ENZYMES TRANSFERRING BIOMOLECULES TO ORGANIC FOREIGN COMPOUNDS: A ROLE FOR GLUCOSYLTRANSFERASE AND GLUTATHIONE S-TRANSFERASE IN PHYTOREMEDIATION

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1. Introduction

In the general enzyme list of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, first published in 1961 and with the last printed edition in 1992) EC 2 is reserved for the enzyme family of transferases. Generally, transferases are enzymes transferring a functional group, for example, methyl- or glycosyl-groups, from one substrate (regarded as donor) to another substrate (regarded as acceptor). Hence, the classification is based on the scheme "donor:acceptor-group transferase". The common names of the enzymes belonging to this group are normally derived from acceptor group-transferase or donor group-transferase. In many cases the donor is a cofactor (coenzyme) carrying the group to be finally transferred.

Whereas most members and subclasses of EC2 are confined to the metabolism of biogenic and natural compounds two subgroups, the glycosyltransferases of EC 2.4 and the aryl/alkyl transferases of EC 2.5, have been recognized as having crucial functions in the metabolism of foreign compounds, xenobiotics, in both animals and plants.

This role is very important, as all organisms are frequently exposed to an array of potentially toxic substances. Organic chemicals are particularly threatening. They may have natural sources e.g. fires, volcano eruptions or processes of biodegradation. They may also be the products of microbial or animal metabolism, or from the secondary metabolism of plants [1]. These organic substances may play a role in defence or in allelopathic reactions. Furthermore, increasing industrialization has provided two novel sources of foreign compounds: (1) through the invention and use of agrichemicals for the protection of crops from pests and weeds, and (2) through the emission of organic xenobiotics in chemical manufacturing processes or the use of synthetic chemicals. The latter compounds of solely anthropogenic origin represent a threat to our environment as these synthetic chemicals are emitted without any control. For plants, the situation is especially difficult as they are rooted in the ground and are dependent on that site for survival. Plants therefore, have to rely on effective detoxification mechanisms.

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The uptake of xenobiotics from polluted media, i.e. air, water or soil, follows the laws of phase distribution and diffusion. Plants therefore, have only limited possibilities to avoid accumulation of xenobiotics in their tissue and the associated detrimental consequences. In recent years, some plant species have been recognized as potent accumulators or detoxifiers of such compounds. These plants are capable of removing these dangerous chemicals from the environment. Hence, they are to be utilized in the green technology of phytoremediation, helping to solve some of our environmental problems in aninexpensive, reliable and natural manner. However, information on the underlying biochemical principles involved in these processes is generally scarce.

2. EC 2.4 Glycosyltransferases

All enzymes transferring glycosyl groups to acceptor molecules belong to this class. Some of these enzymes also catalyse hydrolysis, which can be regarded as transfer of a glycosyl group from a donor molecule to water. Also, inorganic phosphate can act as an acceptor in the case of phosphorylases; phosphorolysis of glycogen is regarded as transfer of one sugar residue from glycogen to phosphate. However, the more usual scenario is the transfer of a sugar from an oligosaccharide or a high-energy compound to another carbohydrate molecule as acceptor. This subclass, EC2.4 is further subdivided, according to the nature of the sugar residue being transferred, into hexosyltransferases (EC 2.4.1), pentosyltransferases (EC 2.4.2) and those transferring other glycosyl groups (EC 2.4.99). This mechanism is widespread in the plant kingdom, and the resulting glycosides represent the largest group of natural substances in plants, contributing factors to whether plants are colourful, tasty, or poisonous. The mechanism of sugar transfer in plants was discovered early in plant biochemistry [2]. The earliest reports of plant glucosides were associated with the metabolism of secondary compounds, such as flavonoids, anthocyanins and phenylpropanoids. Intermediates as well as storage forms of these compounds are frequently glucosylated. Glucosides possess lower reactivity than aglyca [3], they have a high hydrophilicity [4], they are used in detoxification of endogenous products and xenobiotics [5], and they may be compartmentalized in the plant [3]. A physiological role for glucosides is seen in pathogen defence, allelopathy and plant inherent signals [6, 7].

The transfer of glucose to a xenobiotic molecule requires the presence of an acceptor group on the target. Such an acceptor functional group might be an –OH, -NH or –SH function, and correspondingly, the plant glucosyltransferases are named O-glucosyl-, N-glucosyl-, and S-glucosyltransferases.

Molecules that do not bear these functional groups may be conjugated with sugars after chemical activation, i.e. hydroxylation by one of the plant P450 monooxygenases. For a significant number of herbicides, activation by P450 prior to detoxification by O-glycosyltransferases has been reported. Recently the formation of plant cyanoglucosides via stepwise activation by P450 and final glucosylation (e.g. dhurrin or triglochin) has attracted considerable interest [8, 9].

The second requirement for glucosyltransfer reactions is the availability of activated sugars, such as UDP-glucose (uridine[5´]diphospho-[1]-α-D-glucose, Fig. 1A). This metabolite is formed via phosphorylation of glucose in an ATP-driven reaction, to yield

glucose-6-phosphate, followed by conversion to glucose-1-phosphate. Glucose-1-phosphate reacts with UTP (uridine triphosphate), yielding UDP-glucose plus pyrophosphate. The UDP-glucose is the final activated intermediate that donates its glucose residue to the xenobiotic in an energetically favourable reaction. It has to be noted that the reaction of UDP-glucose on hydroxylated ring systems is the most frequently described reaction of sugar transfer in plants. Usually ß-1-D bonds are formed between the sugar moiety and the second substrate, but ß-5 and ß-3-conjugates have also been reported [3].

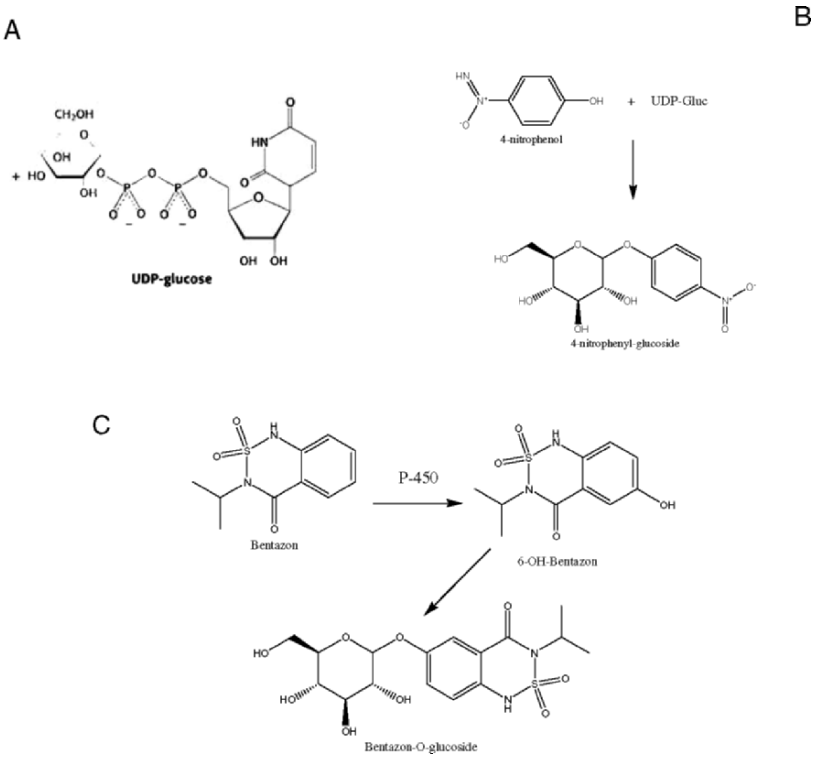

Figure 1. A) UDP-glucose, B) glucosyl transfer to a target molecule; C) conjugation of the herbicide, Bentazon, after activation by P450.

Numerous herbicides are conjugated to sugars via O-glucosyl-transfer or N-glucosyl-transfer in tolerant plants. The non-identity of the responsible enzymes has been demonstrated several times, although overlapping activities have been found in some cases. Conjugation may occur either at OH-groups of the molecule to form O-glucosides or at carboxy-groups to form acylglucosides. For N-glucosyltransfer, coupling to NH_2-groups of the molecule is crucial. From a practical point of view, predominantly phenolic pollutants, as well as components of ammunition (TNT and metabolites) or

pesticide spills, might be candidates for detoxification via glucose transfer in plants. A possible exploitation of these enzymatic mechanisms has recently been reviewed [10].

It has been shown that glucosyl-transferase activities are hardly inducible and might thus represent a class of housekeeping enzymes. Attempts to increase their activity using herbicide safeners are rare. Recently Brazier and co-workers [11] demonstrated the increase of O-GT in black grass after dichlormid or cloquintocet mexyl treatment. In each of the treatments increased activity was found for the conjugation of quercetin but not for xenobiotic compounds. On the other hand, evidence has been found to suggest that the individual enzymes responsible for these reactions might well be under developmental control and that the conjugation of single xenobiotics can not be expected to proceed throughout the plant's life and in every plant part [12, 13]. Of course, this fact has consequences for the practical use of plants in the detoxification of foreign compounds, and it is especially important when considering plants for use in phytoremediation, because it has to be ensured that the detoxification capacity meets with the xenobiotic burden of the system.

Table 1: Examples for xenobiotic substrates of glucosyl transferases in plants (adapted from [14])

O-glucosyl transfer		N-glucosyl transfer	
Direct conjugation	after activation	direct conjugation	after activation
1,2,5-Trichlorophenol	2,4-D	Chloramben	Dinoben
2,4-dichloroanilin	Chlorpropham	Metribuzin	Propanil
4-nitrophenol	Cisanilide		Pyridate
4-nitrophenol	DDT / DDE		
Chloramben	Dicamba		
Clopyralid	Bentazon		
Dimethenamid	Diclofop		
Fenoxaprop ethyl	Diphenamid		
Maleic hydrazide	Methylphenylureas		
MCPA	Perfluridone		
Pentachlorophenol	Sulfonylureas		
Picloram	Terbacil		
Quinclorac			

It is important to note, that glucosyl conjugates may be cleaved by glucosidases and, in the case of acylglucosides (-COOH substitution) by esterases. Both enzyme activities are abundant in plant cells. However, these activities might be compartmentalized or under developmental control. The action of these enzymes will yield the respective aglyca that are spontaneously reprotonated under the conditions of the cytosol. Thus, the original xenobiotic substrate may be regenerated. This reversibility represents a great disadvantage of glucosylation for its practical consideration in phytoremediation, because previously detoxified compounds may regain their toxicity under certain conditions. In oats, the formation of an acylglucoside from Diclofop in the presence of an esterase explains this plant's susceptibility to this herbicide. In wheat, an O-glucoside is formed from Diclofop that is not readily cleaved [15].

This reaction chain of activating, conjugating and releasing a certain compound makes sense in the course of natural compound formation. It has also been shown that

homeostasis of salicylic acid and indole acetic acid (auxin) is maintained in plant cells by this mechanism. For practical application, it has to be ensured that the cleavage of the glycosyl-conjugate happens only under conditions where the aglycon can be inserted into the cell wall and covalently bound to lignin or other polymerous structures.

3. EC 2.8 Glutathione S-transferases

Various xenobiotics possess electrophilic centres, i.e. centres of low electron density that can accept an electron pair to form a covalent bond. This feature makes them dangerous because they can to react spontaneously with corresponding nucleophilic sites of proteins and genetic material, i.e. DNA and RNA and thereby disturb metabolic networks.

The action of such electrophilic xenobiotics appears to be dependent on particular cellular enzymes called glutathione S-transferases (GSTs) [16, 14]. Electrophilic centres necessary for GSH conjugation are found in arene-oxides, aliphatic and arylic halides, in α–β-unsaturated carbonyls, organonitro-esters and organic thiocyanates. Industrial substrates for GSTs are haloalkanes, chlorobenzenes, thiocarbamates, diphenylethers, triazines, chloracetanilides [see 17, 18]. In animals the oxidants acrolein, propenals, lipid hydroperoxides, chlorambucil and fosfomycin are additional substrates [19].

Such compounds will not be conjugated by glucosyl transferases. Instead, the reactions are performed by a somewhat heterogeneous class of enzymes, GSTs, which catalyze the transfer of aliphatic, aromatic, or heterocyclic radicals as well as epoxides and arene oxides to glutathione. The transfer reaction takes place at the sulphur atom and has been annotated as the enzyme class coding EC 2.5.1.18. GST enzymes occur ubiquitously [20]. The binding of the foreign compound and the transfer of glutathione follows two mechanisms catalyzed by glutathione S-transferases [21, 22]:

- (a) Nucleophilic displacement of an alkyl or aryl halogen or a nitro group is the most frequently observed step. Conjugation of many pesticides like atrazine, propachlor or pentachloronitrobenzene (PCNB) are examples of this type of reaction. Halogens or nitrogroups of these molecules are soft electrophiles and react readily with GSH. Further substitution reactions are found in the detoxification of diphenylether herbicides (e.g. fluorodifen, fenoxaprop-ethyl). Here, an ether bond is cleaved and substituted by the thiolate. Moreover, the standard enzyme assays for GST activity use 1-chloro-2,4-dinitrobenzene (CDNB) or 1,2-dinitro-4-chlorobenzene (DCNB) as substrates (see Fig. 2, B).
- (b) Nucleophilic addition (Michael reaction): Addition of the thiolate to carbon-carbon-double bonds is a special type of reactions on compounds with reactive carbon-carbon double bonds neighboured by an electron withdrawing group [23]. Conjugation of tridiphane or cinnamic acid may be examples for this type of reaction [24, 25]. The conjugation on these bonds leads to a labile conjugate that may be sensitive to pH changes.

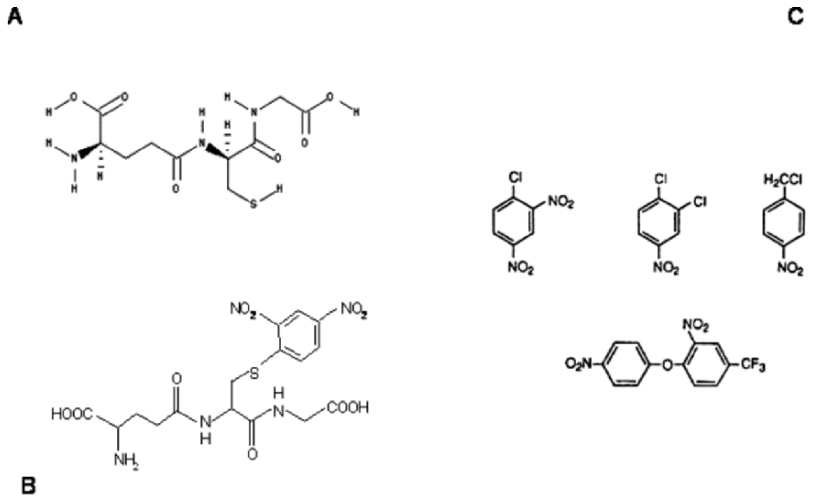

Figure 2. (A) reduced glutathione (γ-glutamyl-cysteinyl-glycin), (B) S-dinitrobenzyl-glutathione; (C) four typical plant GST substrates: chlorodinitrobenzene, dichloronitrobenzene, nitrobenzylchloride, and fluorodifen.

In contrast to the metabolism of glucosyl conjugates, and with the exception of the cleavage of "Michael-reaction"-type conjugates formed at double bonds of molecules (see above), the dissolution of glutathione conjugates does not lead to the liberation of the original toxic or lipophilic foreign compound. This is due to the fact that nucleophilic substitution or displacement removes the significant electrophilic centre from the target molecule to introduce the glutathione thio-function. When cleaved, the electrophilic moiety is lost, and the toxicity of any released parent residue is significantly lowered.

The unravelling of the *Arabidopsis* genome has confirmed the observation that the multiple reactions of GST enzyme activity are attributes to a large number of isoenzymes encoded for by more than 50 GST genes. These genes, many of them formed by gene reduplication in the simple *Arabidopsis* genome, can be clustered in four distinct groups. According to the mammalian GST system, which names the GSTs with Greek letters, a comprehensive nomenclature of plant GSTs has been proposed by Edwards and coworkers [26].

To date, five distinct classes of GSTs, Lambda (and DHAR, dehydroascorbate reductases), Phi, Tau, Theta and Zeta have been identified in plants. According to Edwards & Dixon [27], class Tau, the predominant class, catalyzes the detoxification of xenobiotics by nucleophilic substitution reactions. Class Phi and Tau GSTs, the next most abundant, appear to catalyze reactions with endogenous toxic metabolites or are involved in the metabolism of reactive oxygen species. The two remaining, classes Lambda and Zeta, are unusual, Lambda GSTs occur as monomeric enzymes or enzymes with transmembrane regions, functioning as redox mediators. Zeta GSTs are isomerases.

The multitude of GSTs in plants reflects their ability to conjugate to GSH a large array of different substrates, many of them of anthropogeneous origin. Furthermore, GSTs are inducible by different forms of stress, including xenobiotics [28]. This property also confers herbicide tolerance or resistance in many plant species. Whereas herbicides are designed to kill weeds and leave crops unaffected, organic pollutants might have properties that stress plants in a similar manner. GST activities have been shown to be crucial for the detoxification of a number of these compounds.

4. Physiological roles of conjugates

Summarizing the above results, we must conclude that glutathione and sugar conjugation lead to the formation of detoxified and more or less stable products in the plant cytosol. Upon chemical contact or under conditions of pesticide application, plants may encounter relatively high concentrations of the respective foreign compounds, and, provided the defence enzymes work properly, they will form significant amounts of conjugates from them. Besides eventual chemical lability this accumulation of conjugates will lead to an unfavourable situation as conjugates might inhibit enzymes by feedback mechanisms or may affect specific binding.

Glucosyl- and glutathione-conjugates do not accumulate in the cytosol of plant cell, but are translocated into the vacuole through ABC-transporters [29, 30, 15]. The presence of a conjugate is mandatory for this translocation. An interesting observation is that glucosylconjugates build up in pools inside the plant, whereas glutathione conjugates do not. They undergo rapid and complete metabolism [31]. As well as the ABC-transporters on the tonoplast, ABC-transporters have recently been found in the plasmalemma. They accept the same conjugates and allow for long range transport of these metabolites. Some hints exist that a physiological role for conjugates might be the signalling of pollutant stress, and induction of detoxification reactions has been described after application of xenobiotics to plant cells [32]. In the context of phytoremediation, such conjugates might also play a role in enhancing the plants capacity for detoxification.

5. Conjugation reactions and options for phytoremediation

The use of plants in phytoremediation of organic pollutants has been reviewed thoroughly in the frame of the COST action 837 [33, 34, 10, 14). Literature on the degradation of herbicides in crops demonstrates clearly that xenobiotic conjugates are usually further processed to more complex conjugates [21, 31], or cleaved to form reactive molecules that are excreted from the living cell and reside in the cell wall or the apoplast [35]. In our context of phytoremediation it is, of course, crucial to know which type of primary conjugation occurred, because this determines the final fate of the compound [36, 37].

Further breakdown steps include incorporation of metabolites into the cell wall in the pectin, lignin, hemicellulose and cellulose fraction [35, 38, 39]. This has been demonstrated with numerous cereals, soybean and the respective cell cultures. Few

metabolites have also been found in the rhizosphere, where they might disappear in microbial and mycorrhizal metabolism, and single findings point to the volatilization of metabolites after the action of methyl transferases [31]. One crucial question remaining is how and why the conjugating enzymes detect and meet the foreign compounds attacking the cells. Given the large number of isoforms of detoxification enzymes on the one hand, and the diverse group of xenobiotics on the other, it is hard to imagine how the different reactions occur in the cytosol in an orderly manner. Even if the respective enzymes would occupy distinct positions in a metabolic network, the question would remain how the detoxification is channelled in a cell or a tissue. Our present knowledge is that the conjugating enzymes have low specificity, i.e. that they are able to accept a larger number of endogenous and xenobiotic substrates. Only very specifically designed poisons at higher concentrations will represent a severe threat.

Historically, our present state of knowledge on foreign compound metabolism is focused on crops and a small number of ornamental plants. Only a few reports exist on plants that might be interesting with respect to their potential in phytoremediation. Conjugative metabolism in the outstanding candidates *Arundo donax*, *Brassica juncea*, *Phragmites sp.., Typha sp.., Plantago majus, Populus sp.., Salix sp.*, to name but a few, has not been investigated in any depth. This situation is awkward as there are already numerous existing field sites that seem to be very successful in the removal of xenobiotics from soil and water. Knowing about the mechanisms involved, the efficiency of these systems could probably be improved when methods to increase metabolism rates would be applied. One option could be to utilize gene manipulation methods to over express the desired enzymes. Another option could be to add inducers of herbicide resistance to the plants. Finally it is possible that xenobiotic uptake and transport to tissues with high degradative activity would be enhanced. Each of these attempts would improve commercial and public acceptance of the use of plants to improve the environment with biological methods. Plants have a large potential to cope with their environment [40], and they will also be able to help us solve some of our pollution problems in an environmentally friendly way.

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