG levels may occur and depletion of GSH would no longer be a consideration.

The plasmid pDM7 is derived from the pDM1 plasmid. The DNA region delineated by BssH II and BamH I restriction endonuclease sites on pDM1 were removed using BssH II and BamH I and the plasmid ligated to form the pDM6 plasmid. From pDM6, part of the plasmid containing the DNA encoding for *E. coli* glyoxalase I was excised (Eco RI and BamH I restriction sites) and isolated. Similarly, pUC18 was cut with Eco RI and BamH I and the isolated DNA fragment ligated into pUC18 to generate the pDM7 plasmid.

Tables 23 and 24 summarize only some of our many attempts at the purification of *E. coli* glyoxalase I from DF502 containing pDM7 (Figures 17-22). The purification protocols are nearly identical with the previous systems except that in Table 23 (Figures 17-19), Mono P is used to purify *E. coli* glyoxalase I by chromatofocusing, whereas in Table 24 (Figures 20-22), Mono P is used as an ion-exchange step. It is interesting to compare the specific activities at step 5 for both purifications. There is a greater than 5-fold increase in the specific activity in Table 24 as compared to Table 23. At step 3 in Table 23 there is a slight increase in specific activity and purification whereas in Table 24 at step 3 there is a 9- to 10-fold increase in specific activity. These results may indicate that chromatofocusing or preparative isoelectric focusing is responsible for loss of enzyme

activity. Perhaps exposing *E. coli* glyoxalase I to acidic conditions may result in protonation of residues critical for metal binding or catalysis. Alternatively, loss of enzyme activity may be indicative that *E. coli* glyoxalase I is susceptible to denaturation at pH < 5.

Utilizing the purification scheme in Table 24 (Figures 20-22) generated nearly homogenous *E. coli* glyoxalase I (>95% apparent homogeneity based upon Phast-Gel results). DF502/pDM7 represents a source for the isolation of milligram quantities of *E. coli* glyoxalase I. Experiments pertaining to the physical characterization of *E. coli* glyoxalase I were performed in the enzyme isolated from DF502/pDM7.

Table 23. Purification scheme I for the isolation of *E. coli* glyoxalase I from DF502/pDM7.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	292	29	514	1.76	1.00	100
2. Q-Sepharose Fast Flow	39.9	18	79.8	2.0	1.14	15.5
3. Mono P (chromatofocusing)	8.0	12	20.0	2.5	1.42	3.9
4. Superdex 75	3.8	2	16.3	4.3	2.44	3.2
5. Concentrated solution	3.4	0.5	30.6	9[b]	5.1	5.9

a. Cells lysed by sonication; b. Specific activity determined when stored in 20 mM TRIS, 50% glycerol, pH 7.0.

Table 24. Purification scheme II for the isolation of *E. coli* glyoxalase I from DF502/pDM7.

Stage	Total Protein (mg)	Volume (mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg)	Purification	Yield
1. Supernatant[a]	240	30	456	1.9	1.00	100
2. Q-Sepharose Fast Flow	38.4	18	99.8	2.6	1.37	21.2
3. Mono P (ion-exchange)	3.8	10	74.5	19.6	10.3	16.3
4. Superdex 75	3.0	2	72.3	24.1	12.7	15.8
5. Concentrated solution	2.6	0.5	126.4	48.6[b] 79.4[c]	25.6[b] 41.2[c]	27.7

a. Cells lysed by sonication; b. Specific activity determined when stored in 20 mM TRIS, 50% glycerol, pH 7.0; c. specific activity when stored in 25 mM MOPS, 50% glycerol, pH 7.5.

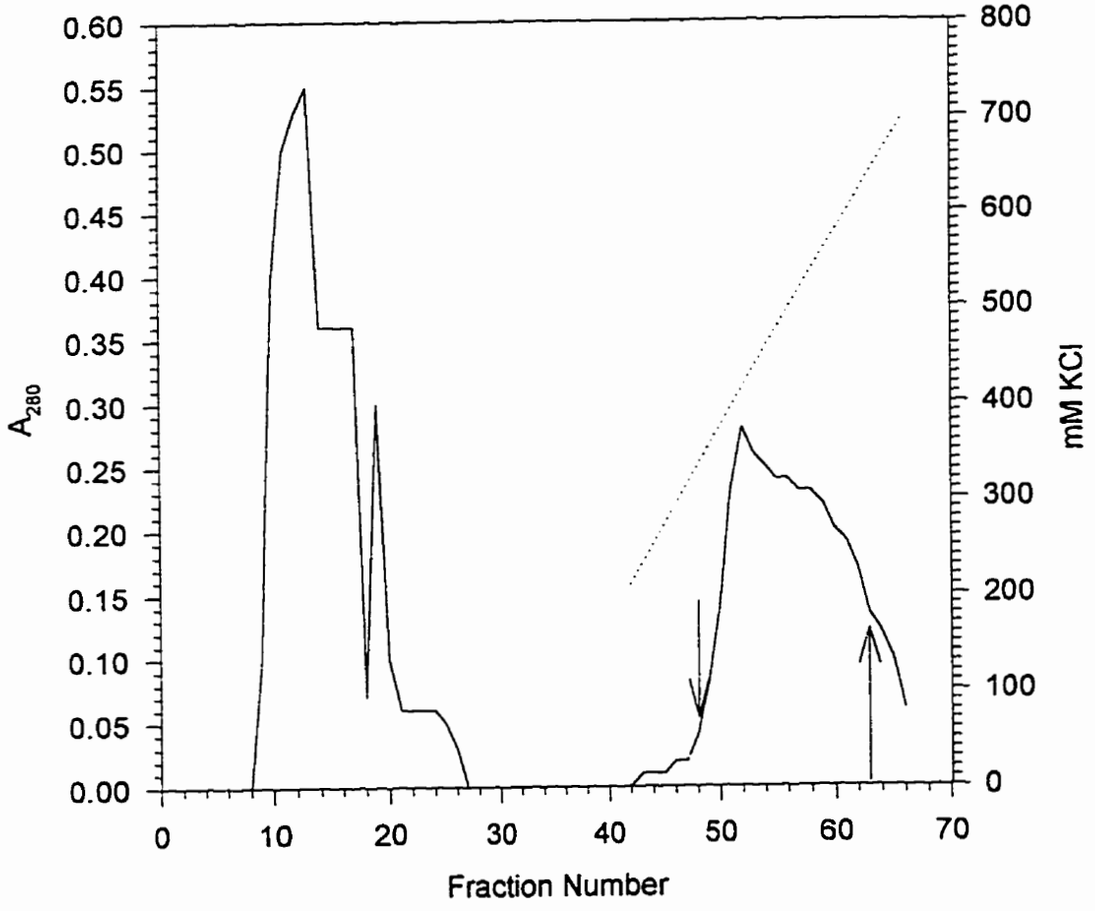

Figure 17. FPLC chromatogram for Q-Sepharose Fast Flow purification scheme I of *E. coli* glyoxalase I from DF502 containing pDM7. The dashed line represents the KCl salt concentration gradient. Active fractions (indicated in the region between the arrows) were collected and pooled.

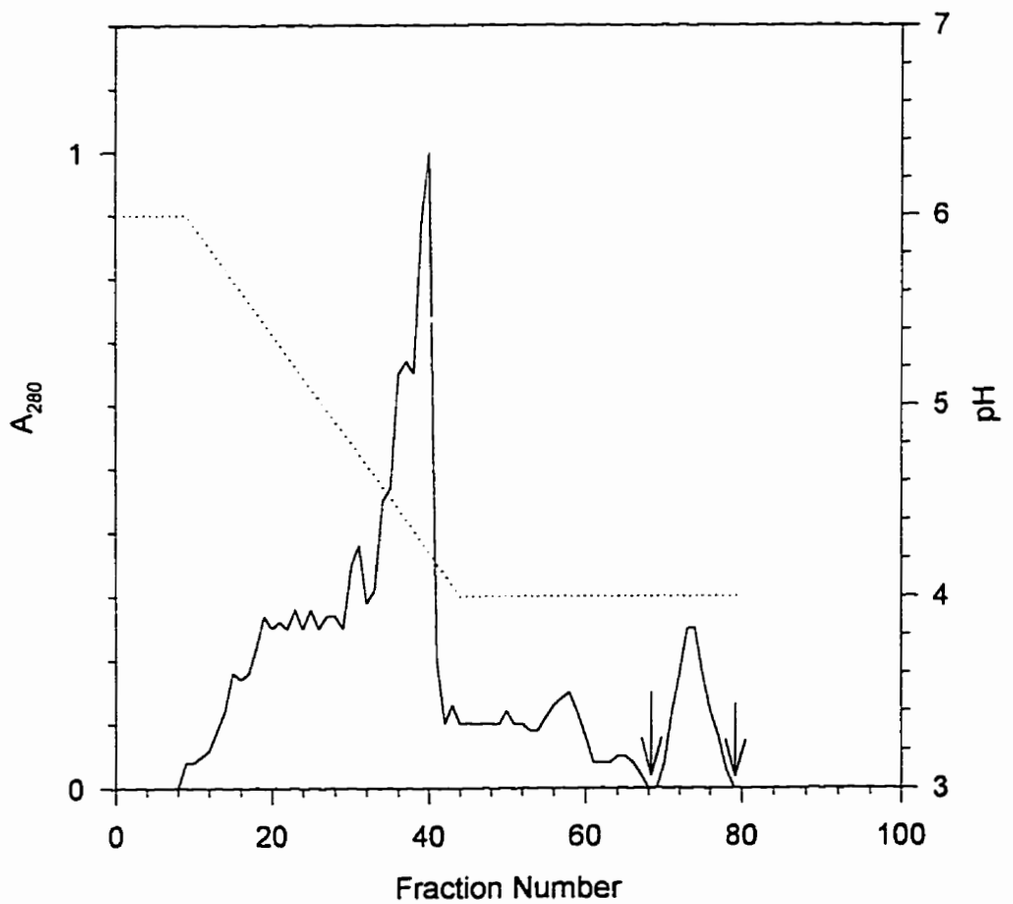

Figure 18. FPLC chromatogram for Mono P (chromatofocusing) purification scheme I of *E. coli* glyoxalase I from DF502 containing pDM7. The dashed line represents the pH gradient. Active fractions (indicated in the region between the arrows) were collected and pooled.

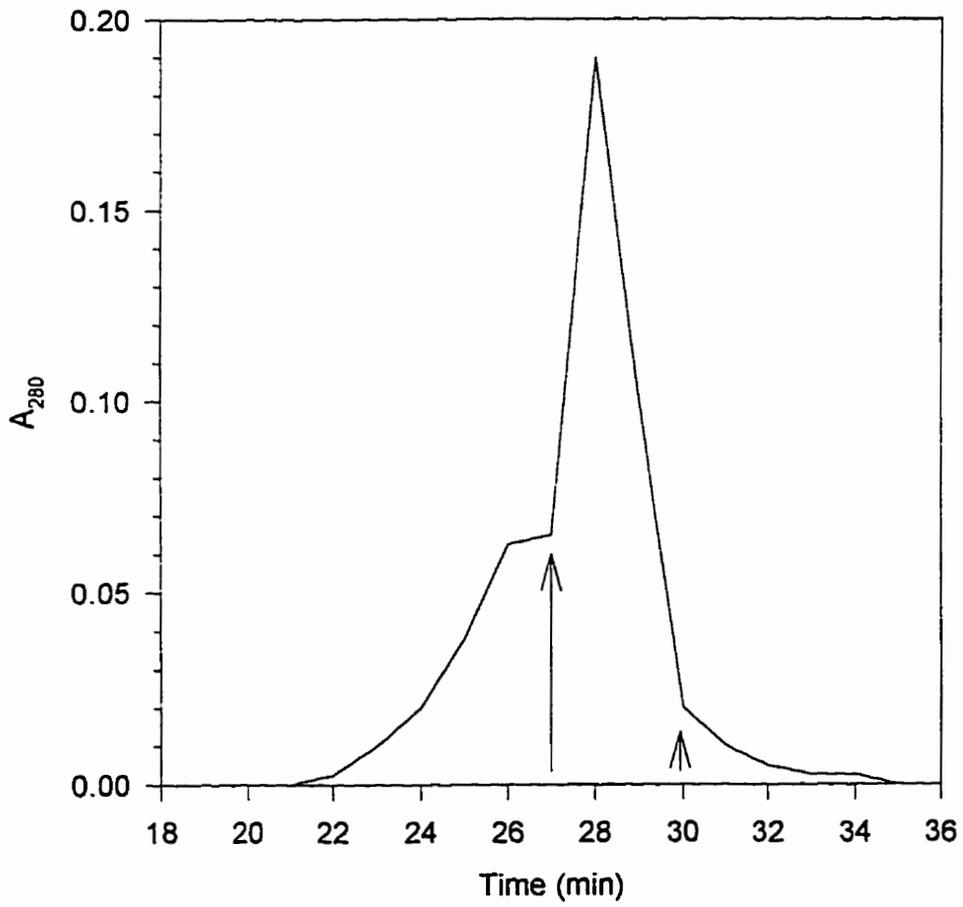

Figure 19. FPLC chromatogram for Superdex 75 purification scheme I of *E. coli* glyoxalase I from DF502 containing pDM7. Active fractions (indicated in the region between the arrows) were collected and pooled.

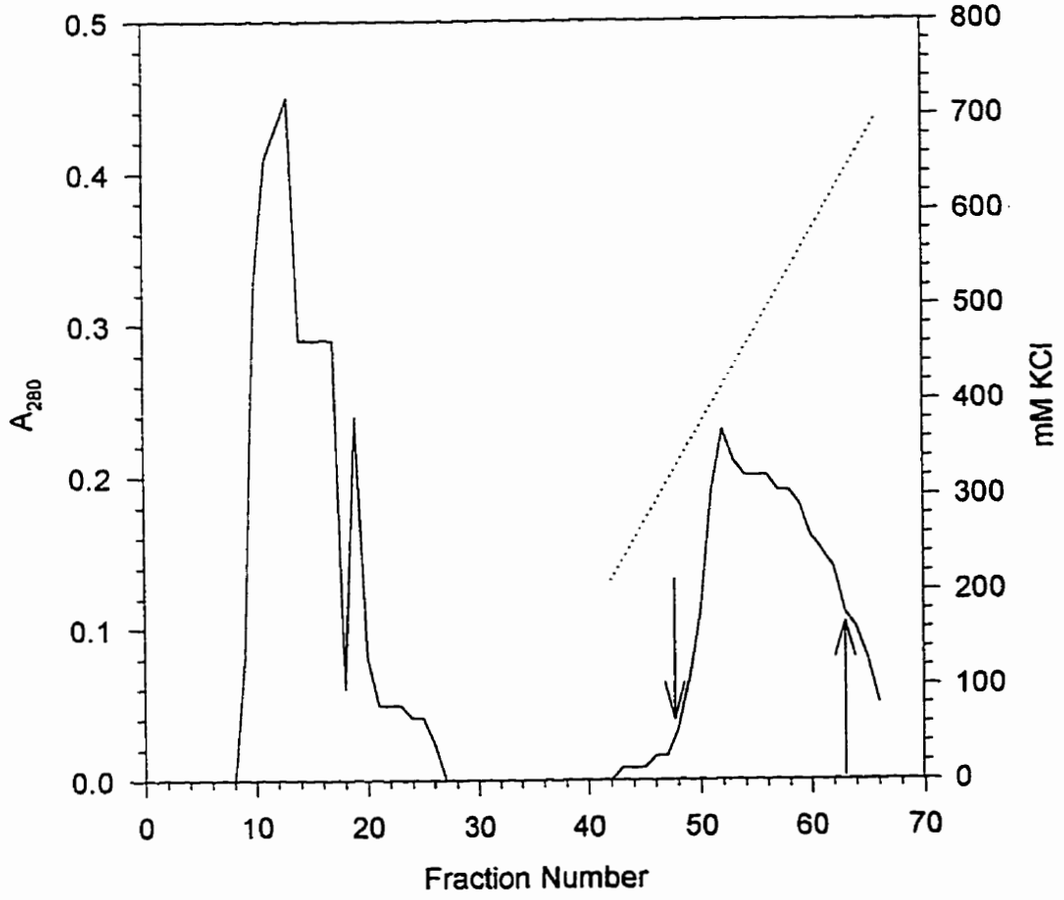

Figure 20. FPLC chromatogram for Q-Sepharose Fast Flow purification scheme II of *E. coli* glyoxalase I from DF502 containing pDM7. The dashed line represents the KCl salt gradient. Active fractions (indicated in the region between the arrows) were collected and pooled.

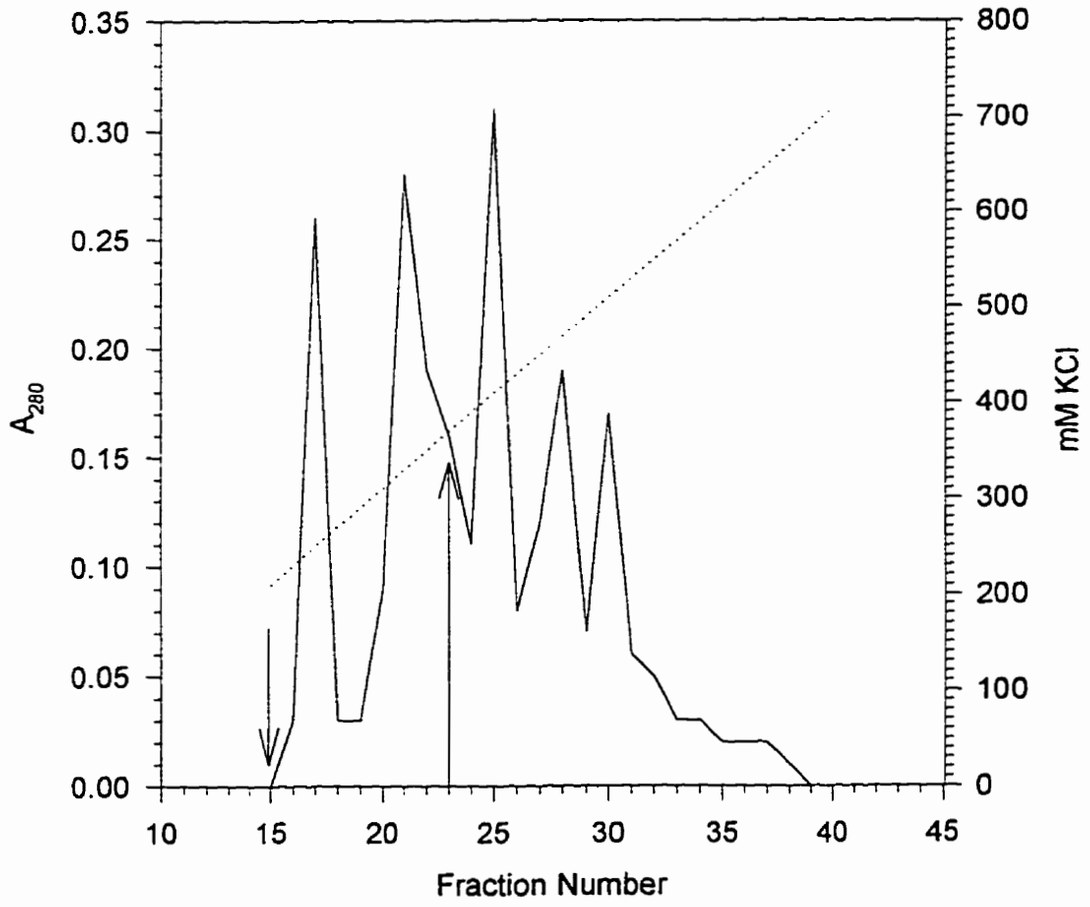

Figure 21. FPLC chromatogram for Mono P (ion-exchange) purification scheme II of *E. coli* glyoxalase I from DF502 containing pDM7. The dashed line represents the KCl salt concentration gradient. Active fractions (indicated in the region between the arrows) were collected and pooled.

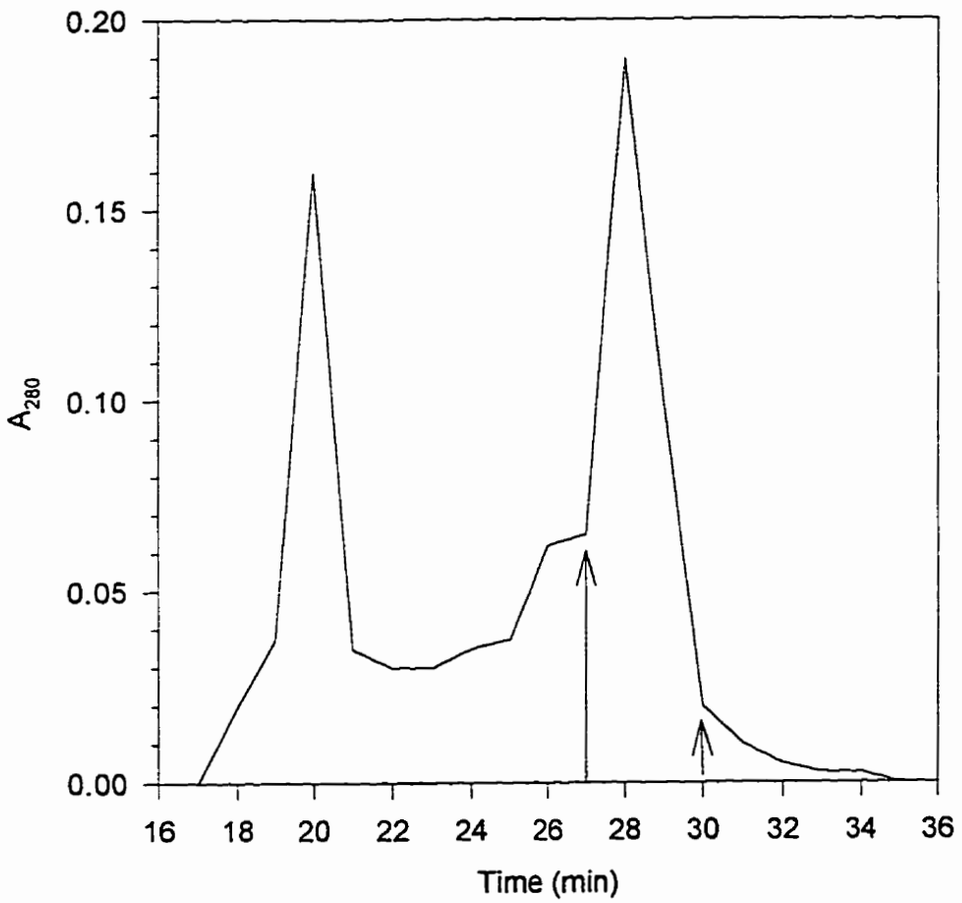

Figure 22. FPLC chromatogram for Superdex 75 purification scheme II of *E. coli* glyoxalase I from DF502 containing pDM7. Active fractions (indicated in the region between the arrows) were collected and pooled.

2.3 PCR Attempts to Overexpress *E. coli* Glyoxalase I

Attempts to overexpress *E. coli* glyoxalase I via polymerase chain reaction (PCR) methodology was performed by Dr. Elisabeth Daub. In brief, the general experimental procedures were as follows:

a. Design primers encoding for the 5′ and 3′ ends of the *E. coli* glyoxalase I gene to be amplified

b. Thermal cycling to promote DNA synthesis

c. Isolate DNA of interest from a low-melt agarose gel

d. Chloroform/phenol purification and ethanol precipitation of DNA at -20 °C

e. Cut pET22B vector and DNA with appropriate restriction enzymes (5′: Nde I; 3′: BamH I) and ligate DNA fragment to vector pET22B

f. Purify DNA fragment on agarose

g. Transform DH5α cells and plate to obtain colonies

h. Isolate plasmid DNA from individual transformants

i. Transform into competent BL21(λDE3) cells

j. Grow and induce cells using isopropylthioglactoside to determine if glyoxalase I has been overexpressed

Various DNA polymerases were used to amplify the DNA encoding for *E. coli* glyoxalase I. No DNA amplification was observed with Vent polymerase. Use of Taq polymerase resulted in amplification of the target DNA but the expressed gene product was unstable and greater than 75% of enzyme activity was lost in 12 hours. Very little expression of the desired gene product was observed when Pfu polymerase was used to amplify the glyoxalase I gene from *E. coli*. Many attempts were made to detect activity in the number of possible clones prepared by Dr. Daub, but to no avail. Since attempts to overexpress *E. coli* glyoxalase I proved troublesome when PCR was used to amplify *E. coli* glyoxalase I DNA this route was abandoned in favor of isolating the enzyme from DF502/pDM7.

3. Properties of *E. coli* Glyoxalase I

3.1 Molecular Weight Determination of *E. coli* Glyoxalase I

The molecular weight of native *E. coli* glyoxalase I was estimated from the results of purifications using a Superdex 75 HR 10/30 column.

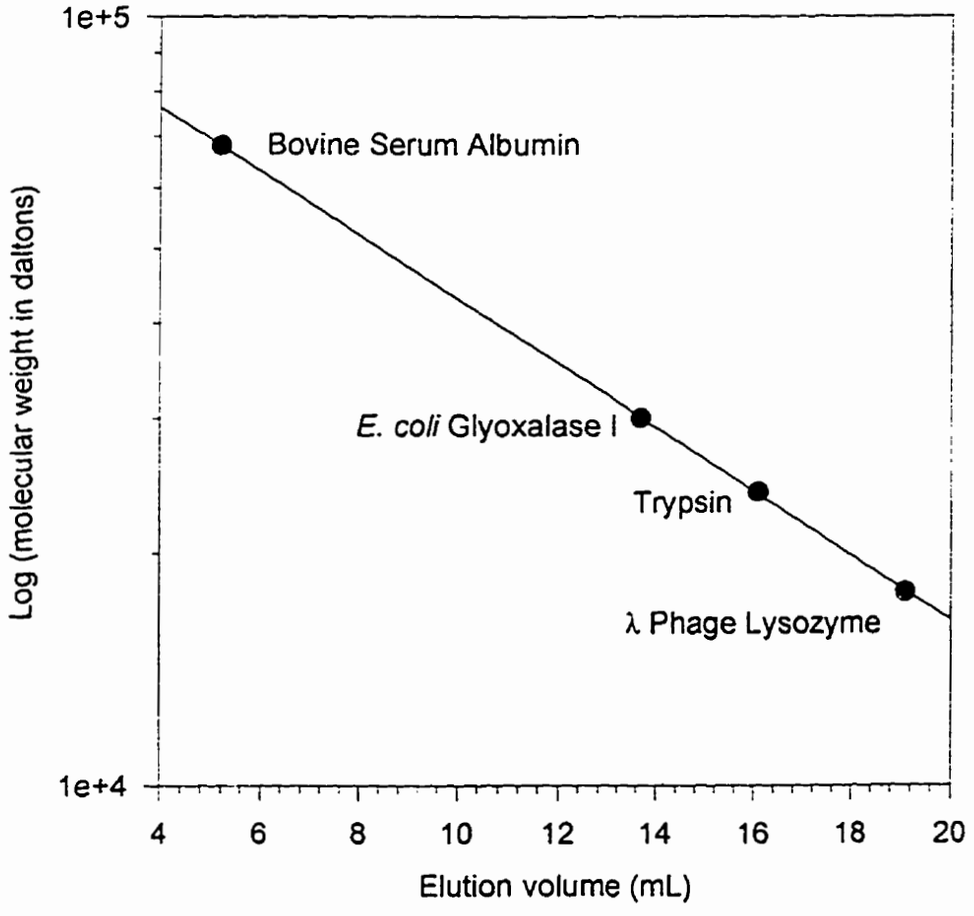

Figure 23. Molecular weight determination for native *E. coli* glyoxalase I.

By comparison of the elution volumes and known molecular weights of 3 other proteins (λ phage lysozyme, 17.8 kDa; trypsin, 24.0 kDa; bovine serum albumin; 68 kDa) with the elution volume of *E. coli* glyoxalase I the molecular weight of the native enzyme was determined to be approximately 30 kDa (Figure 23). Under denaturing conditions on a homogenous 20 Phast Mini-Gel and by comparison with migration profiles for phosphorylase (94 kDa), albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa), the predominant band appeared at approximately 15 kDa indicating that *E. coli* glyoxalase I is an apparent homodimer.

Electrospray mass spectrometry (ESMS) proved exceptionally useful in determining a more accurate molecular mass for the subunit of *E. coli* glyoxalase I. Under denaturing conditions the molecular weight was determined to be 14 921 ± 3 Da (Figure 24). Based upon this result, the molecular weight of the intact dimer can be calculated to be 29 842 Da and is fully consistent with our previous molecular weight studies.

A number of interesting observations were obtained from ESMS analysis. Initially it was believed that the molecular weight for the subunit was 14 992 Da. However this value was in disagreement with the predicted molecular mass of the glyoxalase I subunit based upon the cDNA

sequence. The value of 14 992 Da was determined from a PVDF-membrane electroblot. Before the sample was electroblotted to the membrane it was run on an SDS-PAGE gel. The extent of polymerization in such a gel may be 90% (Chiari *et al.*, 1992) which leaves 10% of reactive acrylamide. Previous studies have indicated that the sulfhydryl group of cysteine is the most likely side chain to be modified by residual, unreacted acrylamide (Chiari *et al.*, 1992; Dirksen and Chambrach, 1992). Modification of cysteine adds 71 Da per cysteine modified (Patterson, 1994). With respect to *E. coli* glyoxalase I there appears to be one cysteine per subunit (Figure 25) and there should be an increase of 71 Da compared to unmodified glyoxalase I and this was indeed found to be the case (14 992-14 921 = 71 Da). A difference of 71 Da is too small to be detected on a denaturing gel or molecular weight sieve but is readily detectable by ESMS where accuracy of 5 Da or less is not uncommon. This example serves as a caveat when molecular weight analysis is performed on a protein sample that has been exposed to an acrylamide gel.

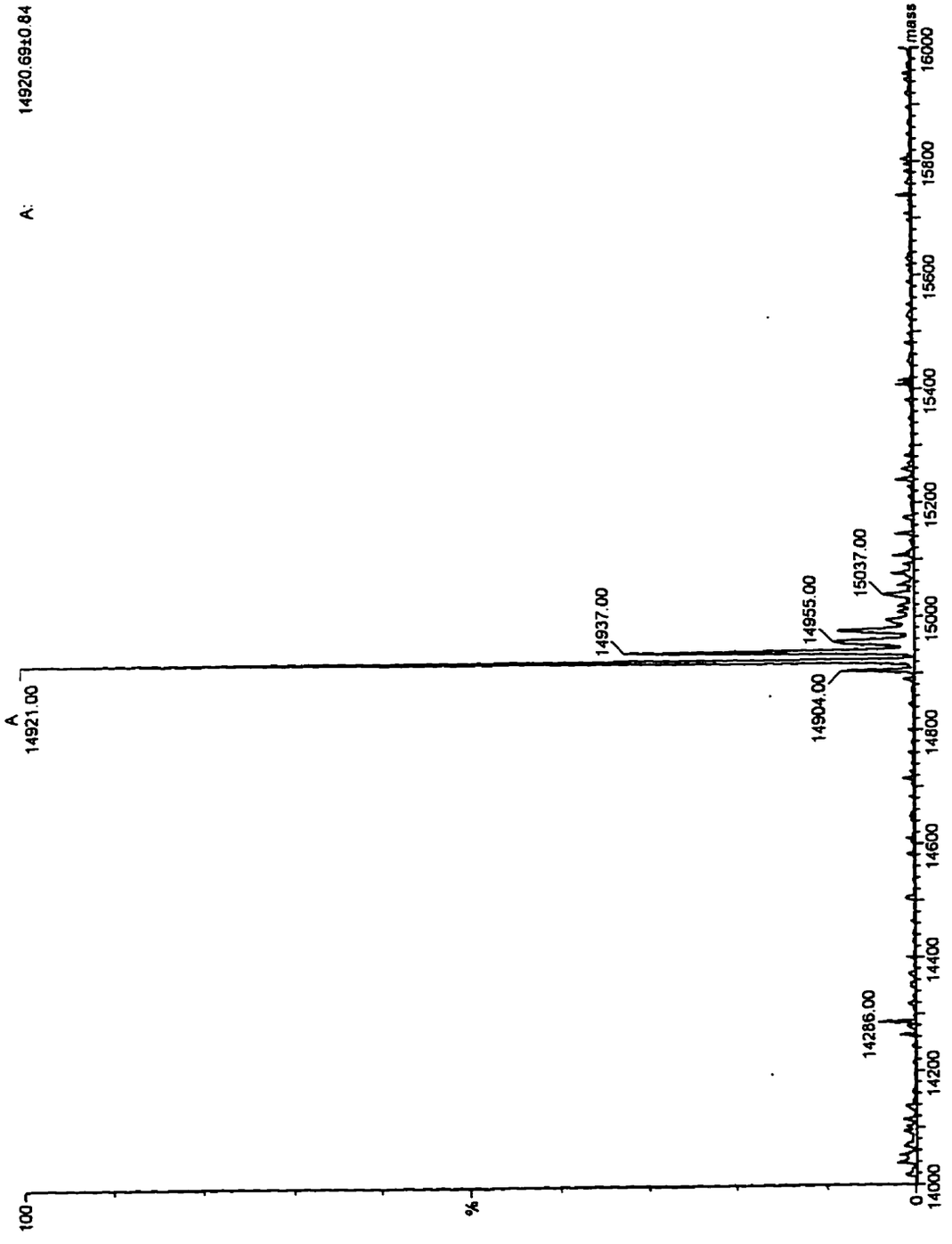

Figure 24. Electrospray-mass spectrum of the subunit of *E. coli* glyoxalase I.

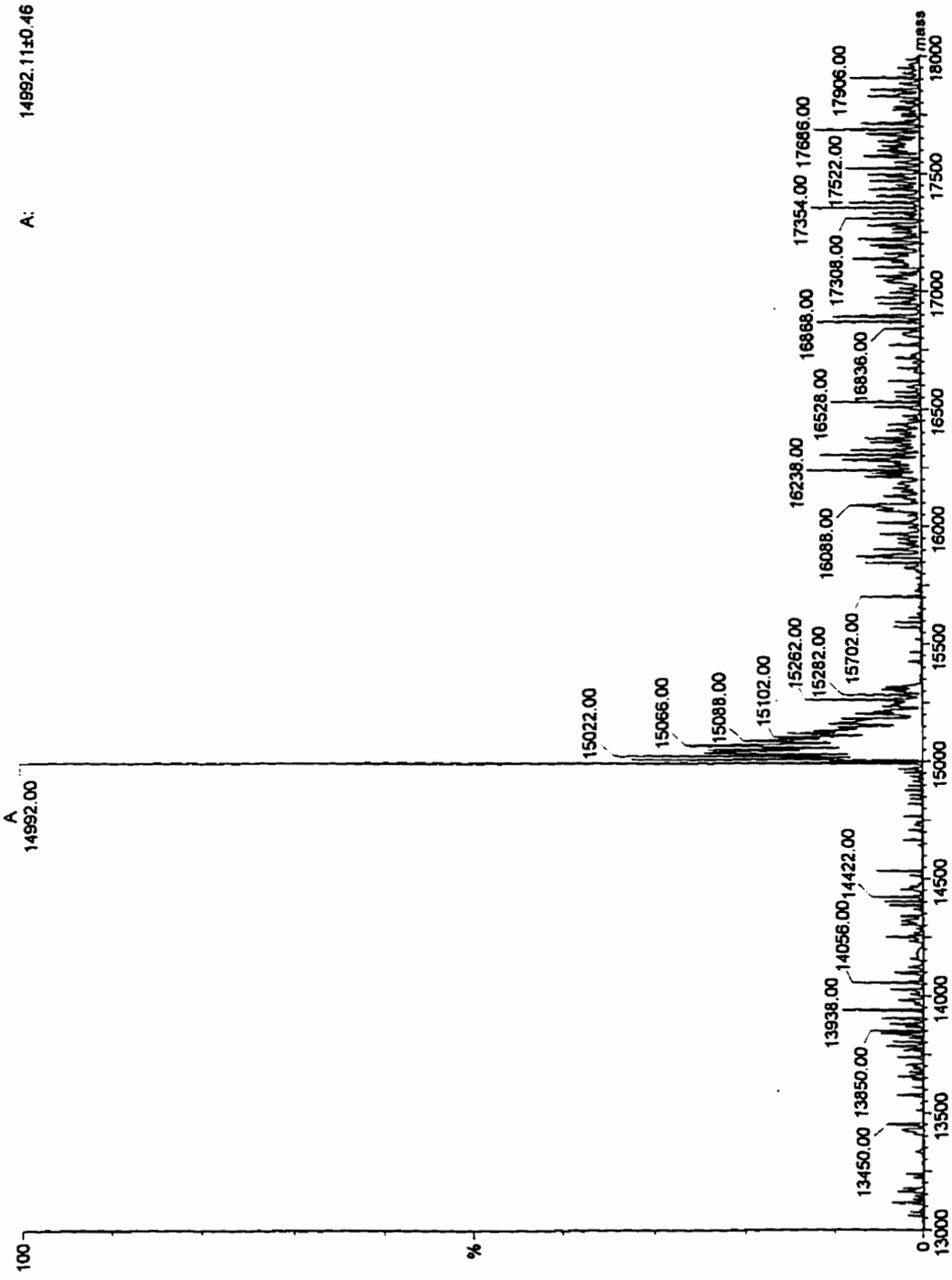

Figure 25. Electrospray-mass spectrum of *E. coli* glyoxalase I-acrylamide adduct.

During one particular purification, ESMS indicated that there was an apparent decrease in the molecular weight of the subunit after the Mono P (ion-exchange) purification step. A reduction from 14 921 Da to 14 578 Da (Figure 26) had occurred. This was unusual in that no such alteration in molecular weight had been observed in prior purifications. The most likely cause for such a decrease would be due to limited proteolysis of the subunit.

There exist at least 12 soluble cytoplasmic and periplasmic endoproteases in *E. coli* (Do, Re, Mi, Fa, So, La, Ci, Pi, protease I, protease II, Deg P and Rec A) (Lazdunski, 1989). Both protease I and protease II demonstrate trypsin-like specificity (Pacaud and Richard, 1975; Pacaud, 1978) and it has been determined that protease II will cleave the carboxymethylated B-chain of porcine insulin between Arg^{22}-Gly^{23}. Glyoxalase I from *E. coli* possesses one such potential Arg-Gly cleavage site (Arg^{131}-Gly^{132}) (Figure 15). Cleavage between these two residues would yield a subunit with molecular mass of 14 578 Da. Based upon the good agreement between the theoretical and empirically determined molecular masses it is likely that a bacterial protease was responsible for the truncation of the *E. coli* glyoxalase I subunit. It is interesting to note that *E. coli* glyoxalase I was fully active in this truncated form as determined by the standard glyoxalase I assay.

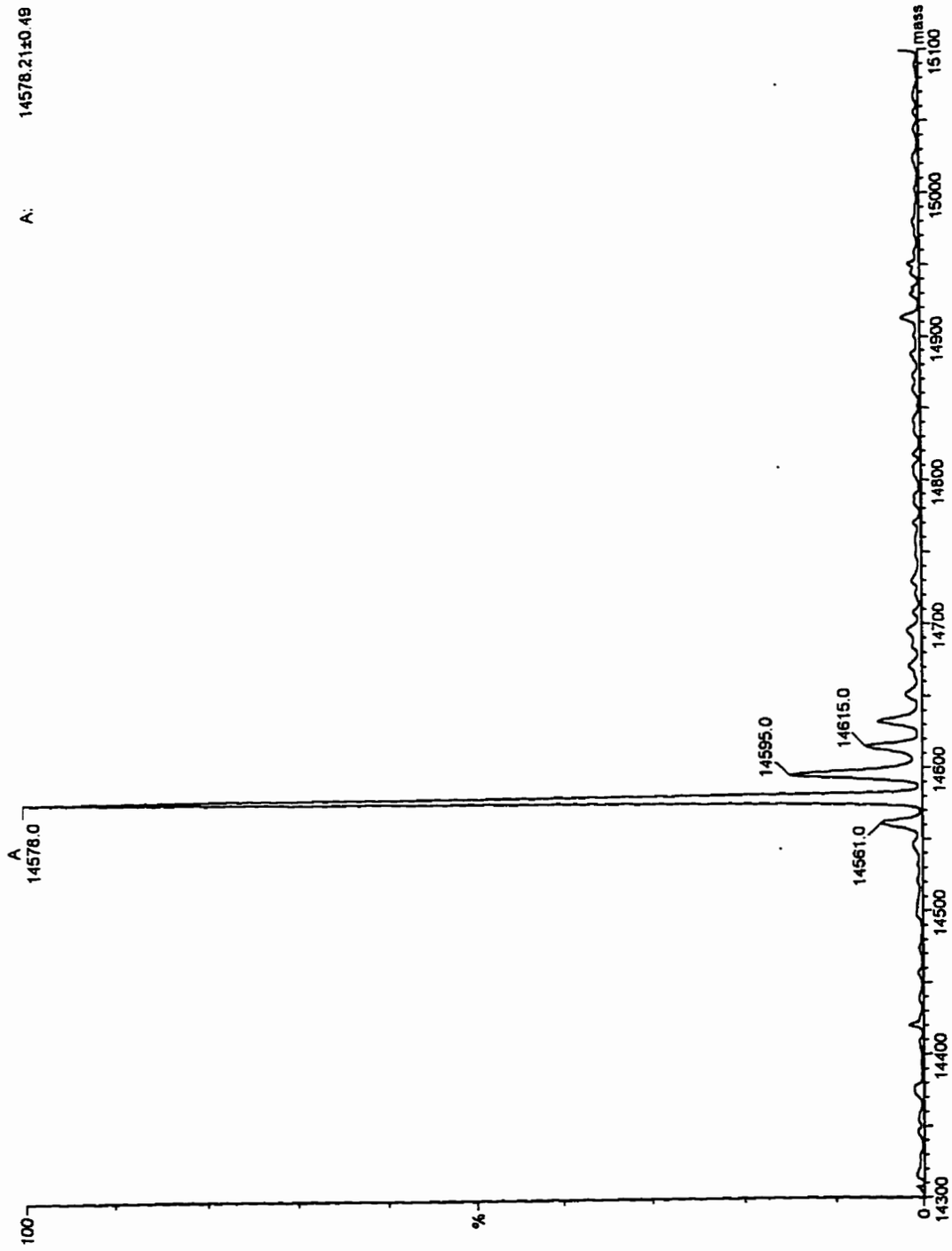

Figure 26. Electrospray-mass spectrum of truncated *E. coli* glyoxalase I.

Azocoll™ consists of an azodye attached to an insoluble, ground collagen matrix and is used to detect the presence of neutral and basic proteases (Chavira *et al.*, 1984). Attempts to use Azocoll™ to detect for the existence of proteases capable of cleaving *E. coli* glyoxalase I in crude extracts (cell lysates) proved unsuccessful. Azocoll™ is not suitable as a substrate for acidic proteases and if the protease responsible for cleavage between Arg^{131}-Gly^{132} is acidic it will not be detected using Azocoll™. During a typical glyoxalase I purification, cells would often be sonicated in 20 mM TRIS, pH 7.0 and 1.0 mM PMSF was added to inhibit serine proteases. For the first dialysis (after Q-Sepharose Fast Flow) another 1.0 mM PMSF was added. To combat further problems which may be due to proteolysis, fresh 1.0 mM PMSF was added every 3 hours during the first overnight dialysis. During subsequent purification no further proteolysis was noted with the new regimen.

3.2 Effect of Buffers and Glycerol on Stability of Glyoxalase I from *E. coli*

Glyoxalase I from *E. coli* was found to lose activity rapidly in a number of buffer systems over the course of two days. For all buffers tested greater than 50% of the initial specific activity was lost during this time (Figure 27). In the case of 25 mM histidine at pH 6.6, greater than 50% of the specific activity was lost within the first hour of incubating glyoxalase I with that particular buffer. Histidine is a known Ni^{2+} chelator (Smith *et al.*, 1988; Ljunquist *et al.*, 1989) and assuming that Ni^{2+} is the endogenous metal cofactor for *E. coli* glyoxalase I, may explain why histidine appears to rapidly inactivate glyoxalase I (see below). Imidazole is often used to help elute proteins possessing histidine tags which are bound to Ni^{2+}-chelator columns (Smith *et al.*, 1988; Ljunquist *et al.*, 1989). The lower binding affinity of imidazole for Ni^{2+} may explain why 25 mM imidazole, pH 7.0 inactivated glyoxalase I approximately 8-fold less rapidly than histidine buffer. Regardless of the buffer tested, 30%-45% of the initial specific activity remained after only 2 days with greatest stability being realized in 20 mM TRIS, pH 7.0 (Figure 28). These experiments were repeated several times, each time confirming the above buffer effects.

The presence of glycerol also had a pronounced effect on specific activity and enzyme lifetime. Even the presence of as little as 5% vol/vol caused an increase in specific activity as well as stabilization of the enzyme over a period of 2 days. The effect of 45% vol/vol glycerol compared to the control was to increase specific activity by a factor of approximately 2.0 over the course of approximately 9 days (Figure 28). Glycerol is a known and commonly used protein stabilization agent and is believed to work by simulating a low-water environment (Scopes, 1987). Glycerol is a small water-soluble molecule capable of forming hydrogen bonds with water thereby increasing the viscosity of water (25% glycerol is twice as viscous as water and 50% glycerol is 6 times as viscous) (Scopes, 1987). It is believed that proteins are less able to unfold against the highly structured glycerol solution than in water (Gekko and Timasheff, 1981) and hence protein stability is maintained. It is not known whether the increase in specific activity is due to the potential presence of trace quantities of metals capable of activating *E. coli* glyoxalase I. The greatest effect on enzyme lifetime was achieved with the greatest glycerol concentration tested. Due to FPLC column pressure limits most stages of purification could handle a maximum of only 15% vol/vol but after enzyme purification was complete the enzyme was stored in buffer containing 50% vol/vol glycerol.

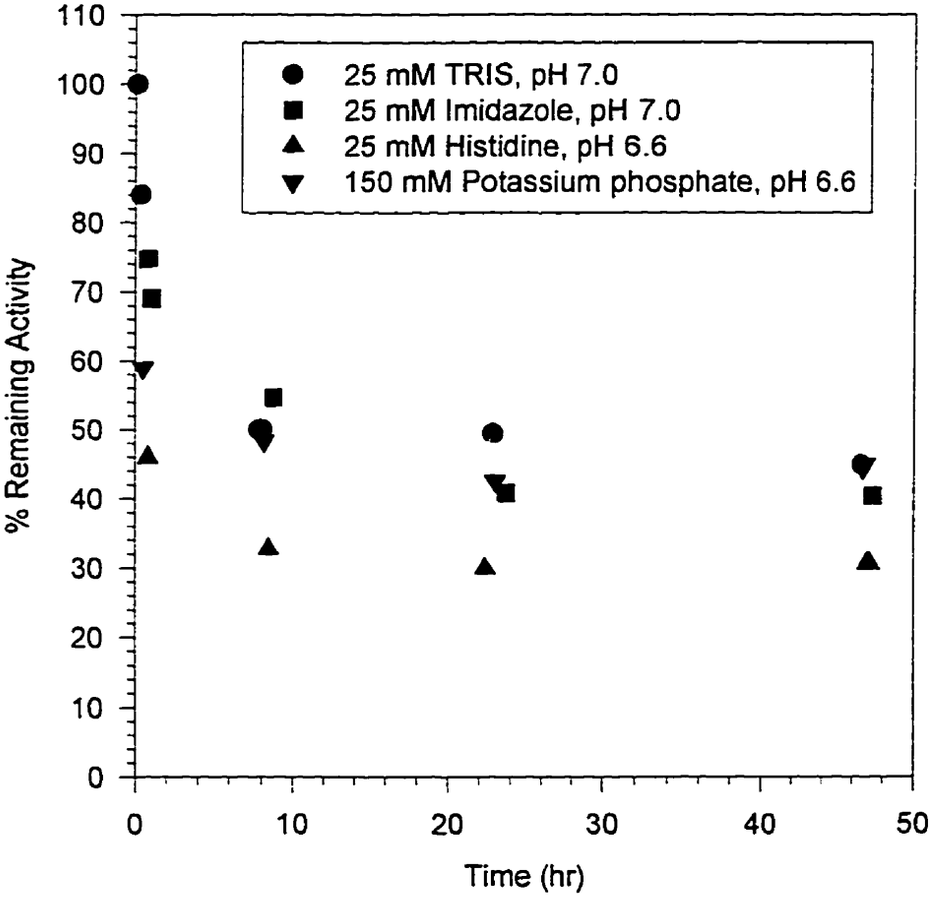

Figure 27. Effect of various buffers on *E. coli* glyoxalase I (PMSF-treated crude lysates) stability.

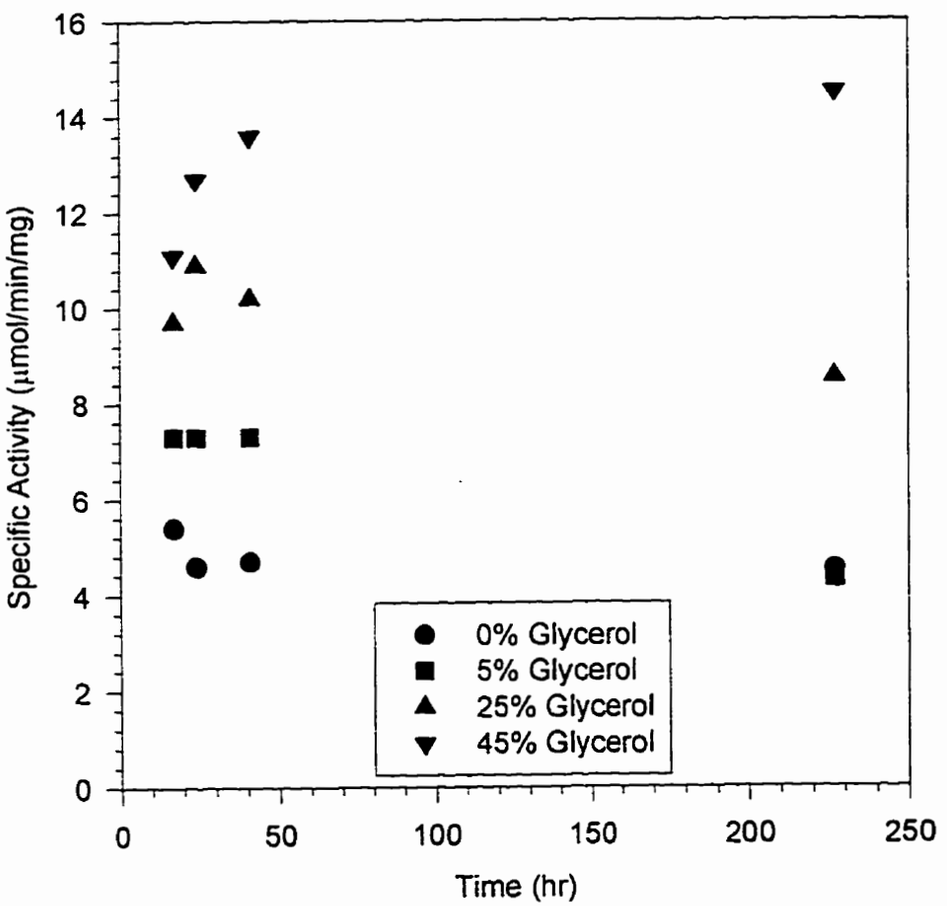

Figure 28. Effect of 0%, 5%, 25% and 45% glycerol on *E. coli* glyoxalase I activity (PMSF-treated crude lysates).

3.3 pH Stability and Isoelectric Point (pI) of *E. coli* Glyoxalase I

A number of investigators have determined that yeast glyoxalase I has a broad pH optimum between pH 6-8 (Racker, 1951; Vander Jagt and Han, 1973; Jerzykowski *et al.*, 1973). In-depth research by Vander Jagt and Han demonstrated that V_{max} was insensitive to pH between pH 4.5-9 but K_m values increased at high and low pH. They interpreted these results to indicate the presence of dissociable groups with pK values of approximately 5.0 and 8.5. Studies on sheep liver glyoxalase I indicated that the enzyme from this source also possessed a broad pH optimum (pH 5.5-7.5) (Uotila and Koisuvalo, 1975). The enzyme from *P. putida* has a pH optimum of 8.0 and at pH 5.0 and pH 9.0 the activity is one-half that at pH 8.0 (Rhee *et al.*, 1986). The enzyme from human erythrocytes has a pH optimum of 7.0 but activity is relatively constant between pH 6.5-7.5 (Aronsson *et al.*, 1979). Studies on glyoxalase I from peas indicated that the pH optimum was achieved at pH 7.5. At pH 7.0 there was a marked increase in activity and at pH 8.0 a decrease in activity was noted (Ramaswany *et al.*, 1983).

The pH profile for glyoxalase I from *E. coli* increases rapidly after pH 5.0 and levels out at approximately pH 8.0 which may indicate the presence of a single crucial ionizable group (Figure 29). A simple analysis of this plot indicates that the pK_a of the amino acid involved

will occur at approximately the mid-point of the curve (approximately pH 6.0; Figure 29). This would seem to suggest the presence of histidine but inactivation studies involving DEPC did not result in inactivation of *E. coli* glyoxalase I. Perhaps the amino acid involved is sufficiently perturbed and possesses an altered pK_a value than that determined in an aqueous environment. In addition, the macroscopic pK_a seen in these plots may not simply yield a microscopic pK_a of a single essential residue but it is a composite of the entire protein pK_a values important in the activity of the enzyme at this pH. This may then implicate a cysteine as a crucial amino acid. Modification studies involving iodoacetamide did not lead to enzyme inactivation whereas $HgCl_2$ led to a 70% decrease in activity. If a crucial cysteine is involved then its modification is dependent upon its surrounding environment.

An interesting point to note is that incubation of glyoxalase I with BICINE buffer between pH 7.81-8.83 and glycine between pH 8.67-10.77 (Figure 30) resulted in considerable loss of activity when compared to preincubating the enzyme with boric acid between pH 8.00-10.15.

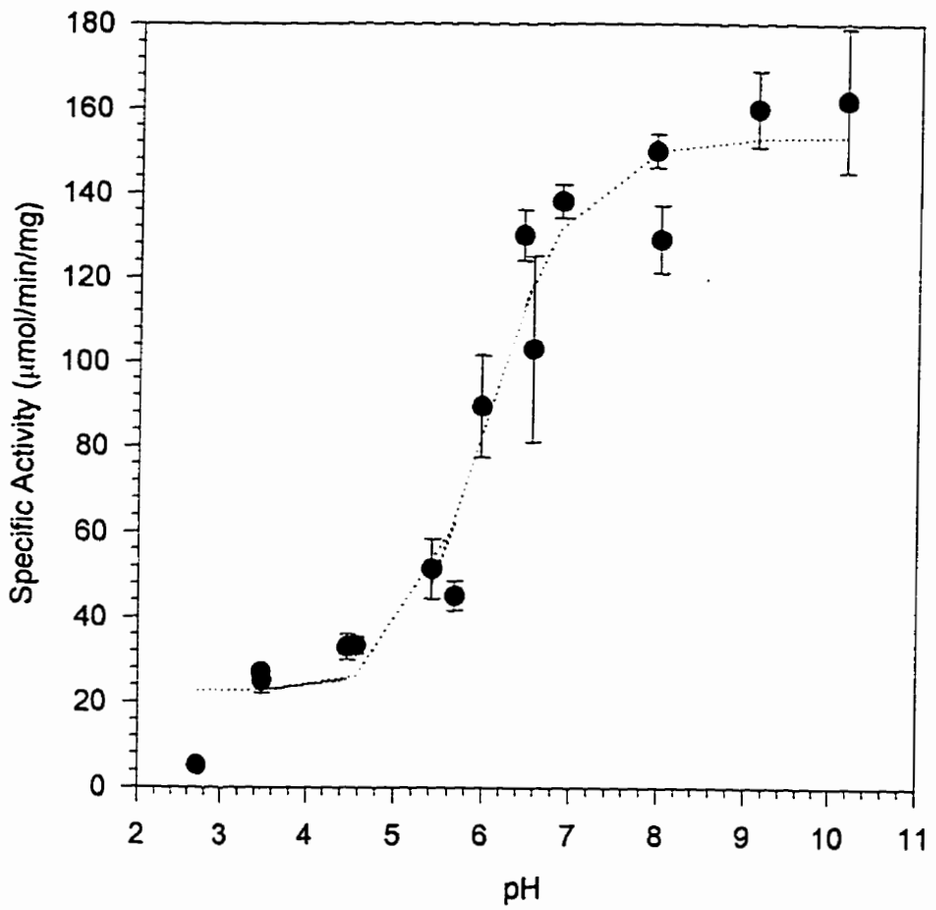

Figure 29. pH profile for *E. coli* glyoxalase I when 50 mM boric acid is used as a buffer between pH 8.0 to 10.15.

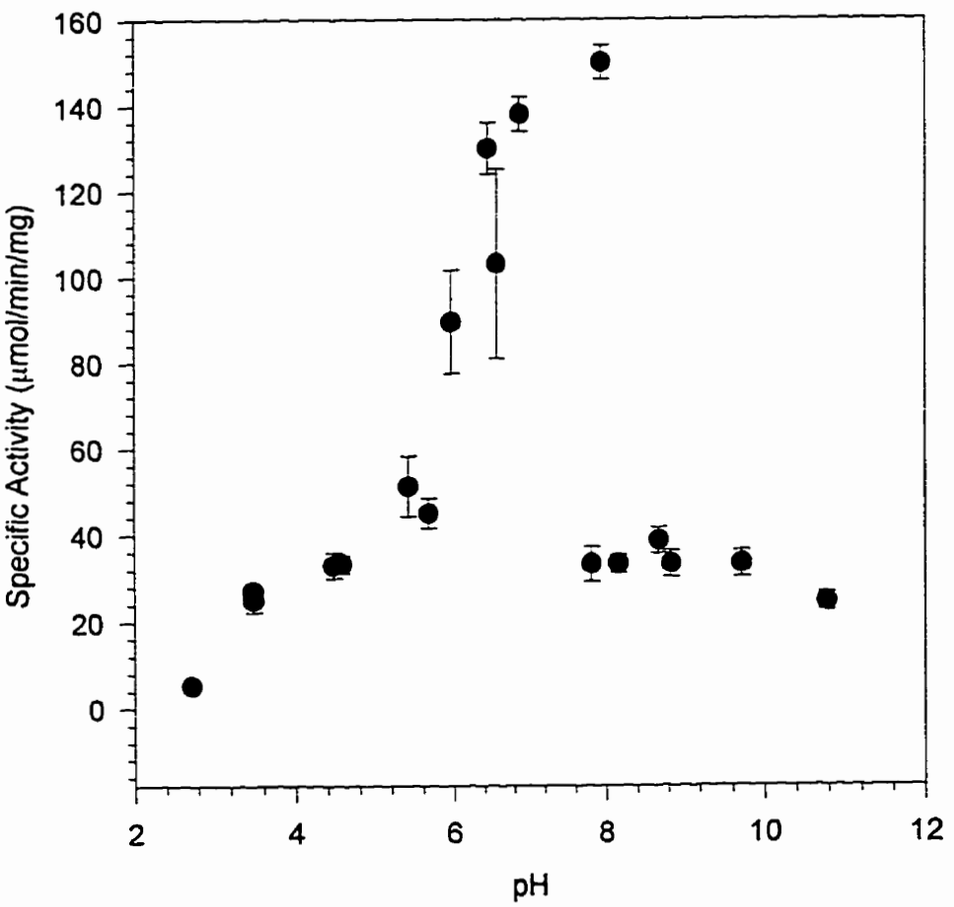

Figure 30. pH profile for *E. coli* glyoxalase I when 50 mM BICINE is used as a buffer between pH 7.81 to 8.83 and 50 mM glycine is used as a buffer between pH 8.67 to 10.77.

Since the pH ranges are comparable then the next most likely cause for loss of activity could be due to glycine and BICINE acting as metal chelators and removing the metal cofactor believed to be necessary for catalysis.

Samples that underwent preparative isoelectric focusing using BioLyte 3-10 had measurable activity covering the pH range 4.15-5.15. This result correlates with what is known concerning *E. coli* glyoxalase I to bind to anionic resins such as Q-Sepharose Fast Flow and Mono Q. However, further refinement of the isoelectric point value is required. A similar result was obtained utilizing the Mono P column as an isoelectric focusing column. With Mono P an isoelectric point of approximately 4.0 was obtained. However, extended elution was required to remove the protein from the column and the pI value of 4.0 may be low due to possible complicating factors such as hydrophobic interactions between the enzyme and the resin matrix. A value of 4.7 for the pI of the protein can be predicted from the amino acid sequence for *E. coli* glyoxalase I. This result is in good agreement with those values determined experimentally from chromatofocusing results.

3.4 Effect of Metals on Glyoxalase I from *E. coli*

Previous studies on human and yeast glyoxalase I have indicated that the holoenzymes from both sources contained zinc (Aronsson *et al.*, 1978). However, the human enzyme could be activated by Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, V^{2+} and Ga^{3+} (Davis and Williams, 1966; Uotila and Koivusalo, 1975; Han *et al.*, 1977; Aronsson *et al.*, 1978; Vander Jagt and Topscott, 1978; Vander Jagt *et al.*, 1980; Sellin *et al.*, 1983; Sellin and Mannervik, 1984). With respect to methylglyoxal as the substrate, the relative k_{cat} of the human enzyme with various metals did not vary by more than a factor of 2 or 3 (Table 6).

A number of studies have been performed on mammal, yeast and *Pseudomonas putida* glyoxalase I enzymes with respect to the effect of metals on the enzyme from these sources. Work by Davis and Williams on the effect of metals on calf-liver glyoxalase I determined that Mg^{2+} and Mn^{2+} (ΔA_{240} of 0.138 and 0.123, respectively due to formation of S-*D*-lactoylglutathione) were more effective than Ni^{2+}, Zn^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} (ΔA_{240} of 0.030, 0.030, 0.024, 0.007 and 0.004, respectively due to formation of S-*D*-lactoyl-glutathione; control = 0.004) (Davis and Williams, 1966).

Studies on the enzyme from sheep liver using N-2-hydroxyethyl piperazine-N-2'-ethane sulfonate (HEPES), pH 6.8 indicated that Mg^{2+} restored full enzymatic activity and that Zn^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+} gave partial reactivation (Uotila and Koivusalo, 1975). However Zn^{2+} had the lowest half-saturation constant (7 μM) while Mg^{2+} had the greatest value (2.8 mM) (Uotila and Koivusalo, 1975).

Experiments involving apoglyoxalase I from pig erythrocytes demonstrated that at 2 hour incubations involving 1 mM M^{2+} that Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} gave 65%, 59%, 62% and 46%, respectively, activity of the holoenzyme (Aronsson and Mannervik, 1977). However, the extent of reactivation was both concentration- and time-dependent. Below 0.1 mM M^{2+} no significant activation was observed while maximal activation was achieved only with 10 mM Mg^{2+}. Metals such as Fe^{2+}, La^{3+}, Ca^{2+} and Cu^{2+} did not reactivate the enzyme (Aronsson and Mannervik, 1977).

Yeast glyoxalase I which had been treated with ethylenediamine tetraacetate (EDTA) was capable of being partially reactivated by Mg^{2+} and Ca^{2+} and only slightly activated by Mn^{2+}. Cations such as Fe^{2+}, Ni^{2+} and Co^{2+} had no effect on the enzyme activity and Zn^{2+} was shown to be inhibitory (Murata *et al.*, 1985).

Rhee and co-workers detected no obvious contribution of metals to enzyme activity of glyoxalase I from *P. putida*. Zinc ions were found to be inhibitory (75% inhibition at 0.100 mM and complete inhibition at 1.0 mM) whereas 1.0 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+} and Hg^{2+} did not affect enzyme activity (Rhee *et al.*, 1986).

Our studies with metals and glyoxalase I from *E. coli* indicate that Ni^{2+} in 100 mM MES, pH 6.0 most effectively activated the enzyme (Table 25). With respect to the enzyme control (no metal preincubation) the order of effectiveness of activation was Ni^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, control, Zn^{2+} and Hg^{2+}. The specific activities determined for calcium and magnesium involved preincubation of the enzyme in 25 mM MOPS, pH 7.6. From the data concerning pH profile (Figure 26) exposing the enzyme to MOPS at pH 7.6 appears to activate the enzyme by 40% more than exposing the enzyme to MES at pH 6.0. If one applies a 40% corrective factor for specific activity to calcium, the result would then place calcium behind manganese in terms of velocity enhancement. Applying a corrective factor to magnesium would place this metal on the same activity as control levels in ability to activate the enzyme. From Tables 25 and 26 it can be seen that there is an approximate correlation between ability to activate *E. coli* glyoxalase I and ionic radius for the various metals tested. The trend is that activation increases with

decreasing ionic radius. Calcium is somewhat anomalous since it possesses the largest ionic radius of all metals tested and is still capable of activating the enzyme.

Nickel is considered to be an essential trace element and is known to be present in jack bean urease, some hydrogenases present in methanogenic bacteria, aerobic hydrogen-oxidizing bacteria, phototropic bacteria and aerobic nitrogen-fixing bacteria. The metal is also present in some acetogenic bacteria and methanogenic bacteria and methyl reductase from methanogens (Cotton and Wilkinson, 1982; Hausinger, 1987). The existence of urease has been known since 1925 and was the first enzyme to be crystallized but the existence of tightly bound nickel was not discovered until 1975 (Cotton and Wilkinson, 1982). The enzyme catalyzes the hydrolysis of urea to ammonia and bicarbonate and the role of the nickel indicates its importance in acting as a Lewis acid in the mechanism of hydrolysis.

Table 25. Effect of various divalent cations on activation of *E. coli* glyoxalase I.

Metal	Ionic Radius (Angstroms)	Specific Activity (μmol/min/mg)[a]
None [b]	-	45 ± 2 (41 ± 3)[d,e]
20 mM $HgCl_2$	0.83[c]	13 ± 2 (13 ± 2)
20 mM $ZnCl_2$	0.74	40 ± 3 (27 ± 2)
20 mM $MnCl_2$	0.80	127 ± 3 (129 ± 3)
20 mM $CoCl_2$	0.72	253 ± 9 (291 ± 6)
20 mM $NiCl_2$	0.69	577 ± 18 (653 ± 60)
2.0 mM $NiCl_2$	0.69	518 ± 29
0.20 mM $NiCl_2$	0.69	338 ± 18

a. 1.55 μg of glyoxalase I used per assay replicate; b. metals and enzyme were preincubated in 100 mM MES, pH 6.0; c. values from Cotton and Wilkinson, 1982; d. specific activities in were generated from 5 minutes of metal-enzyme preincubation. Numbers in parentheses indicate 15 minutes of metal-enzyme preincubation; e. assays were performed in triplicate.

Table 26. Effect of various metals on activation of *E. coli* glyoxalase I.

Metal	Ionic Radius (Angstroms)	Specific Activity (μmol/min/mg)[a]
None[b]	-	54.7 ± 2.3 (48.9 ± 1.0)[d,e]
200 mM NaCl	-	53.5 ± 4 (54.7 ± 2)
200 mM NaBr	-	46.7 ± 2 (44.7 ± 3.2)
200 mM KCl	-	51.3 ± 5 (46.4 ± 3)
200 mM KBr	-	50.7 ± 6 (46 ± 2)
20 mM $CaCl_2$	1.14[c]	198 ± 17 (215 ± 8)
20 mM $MgCl_2$	0.71	82 ± 2 (87 ± 3)

a. 1.55 μg of glyoxalase I used per assay replicate; b. metals and enzyme were preincubated in 25 mM MOPS, pH 7.6; c. values from Cotton and Wilkinson, 1982; d. specific activities in were generated from 5 minutes of metal-enzyme preincubation. Numbers in parentheses indicate 15 minutes of metal-enzyme preincubation; e. assays were performed in triplicate.

Methyl reductase from *Methanobacterium thermoautotrophicum* is an enzyme of molecular weight 300 kDa and consists of 3 subunits of molecular weight 68 kDa, 45 kDa and 38.5 kDa (Rouviere and Wolfe, 1988). It contains a cofactor termed F430 which is a tetrahydrocorphin capable

of binding nickel (II) (Rouviere and Wolfe, 1988). The inactive form of methyl reductase contains Ni^{2+} whereas the active form contains Ni^{+}. Reduction of Ni^{2+} to Ni^{+} requires two proteins designated A2 and A3, adenosine triphosphate (ATP) and a source of donor electrons either H_2 or titanium (III) citrate (Rouviere and Wolfe, 1988). Nickel-containing hydrogenases are found in many bacterium including *E. coli* (Hausinger, 1987; Walsh and Orme-Johnson, 1987). The cofactor content of these hydrogenases also contains iron-sulfur clusters and sometimes FAD. The nickel site may be in the form of nickel tetrathiolate in a distorted tetragonal array and the nickel may be involved in H_2 binding and fragmentation (Hausinger, 1987; Walsh and Orme-Johnson, 1987). Several anaerobic micro-organisms are capable of oxidizing carbon monoxide to carbon dioxide. Some anaerobic acetogens and methanogens possess carbon monoxide dehydrogenases which contain nickel (Hausinger, 1987; Walsh and Orme-Johnson, 1987). The cofactor content also includes zinc and iron-sulfur clusters. The proposed nickel site is postulated to be a mixed iron-nickel cluster and the nickel is believed to be involved in C-C bond formation and cleavage (Hausinger, 1987; Walsh and Orme-Johnson, 1987). In all these cases, except urease, the nickel functions with alteration in its redox state. It is doubtful whether nickel in glyoxalase I alters its redox state from Ni^{2+}. It may be that Ni^{2+} is either acting to polarize the substrate for isomerization similar to the

Lewis acid role it plays in urease or the Ni^{2+} simply plays solely a structural role to maintain glyoxalase I native structure. It is also unknown at the present whether another metal such as zinc is in fact tightly bound to the active site. Metal analysis of the purified protein should aid in the determination of this possibility.

It is believed that there exists a nickel-specific transport system in *E. coli*. The *nik* locus encodes for 5 proteins (NikA-NikE) (Navarro *et al.*, 1993). NikA is the periplasmic binding protein, NikB and NikC are similar to periplasmic permeases and NikD and NikE contain ATP-binding domains (Navarro *et al.*, 1993). Mutations of nik affect nickel-dependent hydrogenase activities. Excess nickel in the growth media was found to reactivate hydrogenase activity (Navarro *et al.*, 1993).

Further work will have to be performed to see if Ni^{2+} is the endogenous metal of *E. coli* glyoxalase I. Dr. Elisabeth Daub has performed studies on the effect on metals on growth of *E. coli* plated onto media that has been treated with methylglyoxal and has determined that the presence of Ni^{2+} can enhance bacterial survivability when Ni^{2+} is present. Similar studies involving Zn^{2+} and Mg^{2+} indicated that those two particular cations did not aid in bacterial survivability in the

presence of MG. Surprisingly Zn^{2+} does not activate the enzyme from *E. coli* in contrast to what has been reported for glyoxalase I from several other organisms. Either Zn^{2+} is not required for this particular enzyme or that Zn^{2+} is tightly bound and is not released from the enzyme, but that a second metal, such as Ni^{2+}, is required for purely structural reasons with the Zn^{2+} being required in the active site as previously suggested for other glyoxalase enzymes.

3.5 Metal-Chelation Studies on Glyoxalase I from *E. coli*

Studies on glyoxalase I from beef liver have determined that 2 mM EDTA was sufficient to completely inactivate the enzyme from that source. In the same study, it was found that yeast glyoxalase I lost 90% of its activity when exposed to 1 mM EDTA for 12 hours or was completely inactivated in 10 minutes by 10 mM *o*-phenanthroline (Williams and Davis, 1966). Work by Murata and co-workers has demonstrated that at 5.0 mM EDTA, yeast glyoxalase I retained 50% of its activity whereas at 15.0 mM the enzyme was completely inhibited (Murata *et al.*, 1985). Studies on the enzyme from *P. putida* have indicated that glyoxalase I from that source was not affected by the addition of 10 mM EDTA (Rhee *et al.*, 1986) indicating that perhaps that particular enzyme is not a metallo-protein or perhaps implying that if a metal is present it must be tightly bound by the enzyme. The effect of 1.0 mM EDTA in 25 mM TRIS pH

7.8 was sufficient to completely inactivate the enzyme from pig erythrocytes (Mannervik *et al.*, 1972). More in-depth studies involving sheep liver glyoxalase I exposed to various concentrations of EDTA, ethyleneglycol-bis(2-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), *o*-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline in either 100 mM imidazole, pH 6.8 or 100 mM HEPES, pH 6.8 indicated that buffer choice in addition to the actual chelator utilized, play an important role in the extent of inactivation of the enzyme (Uotila and Koisuvalo, 1975). For all metal chelators tested the greatest inhibition was observed in the presence of 100 mM imidazole, pH 6.8.

There was a profound difference in the rates of EDTA inactivation of *E. coli* glyoxalase I depending upon the buffer in which the incubation was performed. When 1.0 mM EDTA and 50 mM potassium phosphate, pH 6.6 were incubated with the enzyme, approximately 85% of the activity was lost within 1 minute (Figure 31). When 50 mM MOPS, pH 7.9 was used as a buffer with 1 mM EDTA and 1 mM EGTA the rate of inactivation was considerably decreased. Results with 1 mM EGTA in 50 mM MOPS, pH 7.9 were essentially identical to those obtained for 1.0 mM EDTA (Figure 32).

The rate of inactivation of *E. coli* glyoxalase I for 1.0 mM EDTA in 50 mM MOPS, pH 7.9 is -0.388 ± 0.020 min^{-1} and the constant of inactivation *E. coli* glyoxalase I for 1.0 mM EGTA in 50 mM MOPS, pH 7.9 is -0.351 ± 0.031 min^{-1}. The pKa values for the functional groups in EDTA are 1.99, 2.67, 6.16 and 10.26 (Skoog and West, 1972). For EDTA in 50 mM phosphate, pH 6.6 and 50 mM MOPS, pH 7.9 the first 2 titratable protons are completely removed whereas the fourth titratable proton is protonated. At pH 6.6 the third proton is approximately 73% titrated whereas at pH 7.9 the third proton is approximately 98% removed. It is unlikely that the difference in extent of titration of the third proton is responsible for the dramatic differences in the rate of inactivation. Most alkaline earth metal cations as well as all transition state metal cations form insoluble precipitates with PO_4^{3-} (Cotton and Lynch, 1970) and it may be possible that the phosphate buffer is complexing and precipitating the active site metal of *E. coli* glyoxalase I.

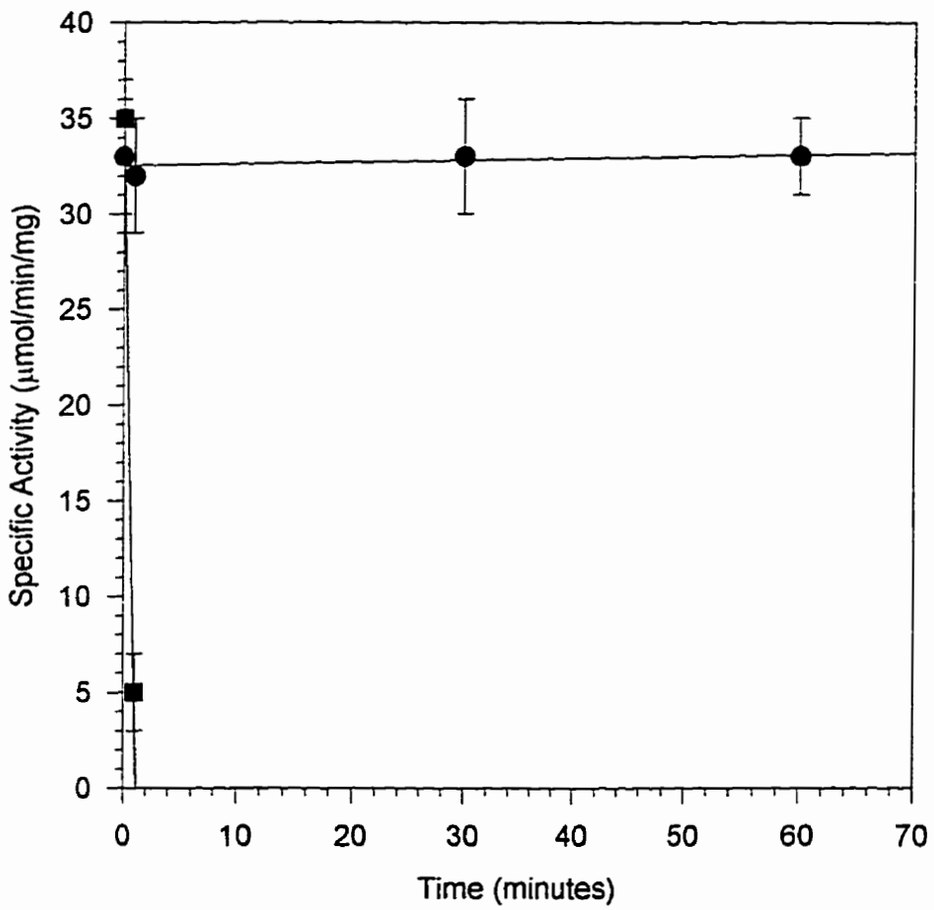

Figure 31. Inactivation of *E. coli* glyoxalase I by 1 mM EDTA (■) (50 mM potassium phosphate, pH 6.6); (●), control curve.

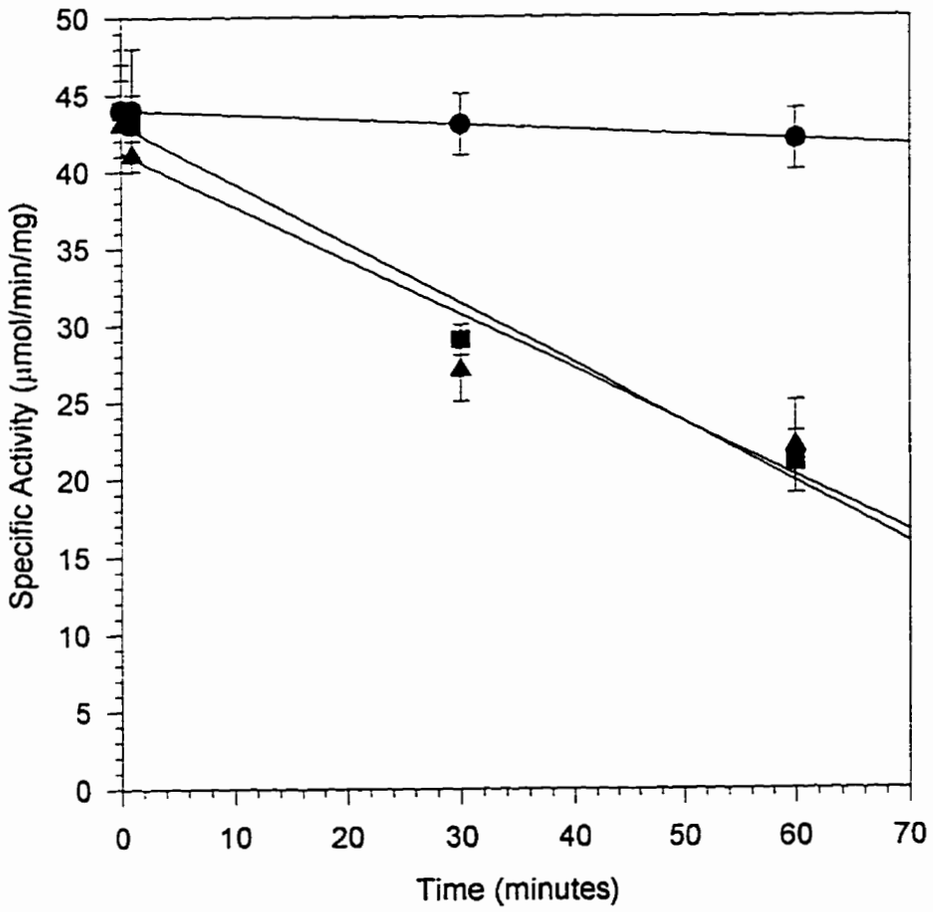

Figure 32. Inactivation of *E. coli* glyoxalase I by 1 mM EDTA (■) and 1 mM EGTA (▲) (50 mM MOPS, pH 7.9); (●), control curve.

3.6 K_m and V_{max} Studies on *E. coli* Glyoxalase I

Kinetic studies on *E. coli* glyoxalase I were done utilizing the hemimercaptal adduct of methylglyoxal and glutathione. The dissociation constant of the hemimercaptal was taken as 3.1 mM (Vince *et al.*, 1971; Vander Jagt *et al.*, 1972). Methylglyoxal and glutathione were preincubated for at least 15 minutes before 1 µL of enzyme was used to initiate the reaction. A plot of velocity versus substrate concentration (Figure 33) shows that at hemimercaptal concentrations of less than 0.6 mM the values generated can be described by a hyperbola. At concentrations greater than 1 mM the velocity is decreasing. This situation is indicative of substrate inhibition and can be analyzed using non-linear regression techniques using the following equation:

$$v = V_{max}[S]/([S]+K_m+[S]^2/K_{si})$$

where V_{max} and K_m are the maximal velocity and Michaelis-Menten constant, respectively, [S] is the hemimercaptal substrate concentration and K_{si} is the substrate inhibition constant (Cornish-Bowden, 1979).

A best-fit analysis of the data was obtained with V_{max} = 0.753 ± 0.033 µmol/min, K_m = 69.1 ± 7.6 µM and K_{si} = 5.82 ± 1.57 mM. With V_{max} = 0.753 µmol/min and 25.2 pmol of glyoxalase I being present, k_{cat} was

calculated to be 498 s^{-1} which is comparable to those reported for glyoxalase I enzymes from other sources (Table 27). The turnover number of *E. coli* glyoxalase I was found to be higher than mammalian or yeast glyoxalase I when methylglyoxal and glutathione hemimercaptal was used as the substrate. The catalytic efficiency (k_{cat}/K_m) varied between $0.95x10^6$-$8.45x10^6$ $M^{-1}\cdot s^{-1}$ with methylglyoxal indicating that glyoxalase I from various sources is an efficient enzyme.

Non-Michaelian rate saturation curves have been observed for yeast (Marmstal *et al.*, 1979), rat liver (Marmstal *et al.*, 1979), pig erythrocytes (Marmstal *et al.*, 1979), human erythrocytes (Marmstal *et al.*, 1979) and sheep liver (Mannervik *et al.*, 1972) glyoxalase I. Glyoxalase I from yeast (Marmstal *et al.*, 1979), rat liver (Marmstal *et al.*, 1979), pig erythrocytes (Marmstal *et al.*, 1979) and human erythrocytes (Marmstal *et al.*, 1979) all exhibited apparent non-linear inhibition with 2 mM GSH (Marmstal *et al.*, 1979). A non-Michaelian saturation curve was observed for *E. coli* glyoxalase I (0.15 mM free GSH) and the fact that non-Michaelian saturation kinetics are observed with glyoxalase I from a number of sources may be indicative of an as yet unknown physiological function.

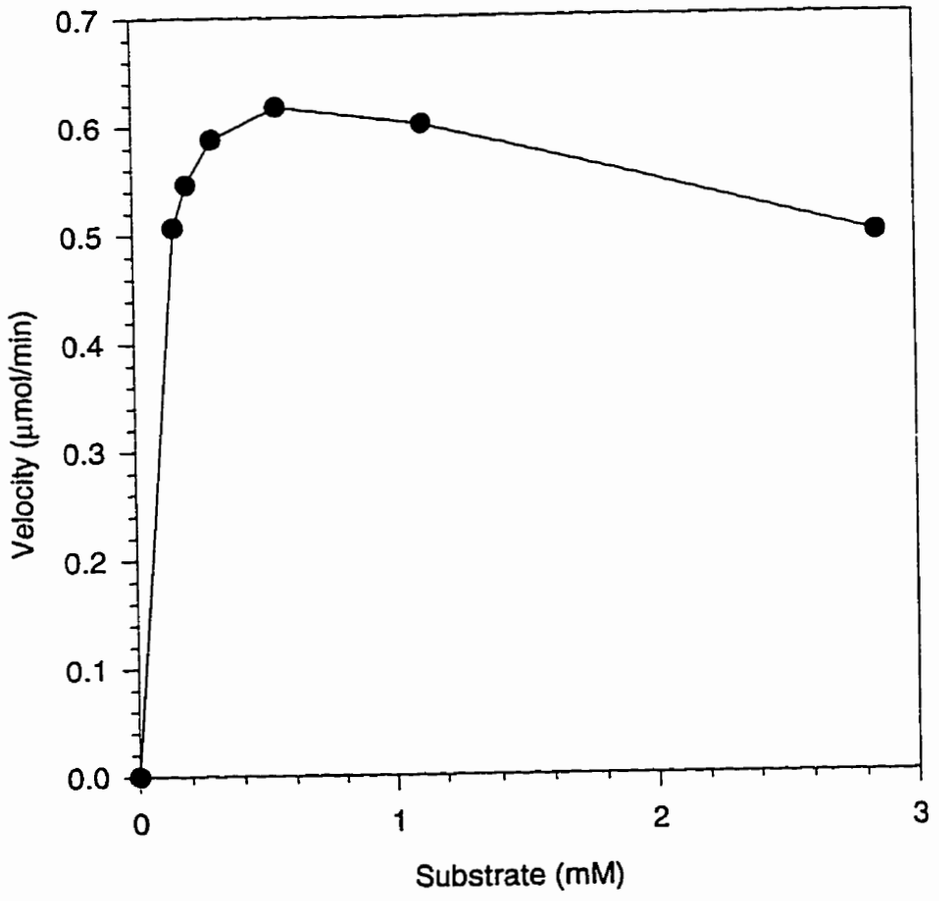

Figure 33. Velocity curve versus substrate (MG-GSH hemimercaptal)for *E. coli* glyoxalase I.

Table 27. Comparison of kinetic parameters for glyoxalase I from various sources.

Source	Kinetic Parameters		
	K_m[e] (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($x10^6$ $M^{-1} \cdot s^{-1}$)
Pig Erythrocyte[a]	120	983	8.19
Human Erythrocyte[b]	192	182	0.95
Rat Liver[a]	140	1 183	8.45
Yeast[a]	530	1 817	3.43
P. putida[c]	360	-	-
E. coli[d]	69	498	7.0

a. Reference: Marmstal *et al.*, 1979. Assays performed in 50 mM potassium phosphate, pH 7.0, 30 °C; b. reference: Han *et al.*, 1976. Assays performed in 50 mM potassium phosphate, pH 6.6, 37 °C; c. reference: Rhee *et al.*, 1986. Assays performed in 10 mM potassium phosphate, pH 7.5, 25 °C; d. assays performed in 50 mM potassium phosphate, pH 6.6, 25 °C; e. MG-GSH hemimercaptal used as the substrate.

3.7 Amino Acid Modification Studies on Glyoxalase I

Surprisingly little information exists concerning amino acid modification of glyoxalase I from various sources. Studies on the enzyme from yeast involving 2,3-butanedione and phenylglyoxal implicate arginine or perhaps lysine may be involved in substrate binding (Schasteen and Reed, 1983; D'Silva, 1986). Further studies have indicated that modification of cysteine leads to inactivation of yeast glyoxalase I but that inhibition is dependent upon the particular thiol-modifying reagent used. Treatment with N-ethylmaleimide partially inactivates the enzyme whereas 4-mercuribenzoate completely inactivates the enzyme (Ekwall and Mannervik, 1970). Histidine may be important for yeast glyoxalase I activity but further studies are required (Hall *et al.*, 1976; Jordan *et al.*, 1983). Tyrosine modification of yeast glyoxalase I using the reagent tetranitromethane (TNM) resulted in greater than 95% inactivation over the course of 60 minutes. Protection against inactivation was afforded by using the competitive inhibitor S-(*p*-bromobenzyl)glutathione (Carrington *et al.*, 1989).

The enzyme from human erythrocytes is believed to possess a tryptophan near the active site zinc (Aronsson *et al.*, 1981). This enzyme was susceptible to inactivation by TNM and a decrease in inactivation achieved by using S-(*p*-bromobenzyl)glutathione (Aronsson *et*

al., 1981).

Glyoxalase I from porcine erythrocytes was inactivated by the amino-group modifying reagent 1-fluoro-2,4-dinitrobenzene sulfonate (Mannervik *et al.*, 1975) but was found to be insensitive to thiol-modifying reagents (Mannervik *et al.*, 1975).

Studies on *E. coli* glyoxalase I using 0.6 mM DEPC (histidine modification) (Figure 34), 50 mM EDC (carboxyl modification) (Figure 35), 20 mM iodoacetamide (Figure 36) and $HgCl_2$ (Table 26) (cysteine modification) indicate that EDC modification of Asp or Glu residues crucial for enzymatic activity may be occuring (Figure 35) and that the rate of inactivation is -0.99 min^{-1}. Loss of activity may be due to modification of carboxyls crucial for substrate binding although future studies using S-(*p*-bromobenzyl)glutathione to protect the active site will need to be performed to test this hypothesis.

Cysteine-modification was dependent upon the reagent used. No inactivation was observed using iodoacetamide but a 71% decrease in activity was observed in 5 minutes 20 mM $HgCl_2$ (Table 25) indicating that the cysteine may be located in an anionic environment or that steric effects favor interaction with the smaller Hg^{2+} but not with

iodoacetamide.

Attempts to modify histidine (Figure 34) indicate that either of the 2 histidines believed to exist per subunit are not accessible to EDC or are not important for enzyme activity.

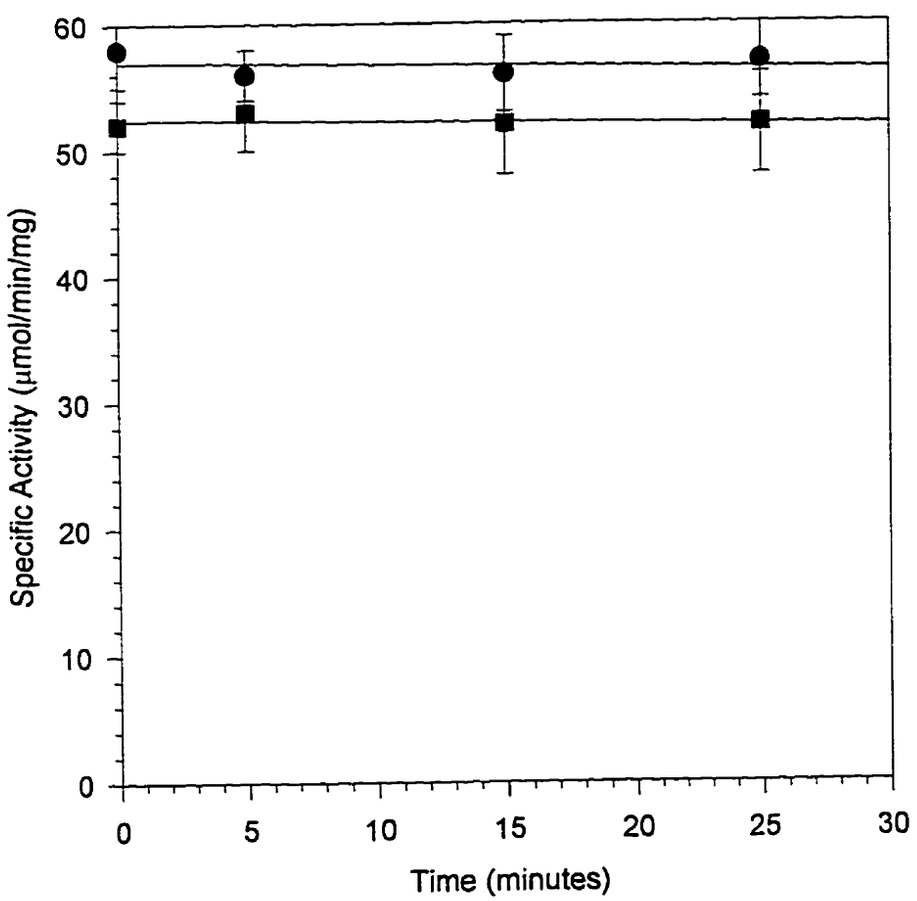

Figure 34. Effect of 0.6 mM DEPC (■)on *E. coli* glyoxalase I activity; (●), control curve. Assays performed in 50 mM potassium phosphate, pH 6.6.

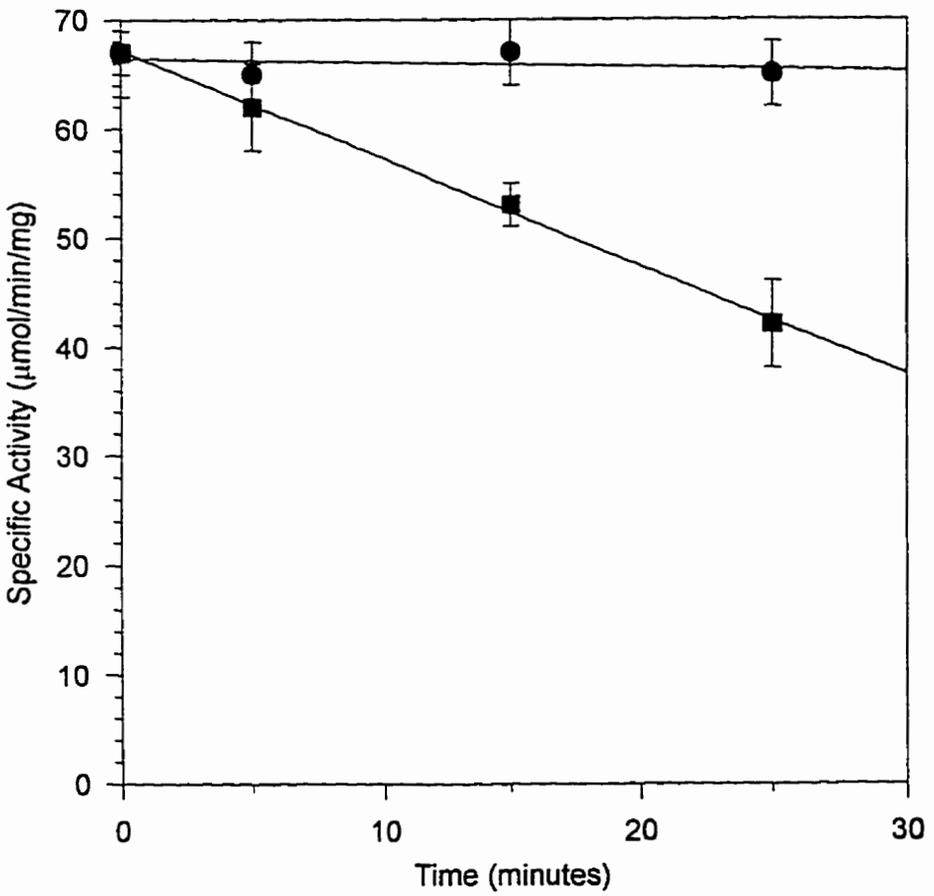

Figure 35. Effect of 50 mM EDC (■) on *E. coli* glyoxalase I activity; (●), control curve. Assays performed in 50 mM potassium phosphate, pH 6.6.

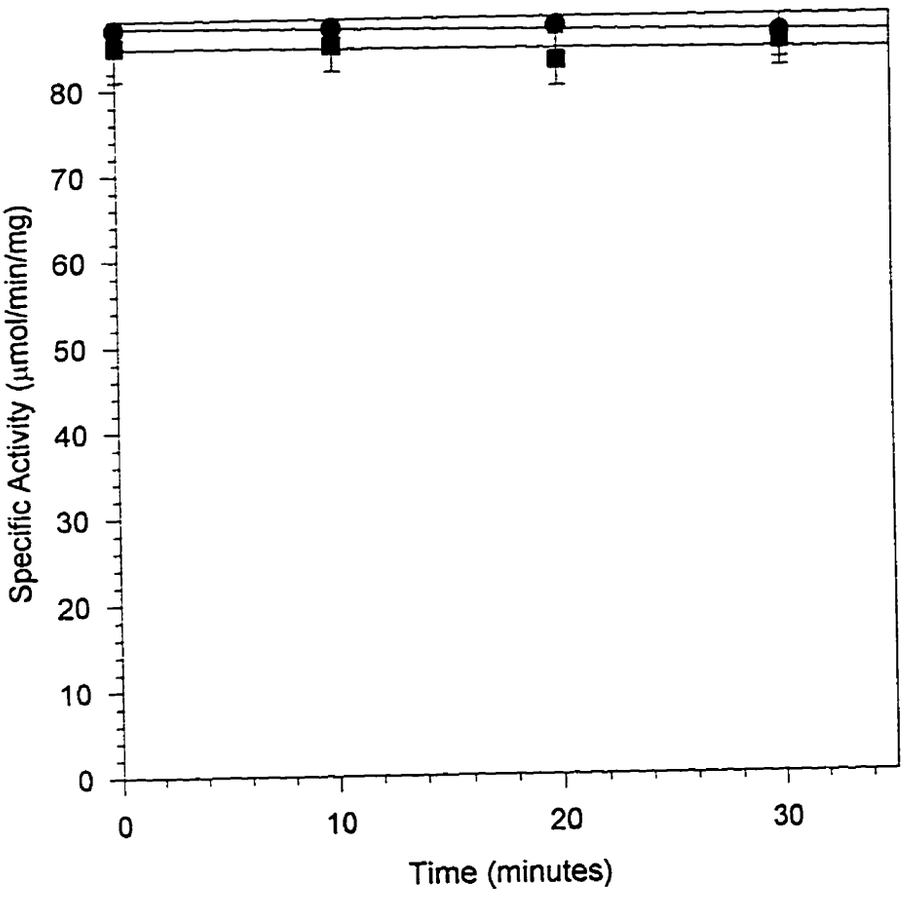

Figure 36. Effect of 20 mM iodoacetamide (■) on *E. coli* glyoxalase I activity; (●), control curve. Assays performed in 50 mM potassium phosphate, pH 6.6.

4. Conclusions and Future Work

The most difficult aspect of research on *E. coli* glyoxalase I lies in the fact that it has an apparent low natural abundance within the wild-type organism (less than 1 μg of glyoxalase I per 12L of cell growth). In order to study *E. coli* glyoxalase I an overexpression system was required. This required knowledge of the *E. coli* DNA sequence for glyoxalase I. Once the DNA sequence for *E. coli* glyoxalase I had been elucidated it was then possible to attempt to overexpress *E. coli* glyoxalase I. The initial overexpression system consisted of the *E. coli* glyoxalase I gene inserted into a pBR322 vector and the resultant plasmid incoporated into DH5α cells. Attempts to isolate *E. coli* glyoxalase I were somewhat successful. While an apparent increase in *E. coli* glyoxalase I was achieved (25 μg per litre of cell growth) this quantity of enzyme was insufficient for extensive studies. PCR attempts to overexpress the enzyme were unsuccessful as the cells tended to generate large quantities of inactive enzyme or very little expression was observed. Successful expression of milligram quantities of *E. coli* glyoxalase I was realized with bacterial strain DF502/pDM7 containing the pDM7 plasmid. The process to achieve sufficient quantities of *E. coli* glyoxalase I required nearly 3.5 years.

During the process of attempting to purify *E. coli* glyoxalase I by different purification schemes it was discovered that subjecting the enzyme to isoelectric focusing or chromatofocusing greatly reduced enzyme activity whereas using protamine sulfate or streptomycin sulfate to remove nucleic acids also seemed to reduce the quantity of enzyme isolated. With these obstacles in mind a purification protocol was implemented such that the time of purification was reduced and enzyme instability was diminished. With the availability of milligram quantities of *E. coli* glyoxalase I it was possible to commence basic biochemical studies on the enzyme.

Glyoxalase I from *E. coli* is an apparent homodimer with a molecular weight of approximately 30 kDa for the intact enzyme. Isoelectric focusing indicates that the enzyme is acidic with an isoelectric pH between 4.15-5.0. The pH stability profile for *E. coli* glyoxalase I increases rapidly above pH 5.0 and levels out at approximately pH 8.0 possibly implicating a single amino acid as crucial for catalytic activity or substrate binding. Amino acid modification studies implicate the importance of aspartic and/or glutamic acid residues for enzyme activity. Studies with metal activation of *E. coli* glyoxalase I indicated that nickel (II) was capable of activating *E. coli* glyoxalase I while metal chelation experiments demonstrated that

loss of activity was buffer-dependent.

Future research on *E. coli* glyoxalase I should include further biochemical characterization with respect to inhibition and chemical modification studies and kinetic analyses. Initial studies have indicated that non-Michaelian kinetics are observed above a MG-GSH hemimercaptal concentration of 0.6 mM. To determine if inhibition is due to the presence of free GSH or S-*D*-lactoylglutathione experiments in which the free GSH concentration is varied or in which various concentrations of S-*D*-lactoylglutathione is present may unequivocally determine which substance is responsible for the deviations from Michaelis-Menten kinetics. Chemical modification studies with or without the presence of known glyoxalase I inhibitors may aid in determining which amino acids are necessary for catalysis and substrate binding. An important and as-of-yet unanswered question pertains to which metal is endogenous to *E. coli* glyoxalase I. Metal ion determination is standard procedure via atomic absorption but it would be necessary to treat all solutions used to purify the enzyme with Chelex to remove metal ions in solution. A number of substrate and transition state inhibitors of glyoxalase I (from various sources) are known to exist. Testing of these compounds against *E. coli* glyoxalase I may result in the discovery of novel anti-bacterial agents targetting the bacterial glyoxalase I.

Computer modelling may then be used to predict and enhance the efficacy of known glyoxalase I inhibitors.

Site-directed mutagenesis studies to generate mutants will be important to determine which amino acids are crucial for catalysis. Since considerable quantities of the enzyme can be isolated, crystallographic studies can be considered as being feasible.

CHAPTER 4

REFERENCES

Alber, T. and Kowasaki, G, (1982) *J. Mol. Appl. Genet.* 1, 419-434.

Allen, R.E., Lo, T.W.C. and Thornalley, P.J. (1993a) *J. Protein Chem.* 12, 111-119.

Allen, R.E., Lo, T.W.C. and Thornalley, P.J. (1993b) *Biochem. Soc. Trans.* 21, 535-540.

Anderson, A. and Cooper, R.A. (1969) *FEBS Lett.* 4, 19-20.

Aronsson, A.-C. and Mannervik, B. (1977) *Biochem. J.* 165, 503-509.

Aronsson, A.-C., Marmstal, E. and Mannervik, B. (1978) *Biochem. Biophys. Res. Commun.* 81, 1235-1240.

Aronsson, A.-C., Tibbelin, G. and Mannervik, B. (1979) *Anal. Biochem.* 92, 390-393.

Aronsson, A.-C., Sellin, S., Tibbelin, G, and Mannervik, B. (1981) *Biochem. J.* 197, 67-75.

Azari, M.H. and Wiseman, A. (1990) *Anal. Biochem.* 122, 129-138.

Babel, W. and Hofmann, K.H. (1981) *FEMS Microbiol. Lett.* 10, 133-136.

Babul, J. (1978) *J. Biol. Chem.* 253, 4350-4355.

Ball, J.C. and Vander Jagt, D.L. (1980) *Anal. Biochem.* 98, 472-477.

Ball, J.C. and Vander Jagt, D.L. (1981) *Biochemistry* 20, 899-905.

Bagster, I.A. and Parr, C.W. (1975) *J. Physiol. (Lond)* 256, 56P-57P.

Barman, T.E. (1985) in Enzyme Handbook, Barman, T.E. (Ed.) Suppl. I, p.441.

Barnard, J.F and Honek, J.F. (1989) *Biochem. Biophys. Res. Commun.* 165, 118-124.

Barnard, J.F., Vander Jagt, D.L. and Honek, J.F. (1994) *Biochim. Biophys. Acta* 1208, 127-135.

de Beck, C.A., Brug, J. and Walor, J.N. (1957) *Nature* 179, 706-707.

Behrers, O.K. (1941) *J. Biol. Chem.* 141, 503-508.

Bernardi, G., Givo, M.G. and Gaillard, C. (1972) *Biochim. Biophys. Acta* 278, 409-420.

Bradford, M.M (1976) *Anal. Biochem.* 72, 248-254.

Brown, C., Douglas, K.T. and Ghobt-Sharif, J. (1981) *J. Chem. Soc. Chem. Commun.* 18, 944-946.

Bush, P.E. and Norton, S.J. (1985) *J. Med. Chem.* 28, 828-830.

Caccuri, A.M., Risitano, A., Marmucchi, F., Venardi, G., Aureli, G., Petruzelli, R. and Desideri, A. (1993) *Biochim. Biophys. Acta* 1202, 157-160.

Canuto, R.A., Biocca, M.E., Muzio, G., Gariea, R. and Diazani, M.U. (1985) *Cell. Biochem. Funct.* 3, 3-8.

Carnegie, P.R. (1963) *Biochem. J.* 89, 471-478.

Carrington, S.J. and Douglas, K.T. (1985) *IRCS Med. Sci.* 31, 883-884.

Carrington, S.J., Fetherbe, D. and Douglas, K.T. (1989) *Int. J. Biochem.* 23, 901-908.

Chari, R.V.J. and Kozarich, J.W. (1983) *J. Am. Chem. Soc.* 103, 7169-7171.

Chavira, R., Burnett, T.J. and Hageman, J.H. (1984) *Anal. Biochem.* 136, 446-450.

Chiari, M., Rhigetti, P.G., Negri, A., Ceciliani, F. and Ronchi, S. (1992) *Electrophoresis* 13, 882-884.

Chiu, T-H and Feingold, D.S. (1969) *Biochemistry* 8, 98-108.

Clarke, A.J. and Yaguchi, M. (1985) *Eur. J. Biochem.* 149, 233-238.

Collier, B.H. (1973) *Anal. Biochem.* 56, 310-311.

Cooper, R.A. and Anderson, A. (1970) *FEBS Lett.* 11, 273-276.

Cooper, R.A. (1974) *Eur. J. Biochem.* 44, 81-86.

Cooper, R.A. (1975) *Biochem. Soc. Trans.* 3, 837-840.

Cornish-Bowden, A. (1979) in *Fundamentals of Enzyme-Kinetics*. Buttersworths, London.

Cotton, F.A and Lynch, L.D. (1970) in *Chemistry An Investigative Approach*, Cotton, F.A and Lynch, L.D. (Eds.), pp. 1375-1376, 1385-1388. Houghton Mifflin Co., Boston.

Cotton, F.A. and Wilkinson, G. (1982) in *Advanced Inorganic Chemistry*, Cotton, F.A and Wilkinson, G. (Eds.), pp. 1375-1376, 1385-1388. Wiley-Interscience, New York.

Dakin, H.D. and Dudley, H.W. (1913) *J. Biol. Chem.* 14, 155-157.

Daub, E., Kinach, R., Miedema, D., Barnard, J.F.J., Clugstron, S.L. and Honek, J.F. (1996). *Escherichia coli* glyoxalase I. Genbank accession number U57363.

Davis, K.A. and Williams, G.R. (1966) *Biochim. Biophys. Acta* 113, 393-395.

Diazani, M.U. (1985) *Chem.-Biol. Interact.* 53, 209-217.

Dirksen, M.L. and Chambrach, A. (1992) *Sep. Sci.* 7, 744-772.

Douglas, K.T. and Keysworth, L.T.A. (1994) *Med. Sci. Res.* 22, 641-642.

Douglas, K.T., Quilter, A.J., Shinkai, S. and Ueda, K. (1985a) *Biochim. Biophys. Acta* 829, 119-126.

Douglas, K.T., Gohel, D.I., Nadvi, I.N., Quilter, A.J. and Seddon, A.P. (1985b) *Biochim. Biophys. Acta* 829, 109-118.

D'Silva, C. (1986) *FEBS Lett.* 202, 240-244.

Ekwall, K. and Mannervik, B. (1970) *Archs. Biochem. Biophys.* 137, 128-132.

Elango, N., Janaki, S. and Rao, A.R. (1978) *Biochem. Biophys. Res. Commun.* 83, 1388-1395.

Elliot, W.H. (1959) *Nature* 183, 1051-1052.

Ellman, G.L. (1959) *Arch. Biochem. Biophys.* 82, 70-77.

Entner, N. and Doudoroff, M. (1952) *J. Biol. Chem.* 196, 853-862.

Freedberg, W.B., Kistler, W.S. and Lin, E.C.C. (1971) *J. Bacteriol.* 108, 137-144.

French, F.A. and Freedlander, B.L. (1958) *Cancer Res.* 18, 172-175.

Garcia-Iniquez, L., Powers, L., Chance, B., Sellin, S., Mannervik, B. and Mildvan, S. (1984) *Biochemistry* 23, 685-689.

Gawehn, K. (1985) in *Methods of Enzymatic Analysis*, Third Edition, Bergmeyer, H.U. (Ed.), vol. VI, p. 593-596. VCH Publishers, Deerfield Beach, Fl.

Gekko, K. and Timasheff, S.N. (1981) *Biochemistry* 20, 4677-4686.

Gillespie, E. (1975) *Fedn. Proc.* 34, 541.

Griffis, C.E.F., Ong, L.H., Buettner, L. and Creighton, D.J. (1983) *Biochemistry* 22, 2945-2951.

Guha, M.K., Vander Jagt, D.L. and Creighton, D.J. (1988) *Biochemistry* 27, 8818-8822.

Hall, S.S., Doweyko, A.M. and Jordan, F. (1976) *J. Am. Chem. Soc.* 98, 7460-7461.

Hamilton, D.S. and Creighton, D.J. (1992) *Biochim. Biophys Acta* 1159, 203-208.

Han, L.-P.B., Davison, L. and Vander Jagt, D.L. (1976) *Biochim. Biophys Acta* 445, 486-499.

Han, L.-P.B., Schimandle, C., Davison, L.M. and Vander Jagt, D.L. (1977) *Biochemistry* 16, 5478-5484.

Hausinger, R. (1987) *Microbiol. Rev.* 51, 22-42.

Hemmerich, P., Massey, V. and Fenner, H. (1977) *FEBS Lett.* 84, 5-21.

Heukeshoven, J. and Dernick, R. (1988) *Electrophoresis* 9, 28-32.

Hillman, J.D. and Fraenkel, D.G. (1975) *J. Bacteriol.* 122, 1175-1179.

Hopkins, F.G. and Morgan, E.J. (1945) *Biochem. J.* 39, 320-324.

Hopkins, F.G. and Morgan, E.J. (1948) *Biochem. J.* 42, 23-27.

Hopper, D.J. and Cooper, R.A. (1971) *FEBS Lett.* 13, 213-216.

Hopper, D.J. and Cooper, R.A. (1972) *Biochem.* J. 128, 321-329.

Hsu, Y.R and Norton, S.J. (1983) *J. Med. Chem.* 26, 1784-1785.

Inoue, Y., Watanabe, K., Shimosaka, M., Saikusa, T., Fukuda, F., Murata, K. and Kimura, A (1985) *Eur. J. Biochem.* 53, 243-247.

Inoue, Y., Rhee, H.I., Watanabe, K., Murata, K. and Kimura, A. (1988) Eur. *J. Biochem.* 171, 213-218.

Inoue, Y., Feng, L., Bong-Young, C., Ginya, H., Murata, K. and Kimura, A. (1990) *Biotechnol. Appl. Biochem.* 12, 341-345.

Inoue, Y. and Kimura, A. (1992) *J. Ferment. Bioeng.* 73, 271-276.

Irani, M. and Maitra, P.K. (1974) *Biochem. Biophys. Res. Commun.* 56, 127-133.

Jensen, K.J. (1995) *J.Bacteriol.* 175, 3401-3407.

Jeryzkowski, T., Winter, R. and Matuszewski, W. (1973) *Biochem. J.* 135, 713-719.

Jerzykowski, T., Winter, R., Matuszewski, W. and Piskura, D. (1978) *Int. J. Biochem.* 9, 853-860.

Jordan, F., Cohen, J.F. Wang, C.-T., Wilmott, J.M. and Hall, S.S. (1983) *Drug Metabolism Reviews* 14, 723-741.

Kaplan, J.C. and Grossman, L. (1971) in *Methods of Enzymology*, Grossman, L. and Moldave, K. (Eds.), vol XXI, pp. 249-269. Academic Press, New York.

Kaback, H.R. and Milner, L.S. (1970) *Proc. Nat. Acad. Sci. USA* 66, 1008-1015.

Kaback, H.R. (1974) *Science* 186, 882-892.

Kato, H., Liang, Z.-Q., Nishimura, T. and Hayase, F. (1988) *Agric. Biol. Chem.* 52, 2675-2677.

Kermack, W.O. and Matheson, N.A. (1957) *Biochem. J.* 65, 48-58.

Kim, N.-S., Umezawa, Y., Ohmura, S. and Kato, S. (1993) *J. Biol. Chem.* 268, 11217-11221.

Kim, N.-S., Sekine, S., Kimuchi, N. and Sato, K. (1995) *J. Biochem.* 117, 359-361.

King, D.J., Azari, M.R. and Wiseman, A. (1984) *Xenobiotica* 14, 187-206.

Kline, E.S. and Mahler, H.R. (1965) *Ann. NY Acad. Sci.* 119, 905-919.

Kompf, J., Bissbort, S., Gussman, S. and Ritter, H. (1975) *Humangenetik* 27, 141-143.

Kozarich, J.W. and Chari, R.V.J. (1982) *J. Am. Chem. Soc.* 104, 2655-2657.

Kozarich, J.W., Chari, R.V.J., Wu, J.C. and Lawrence, J. (1981) *J. Am. Chem. Soc.* 103, 4593-4595.

Landro, J.A., Brush, E.J. and Kozarich, J.W. (1992) *Biochemistry* 31, 6069-6077.

Larsen, K., Aronsson, A.-C., Marmstal, E. and Mannervik, B. (1985) *Comp. Biochem. Physiol.* 82B, 625.

Lazdunski, A.M. (1989) *FEMS Microbiol. Rev.* 63, 265-276.

LeGendre, N. and Matsudaira, P. (1989) in *A Practical Guide to Protein Purification for Microsequencing*, Matsudaira, P. (Ed.), pp. 49-69. Academic Press, New York.

Ljunquist, C., Breitholtz, A., Brink-Nilsson, H., Moks, T., Uhlen, M. and Nilsson, B. (1989) *Eur. J. Biochem.* 186, 563-569.

Lohmann, K. (1932) *Biochem. Z.* 254, 332-354.

Lu, T., Creighton, D.J. Antoine, M., Fenselau, C. and Lovetti, P.S. (1994) *Gene* 150, 93-96.

Mannervik, B., Lindstrom, L. and Bartfai, T. (1972) *Eur. J. Biochem.* 29, 276-281.

Mannervik, B., Marmstal, E., Ekwall, E. and Gorna-Hall, B. (1975) *Eur. J. Biochem.* 53, 327-333.

Mannervik, B., Aronsson, A.-C., Marmstal, E. and Tibbelin, G. (1981) *Meth. Enzymol.* 77, 297-301.

Marmstal, E. and Mannervik, B. (1979) *Biochim. Biophys. Acta* 566, 362-370.

Marmstal, E., Akerlund, A.-C. and Mannervik, B. (1975) Abstr. FEBS Meet. 10th, abstr. No. 598.

Marmstal, E., Aronsson, A.-C. and Mannervik, (1979) *Biochem. J.* 183, 23-30.

Meo, T., Douglas, K.T. and Rijnbeek, A.-M. (1977) *Science* 198, 311-313.

Meyerhof, O. (1948) *Experentia* 4, 169-176.

Misra, K., Bannerjee, A.B., Ray, S. and Ray, M. (1995) *Biochem. J.* 305, 999-1003.

Monks, T.J., Lau, S.S., Highet, R.J. and Gillette, J.R. (1985) *Drug. Met. Disp.* 13, 533-539.

Monks, T.J. and Lau, S.S. (1987) *Drug. Met. Disp.* 15, 437-441.

Murata, K., Fukuda, Y., Shimosaka, M., Watanabe, K., Saikusa, T. and Kimura, A. (1985a) *Appl. Environ. Microbiol.* 50, 1200-1207.

Murata, K., Fukuda, F., Shimosaka, M., Watanabe, K., Saikusa, T. and Kimura, A. (1985b) *Eur. J. Biochem.* 151, 631-636.

Murata, K., Saikusa, T., Watanabe, K., Inoue, Y., Fukuda, Y., Shimosaka, M. and Kimura, A. (1985c) *Agric. Biol. Chem.* 50, 2381-2383.

Murata, K., Inoue, Y., Watanabe, K., Fukuda, Y., Saikusa, T., Shimosaka, M. and Kimura, A. (1986) *Agric. Biol. Chem.* 50, 135-142.

Murata, K., Saikusa, T., Inoue, Y. and Kimura, A. (1988) *J. Ferment. Technol.* 66, 495-500.

Navarro, C., Wu, L.-F. And Mandrand-Berthelot, M.-A. (1993) *Mol. Microbiol.* 9, 1181-1191.

Neben, I., Sahm, H. and Kula, M.-R. (1980) *Biochem. Biophys. Acta* 614, 81-91.

Neuberg, C. (1913) *Biochem. Z.* 49, 502-506.

Neuberg, C. and Gorr, G. (1925) *Biochem. Z.* 162, 490-495.

Neuberg, C. and Varcellone, A. (1953) *Biochem. Z.* 279, 140-142.

Oesch, F. (1980) in *Enzymatic Basis of Detoxification*, Jakoby, W.H. (Ed.) vol. 2, p.277. Academic Press, New York.

Oray, B. and Norton, S.J. (1977) *Biochim. Biophys. Acta* 483, 203-209.

Oray. B. and Norton, S. J. (1980) *Biochim. Biophys. Acta* 611, 168-173.

Pacaud, M. (1978) *Eur. J. Biochem.* 82, 439-451.

Pacaud, M. and Richaud, C (1975) *J. Biol. Chem.* 250, 7771-7779.

Pahel, G., Bloom, F.R. and Tyler, B. (1979) *J. Bacteriol.* 138, 653-656.

Paradini, L., Pargini, C., Parola, M., Barrera, M. and Diazini, M.U. (1985) *Chem.-Biol. Interact.* 53, 209-217.

Patterson, S.D. (1994) *Anal. Biochem.* 221, 1-15.

Pegg, A.E. (1973) *Biochem. J.* 132, 537-540.

Pompliano, D.L., Peynman, A. and Knowles, J.R. (1980) *Biochemistry* 29, 3186-3194.

Porath, J. and Flodin, P. (1957) *Nature* 83, 1657-1659.

Phillips, S.A. and Thornalley, P.J. (1992) *Eur. J. Biochem.* 92, 1436-1440.

Principato, G.B, Rosi, G., Talesa, V., Giovanni, E. and Norton, S.J. (1987a) *Enzyme* 37, 164-168.

Principato, G.B., Rose, G., Talesa, V., Giovannini, E. and Uotila, L. (1987b) *Biochim. Biophys. Acta* 911, 349-355.

Racker, E. (1951) *J. Biol. Chem.* 190, 685-696.

Rae, C., O'Donoghue, I., Bubb, W.A. and Kuchel, P.W. (1994) *Biochemistry* 33, 3548-3559.

Ramaswany, O., Pal, S., Guha-Mukherjee, S. and Sopory, S.K. (1983) *Biochem. Int.* 7, 307-318.

Ranganathan, S. and Tew, K.D. (1993) *Biochim. Biophys. Acta* 1182, 311-316.

Ranganathan, S., Walsh. E.S., Godwin, A.K. and Tew, K.D. (1993) *J. Biol. Chem.* 268, 5661-5667.

Ray, S. and Ray, M. (1981) *J. Biol. Chem.* 256, 6230-6233.

Ray, S. and Ray, M. (1982) *J. Biol. Chem.* 257, 10566-10577.

Ray, M. and Ray, S. (1984) *Biochem. Biophys. Acta* 802, 128-134.

Ray, M. and Ray, S. (1987) *J. Biol. Chem.* 262, 5974-5977.

Rhee, H.-I., Murata, K. and Kimura, A. (1986) *Biochem. Biophys. Res. Commun.* 141, 993-999.

Rhee, H.I., Watanabe, K., Murata, K. and Kimura, A. (1987a) *Agric. Biol. Chem.* 51, 1059-1066.

Rhee, H.-I., Murata, K. and Kimura, A. (1987b) *Biochem. Biophys. Res. Commun.* 147, 831-838.

Richards, J.P. (1991) *Biochemistry* 30, 4581-4585.

Ridderstrom, M., Saccucci, F., Hellman, U., Bergman, T., Principato, G. and Mannervik, B. (1996) *J. Biol. Chem.* 271, 319-323.

Riddle, V. and Lorez, F.W. (1973) *Biochem. Biophys. Res. Commun.* 50, 27-34.

Rose, I.A. (1957) *Biochim. Biophys. Acta* 25, 214-215.

Rose, I.A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* 43, 491-517.

Rouviere, P.E. and Wolfe, R.S. (1988) *J. Biol. Chem.* 263, 7913-7916.

Sandman, R.P. and Miller, O.N. (1958) *J. Biol. Chem.* 230, 361-368.

Sato, J., Wang, Y.-M. and van Eyre, J. (1980) *J. Biol. Chem.* 256, 2046-2050.

Schasteen, C.S. and Reed, D.J. (1983) *Biochim. Biophys. Acta* 742, 419-425.

Schimandle, C.M and Vander Jagt, D.L. (1979) *Arch. Biochem. Biophys.* 195, 261-268.

Schuetz, E.G. and Guzelian, P.S. (1984) *J. Biol. Chem.* 259, 2007-2012.

Schutte, H., Kula, M.-R. and Sahm, H. (1982) *Meth. Enzymol.* 89, 527-531.

Scopes, R.K. (1987) in *Protein Purification Principles and Practices*, Second Edition, Cantor, C.R. (Ed.). Springer-Verlag, New York.

Sellin, S., Rosevear, P.R., Mannervik, B. and Mildvan, A.S. (1982a) *J. Biol. Chem.* 257, 10023-10029.

Sellin, S., Eriksson, L.E.G. and Mannervik, B. (1982b) *Biochemistry* 21, 4850-4857.

Sellin, S., Eriksson, L.E.G., Aronsson, A.-C. and Mannervik, B.(1983) *J. Biol. Chem.* 258, 2091-2093.

Sellin, S. and Mannervik, B. (1984) *J. Biol. Chem.* 259, 11426-11429.

Shinkai, S., Yamashita, S. and Manabe, O. (1970) *J. Chem. Soc. Chem. Commun.*, 301-302.

Shinkai, S., Kunitake, T. and Bruice, T.C. (1974) *J. Am. Chem. Soc.* 96, 7140-7141.

Shinkai, S., Yamashita, S., Kusano, Y. and Manabe, O. (1981) *J. Am. Chem. Soc.* 103, 2070-2074.

Skoog, D.A. and West, D.M. (1972) in *Fundamentals of Analytical Chemistry*, Vondeling, J., Smith, P.C. and Kesselring, L. (Eds.), p. 283. Saunders College Publishing, New York.

Smith, M.C., Furman, T.C. Ingolia, T.D. and Pidgeon, C. (1988) *J. Biol. Chem.* 263, 7211-7215.

Sridhara, S. and Wu, T.T. (1969) *J. Biol. Chem.* 244, 5233-5238.

Stafforini, D.M., Kushner, J.P. and Burnham, B.F. (1985) *Biochem. Biophys. Acta* 839, 191-198.

Stern, M. (1956) *Science* 124, 1292-1296.

Stevens, J., Hayden, P. and Taylor, G. (1986) *J. Biol. Chem.* 263, 3325-3332.

Still, J.L. (1941) *Biochem. J.* 35, 390-391.

Strauss, D. and Gilbert, W. (1985) *Proc. Natl. Acad. Sci.* 82, 2014-2018.

Summers, M.C and Rose, I.A. (1975) *J. Am. Chem. Soc.* 99, 4475-4478.

Sundstrom, J. (1967) *J. Phys. Chem.* 71, 2318-2325.

Szent-Gyorgi, A. and Egyund, L. (1967) *Proc. Natl. Acad. Sci. USA* 57, 1644-1650.

Talesa, V., Rossi, G., Bistoni, F., Marconi, P., Norton, S.J. and Principato, G.B (1990) *Biochem. Int.* 21, 397-403.

Taylor, D.G., Trudgill, P.W., Cripps, R.E. and Harris, P.R. (1980) *J. Gen. Microbiol.* 118, 159-170.

Thornalley, P.J. (1995) *Clin. Rev. Oncology/Hematology* 20, 99-128.

Tsai, P.-K. and Gracy, R.W. (1976) *J. Biol. Chem.* 251, 364-367.

Ueda, K., Shinkai, S. and Douglas, K.T. (1984) *J. Chem. Soc. Chem. Commun.*, 371-372.

Uotila, L. (1973) *Biochemistry* 12, 3944-3951.

Uotila, L. and Koivusalo, M. (1974) *J. Biol. Chem.* 249, 7664-7672.

Uotila, L. and Koisuvalo, M. (1975) *Eur. J. Biochem.* 52, 493-503.

Urata, G. and Granick, S. (1963) *J. Biol. Chem.* 238, 811-820.

Vander Jagt, D.L. (1975) *J. Med. Chem.* 18, 1155-1158.

Vander Jagt, D.L. and Han, L.-P. (1973) *Biochemistry* 12, 5161-5167.

Vander Jagt, D.L., Dean, V.L. and Ball, J.C. (1980) *Fedn. Proc.* 39, 1324.

Vander Jagt, D.L., Han, L.-P.B. and Lehman, C.H. (1972) *Biochemistry* 11, 3735-3740.

Vander Jagt, D.L., Daub, E., Krohn, J.A. and Han, L.-P.B. (1975) *Biochemistry* 14, 3669-3675.

Vander Jagt, D.L. and Tapscott, R.E. (1978) *Fedn. Proc.* 37, 827.

Vince, R., Daluge, S. and Wadd, W.B. (1971) *J. Med. Chem.* 14, 402-404.

Walsh, C.T. and Orme-Johnson, W.H. (1987) *Biochemistry* 26, 4901-4905.

Wang, S.-L., Chen, J.-P. and Shen, S.-C. (1964) *Scientia Sinica* 13, 167-168.

Weaver, R.H. and Lardy, H.A. (1961) *J. Biol. Chem.* 236, 313-319.

White, J.S. and Rees, K.R. (1982) *Chem.-Biol. Interact.* 38, 339-347.

Widmann, E. (1929) *Biochem. Z.* 216, 475-478.

Wieland, T., Pfleiderer, G. and Lau, H.H. (1956) *Biochem. Z.* 327, 393-407.

Wislocki, P.G., Miwa, G.T. and Lu, A.Y.M. (1980) in Enzymatic Basis of Detoxification, Jakoby, W.H. (Ed.) vol. 1, p.135. Academic Press, New York.

Wiseman, A. and Woods, F.J. (1979) *J. Chem. Tech. Bioltech.* 29, 320-324.

Woodward, G.E. and Reinhardt, F.E. (1942) *J. Biol. Chem.* 145, 471-476.

Woods, L.F.J. and Wiseman, A. (1980) *Biochim. Biophys. Acta* 613, 52-61.

Xie, X. and Creighton, D.J. (1991) *Biochem. Biophys. Res. Commun.* 177, 252-258.

Zubay, G. (1988) in *Biochemistry*, Second Edition, Rogers, R. (Ed.), p. 272. Macmillan Publishing Co., New York