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Novel Sources of Mammalian C-S Lyase Activity

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Abstract

The C-S lysis of L-cysteine conjugates is one biotransformation pathway which is responsible for the generation of mutagenic and cytotoxic metabolic species. Thirteen cysteine *S*-conjugates were synthesized in our laboratories and incubated with aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) enzymes from porcine heart tissue. The C-S lyase (CSL) activity for each enzyme-substrate combination was determined.

ASAT and ALAT were shown to exhibit CSL activity and it was also demonstrated that this activity was inhibited in the presence of the pyridoxal phosphate-dependent enzyme inhibitor amino(oxyacetic acid) confirming the pyridoxal phosphate-dependent mechanism by which C-S lysis is known to take place. This finding has potentially important implications for the risk assessment of compounds which produce L-cysteine conjugates during their biotransformation.

Glutathione conjugation is a classical pathway for the detoxification of electrophilic xenobiotics. Enzymatic degradation of the glutathione conjugate leads to the corresponding cysteine conjugate which can be excreted via the kidney following *N*-acetylation. Aberrant metabolism of the cysteine conjugate by enzyme-catalysed C-S lysis (CSL) results in the production of equimolar amounts of pyruvate, ammonia, and a reactive thiolic species (Scheme 1). Where this thiolic species is able to rearrange, such as with 1-chlorovinyl conjugates (Scheme 2), it can be responsible for the mutagenic and cytotoxic consequences which occur following exposure to halogenated hydrocarbons.

C-S lyases have an important physiological role in the pathways of amino acid metabolism, and enzymes exhibiting C-S lyase activity include glutamine transaminase K (human and rat kidney), kynureninase (rat liver) and kynurenine aminotransferase (human liver) (Stevens 1985; Buckberry et al 1990, 1992). These enzymes are pyridoxal phosphate-dependent, pyridoxal phosphate being employed in an electrophilic covalent catalytic system allowing, in effect, facile abstraction of the α-H of the substrate through provision of a low-energy reaction pathway (Scheme 1). In this way, C-S lysis can be seen as competing with the physiological role of the enzyme, such as transamination, branching from the conventional pathway following formation of a Schiff base between the α-amino group of the amino acid and the carbonyl group of pyridoxal phosphate. This stereoelectronically assists the proton abstraction by delocalisation of the electron pair of the α-C to α-H bond mesomerically with the positively charged pyridinium nitrogen. Transamination would proceed through protonation of the resultant quinoid intermediate to give a ketimine which, with subsequent hydrolysis, yields pyridoxamine phosphate and the α-keto acid. The full transamination cycle is completed using an α-keto acid co-substrate in a reaction which is mechanistically the reverse of the above (Braunstein & Shemyakin 1953). In the absence of further substrate the pyridoxal phosphate forms a Schiff base with a lysine residue at the active site (Buckberry et al 1993 a,b). Alternatively, in the presence of a highly electronegative S-conjugate side chain a β-elimination reaction can take place following formation of the quinoid intermediate. This elimination results in the formation of thiolate anions and an equivalent of dehydroalanine which hydrolyses to pyruvate and ammonia. Toxicity occurs as the result of rearrangement of the thiolate anion to give reactive thiones, such as thioketenes and thioacyl halides (Scheme 2). It is thus reasonable to postulate that C-S lysis occurs as a natural consequence of the mechanism of action of pyridoxal phosphate-dependent enzymes acting upon cysteine S-conjugates.

Amino transferases are important pyridoxal phosphate-dependent enzymes whose active sites specifically recognise the α-amino group of amino acids, but shows broad specificity for amino acids as a whole, often reacting with groups with broadly related side chain chemistries and as such may be predicted to act as C-S lyases. Two of the most extensively characterized transaminases are aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), which are found at high levels in many tissues including the liver, heart, kidney and lung (Braunstein & Shemyakin 1953). The physiological role of these transaminase enzymes involves the interconversion of α-ketoacids and α-amino acids, specifically, alanine and pyruvate (ALAT), and aspartate and oxaloacetate (ASAT), thereby playing a key role in cellular respiration. Despite their ubiquitous presence in man, no previous reports have examined these transaminase enzymes for C-S lyase activity.

The work reported here represents the first examination of ASAT and ALAT as C-S lyases. Porcine heart ASAT and

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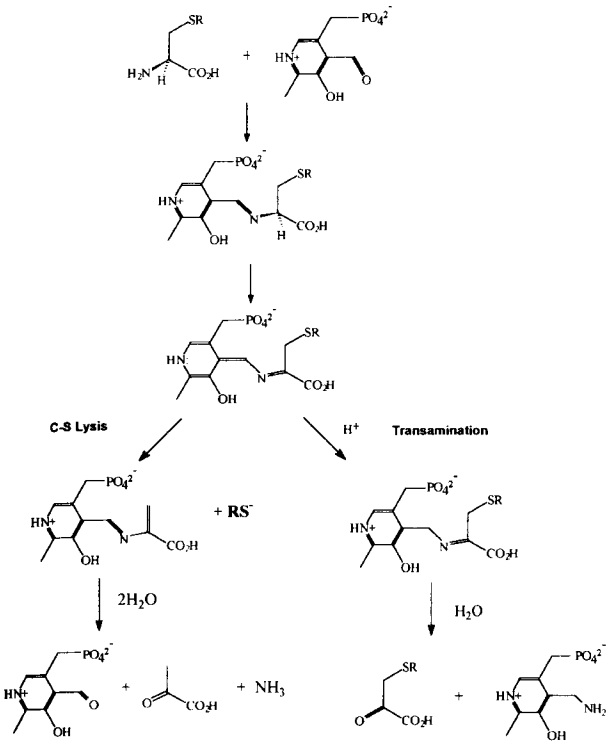
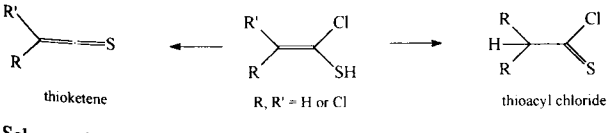


Scheme 1. The competing pathways of transamination and C-S lysis.

ALAT were employed as model systems since both are commercially available. Thirteen known and novel L-cysteine-S-conjugates were used to provide structure-activity information for the C-S lyase activity of each enzyme.

Materials and Methods

S-(1,2-dichlorovinyl)-L-cysteine (DCVC), *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC) and *S*-(benzothiazolyl)-L-cysteine (BTC) were all synthesized according to the method of McKinney et al (1959). *S*-(2-chloro-1,2-difluoroethyl)-L-cysteine (CDFC) and *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFC) were synthesized according to the method of Gandolfi et al (1981). *S*-(Phenyl)-L-cysteine (PC), *S*-(4-chlorophenyl)-L-cysteine (CPC), *S*-(2,4-dichlorophenyl)-L-cysteine (DCPC), *S*-(4-bromophenyl)-L-cysteine (BPC), *S*-(2,4-dibromophenyl)-L-cysteine (DBPC), *S*-(4-methoxyphenyl)-L-cysteine (MPC), *S*-(4-methoxy-2-methylphenyl)-L-cysteine (MMPC) and *S*-(3-nitrophenyl)-L-cysteine (NPC) were synthesized according to the method of Parke & Williams (1951). Porcine heart ASAT and ALAT were obtained as lyophilized preparations from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). All other reagents used were of analytical grade and obtained commercially from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). C-S lyase activity (nmol pyruvate produced min^{-1} (mg protein)$^{-1}$) for each enzyme-substrate combination was determined using the method of Gutman & Wahlefeld (1974). The substrate concentration was 2·5 mM. C-S lyase activity in the presence of amino-oxyacetic acid (AOAA) and α-keto-γ-methiolbutyric acid was assayed according to the method of Buckberry et al (1993) due to the inhibition of lactate dehydrogenase (used in the method reported by Buckberry et al) by AOAA. Molecular modelling using INSIGHT and DISCOVER from Biosym Technologies was based on the crystal structure of chicken heart mitochondrial aspartate aminotransferase, which is known to have a very high degree of structural similarity to the porcine enzyme (McPhalen et al 1992; Malashkevich & Jansonius 1993) available from the Brookhaven database. TCVC was modelled in the active site using the aspartate-pyridoxal phosphate ketimine adduct from the crystal structure as a template and minimized using a conjugate gradient algorithm to RMS derivative of 0·0000239 kcal mol^{-1} $Å^{-1}$, allowing only the TCVC-pyridoxal phosphate adduct and residues within 3.5 Å of it to vary from their crystal structure coordinates.



Scheme 2. Rearrangement of 1-chlorovinyl thiolate anions to thioacyl chlorides and thioketenes.

Results

The optimum substrates for ASAT were shown to be DCVC and TCVC (106·29 ± 1·80 and 134·4 ± 13·25 nmol pyruvate min^{-1} (mg protein)$^{-1}$, respectively). The haloalkane-L-cysteine conjugates, CDFC and CTFC, and the heterocyclic conjugate BTC, were approximately 60% as active as the DCVC and TCVC (33·39 ± 5·80, 38·5 ± 10·46 and 40·56 ± 1·67 nmol min^{-1} mg^{-1}, respectively) (Table 1). Substituted *S*-(phenyl)-L-cysteine conjugates demonstrated C-S lyase activity below 30 nmol min^{-1} mg^{-1}. However, the overall C-S lyase activity of porcine heart ASAT was tenfold that observed previously with human or bovine tissues from any source. No significant change in activity was observed when the two optimum substrates were incubated with the transaminase co-substrate α-keto-γ-methiolbutyric acid (200 μM), producing only a 2 and a 1% increase in activity

Table 1. C-S lyase activity (nmol pyruvate min^{-1} (mg protein)$^{-1}$) of aspartate and alanine amino transferase.

L-cysteine conjugate	ASAT	ALAT
TCVC	134·4 ± 13·25	10·24 ± 1·64
DCVC	106·29 ± 1·80	4·32 ± 0·26
BTC	40·56 ± 1·67	6·46 ± 0·63
CTFC	38·5 ± 10·46	3·33 ± 0·02
CDFC	33·39 ± 5·8	2·35 ± 0·29
BPC	29·00 ± 0·46	2·7 ± 10·18
MPC	28·84 ± 0·55	4·36 ± 0·74
PC	28·43 ± 0·40	2·74 ± 0·93
DCPC	22·18 ± 3·59	2·25 ± 0·09
DBPC	15·14 ± 0·55	1·59 ± 0·10
MMPC	14·01 ± 0·66	2·62 ± 0·10
NPC	12·24 ± 0·56	3·46 ± 0·29
CPC	5·96 ± 1·89	3·09 ± 0·92

with DCVC and TCVC, respectively. The presence of the enzyme inhibitor, amino-oxyacetic acid (200 μM), resulted in the complete abolition of C-S lyase activity with DCVC and TCVC.

S-(1,2,2-Trichlorovinyl)-L-cysteine was observed to produce the greatest C-S lyase activity with ALAT ($10{\cdot}24 \pm 1{\cdot}64$ nmol pyruvate min^{-1} (mg protein)$^{-1}$). The other conjugates tested all exhibited activity between $6{\cdot}46 \pm 0{\cdot}63$ (BTC) and $1{\cdot}59 \pm 0{\cdot}10$ nmol min^{-1} mg^{-1} (DBPC) (Table 1). This level of activity is in agreement with those results previously obtained using human and bovine sources of C-S lyase (Buckberry et al 1990, 1992). No notable change in activity was observed when the two optimum substrates were incubated with the transaminase co-substrate α-keto-γ-methiolbutyric acid (200 mM), although the presence of the pyridoxal phosphate dependent enzyme inhibitor, amino-oxyacetic acid (200 μM), yielded a 97 and 89% decrease in CSL activity for DCVC and TCVC, respectively.

ASAT is a homodimeric enzyme with residues from both subunits important in each active site. Molecular modelling indicates that the active site of the enzyme is able to accommodate TCVC although there is appreciable Van der Waals overlap (35%) between the side-chain oxygen of the proximal serine residue (Ser 296) of the alternative subunit and one of the chlorine atoms of TCVC. Results from the molecular modelling are presented in Fig. 1.

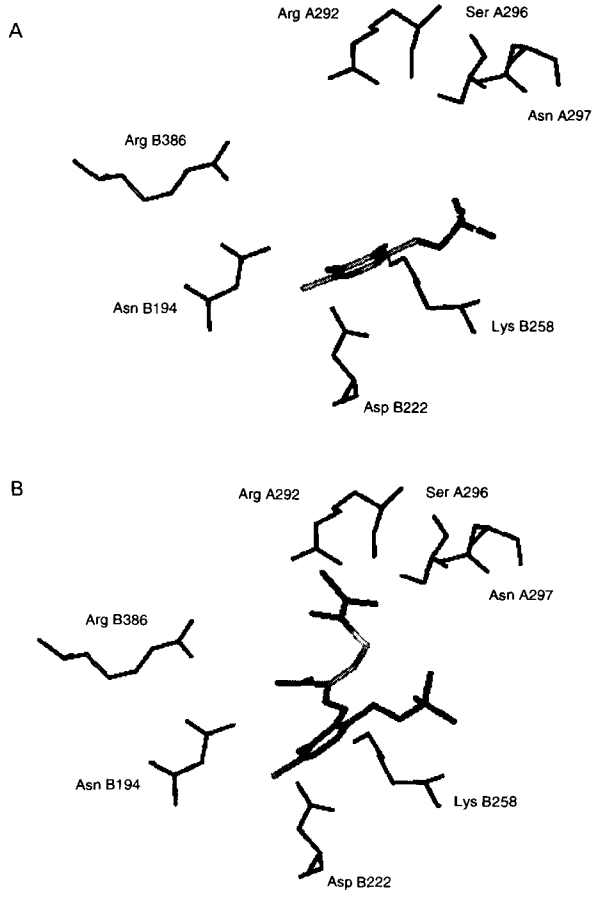


FIG. 1. The active site residues of ASAT in the absence of a substrate (A) and in the presence of TCVC (B).

Discussion

The ability of both ASAT and ALAT to act as C-S lyases has been firmly established and rationalized. The optimum substrates, TCVC and DCVC, are both 1-chlorovinyl cysteine conjugates which, as indicated above, are able to rearrange according to Scheme 2. The resulting thioacyl halide or thioketene is highly electrophilic and the most immediate biochemical outcome is the formation of adducts with cellular nucleophiles. Terminal amino groups and the nucleophilic side chains of lysyl, histidinyl and cysteinyl protein residues are obvious targets as well as the purine and pyrimidine bases of DNA (Farmer et al 1987). The result of such adduct formation being cytotoxicity and mutagenicity, respectively.

Whilst adduct formation onto the N-terminal valine residue of haemoglobin and cysteinyl thiols associated with the active sites of enzymes such as aldehyde oxidase are well documented (Farmer et al 1987), the highly reactive electrophilic species generated by C-S lysis would have a short life-time once released. Therefore the ASAT and ALAT amino acid sidechains would be the most immediate nucleophilic target for lyase mediated adduct formation, particularly the active site lysine, arginines or serine. Perturbation of ASAT and ALAT activity through binding coupled with the ubiquitous nature of these enzymes has serious implications for the respiratory capacity of cardiac, hepatic, renal and other metabolically active tissue.

Transamination reactions are known to be of central importance in linking amino acid metabolism and cellular respiration through the generation of α-keto acids (Miles 1985). Both isoforms of ASAT, that found in the cytosol and that found in the mitochondrion, are involved in the malate-aspartate shuttle (Price & Stevens 1987). The function of this complex pathway is to generate and transport reducing equivalents, in the form of NADH, maintaining mitochondrial redox status. The role of ASAT within the shuttle is the conversion of oxalacetate to 2-oxoglutarate which can be transported back out of the mitochondrion to complete the cycle with the aid of reconversion by cytosolic ASAT to oxalacetate. The malate-aspartate shuttle is the principal method by which hepatic and cardiac cells can translocate NADH into the mitochondrion.

The literature is in agreement (Stevens et al 1986) that the putative thioketene or thioacylating product formed by C-S lysis of DCVC is an extremely energetic, short-lived electrophile. It is therefore likely that such a product would damage targets in the microenvironment within which it was produced. Stonard & Parker (1971) demonstrated inhibition of malate dehydrogenase when rat-liver mitochondria were incubated with substrates in the presence of DCVC; other membrane components affected may be the calcium-transport systems which we have shown to be involved in DCVC-mediated cytotoxicity (Gaskin & Shaw 1994). Finally, the enzyme ASAT could itself be a target for suicide inhibition. In this scenario the energy and redox status of the mitochondrion would quickly be compromised leading to oxidative stress within the mitochondrion.

Similarly, ALAT is involved in energy production within the cell by the regulation of pyruvate levels through its interconversion with alanine. The provision of pyruvate to link the second stage energy metabolism of lipids, proteins and carbohydrates in the cellular cytosol, prior to its conversion into acetyl Co-A and entry into the TCA cycle highlights the importance of this enzyme in normal metabolic activity. Clearly perturbation of the action of both of these enzymes would have serious consequences for the cellular chemistry of cardiac and other metabolically active tissue.

The multitarget nature of DCVC toxicity has been demonstrated as nephrotoxicity (Gandolfi et al 1981), more recently as neurotoxicity (Patel et al 1984) and also as cytotoxicity of L-cysteine conjugates in-vitro in human Chang liver cells (Buckberry et al 1993b). The observation of ASAT and ALAT C-S lyase activity which we report here must therefore have a direct bearing on the risk assessment of any compounds metabolized by this route. This activity is likely not to be confined solely to these two aminotransferase enzymes, but may be a property of other pyridoxal phosphate dependent enzymes. This finding has two important implications. Firstly, that of computationally-aided structure-activity relationship studies permitting the prediction of cysteine conjugates which are likely to be cleaved by C-S lysis yielding a potentially reactive species, and secondly that C-S lyase enzymes may be more ubiquitous than was previously believed.

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