Reaction of isothiazolinones with glutathione

1. Introduction

Reactive substances and how cells deal with them

Cells are the smallest biological entities performing active metabolism and therefore they have to deal with a couple of reactive substances, intermediates and metabolites.

There are different sources of such substances in biological systems. The major part comprises intermediates and metabolites produced during the "house-hold" cell metabolism (e.g. aldehydes and ketones in the citric-acid cycle) wich are not toxicologically relevant under normal conditions.

But of course there is also an extracellular input of highly reactive substances chemicals into organisms, tissue and cells. Once passed through the cell membrane reactive chemicals can directly interact with proteins leading to inactivation of, for example, enzymes ore even worse they can migrate into the nucleus and form adducts with DNA. Additionally, during the metabolic degradation of xenobiotics in cells it is possible that highly reactive metabolites and intermediates (e.g. epoxydes or alkylating electrophiles) are formed.

To deal with that problem different protection mechanisms have evolved in cells to absorb, inactivate and excrete reactive substances. Among these the most prominent and ubiquitous is the glutathione system. Glutathione (GSH, Figure 1) is a tripetide which is synthesized in the cytosol of the three amino acids cysteine, glycine and glutamate.

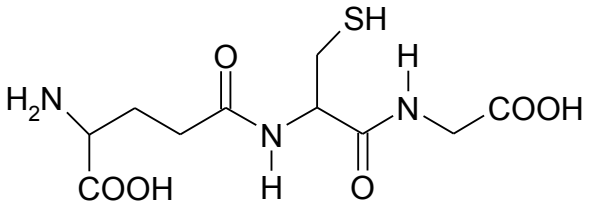

Figure 1: Structure of the glutathione tripeptide

The core of this biomolecule is formed by the cysteine residue in which particularly the thiol group plays a crucial role in the detoxification process of xenobiotics. This process can be divided into three major steps catalyzed by certain metabolic enzymes (Figure 2). According to its relatively pK_a value of 8.75 the thiol group of GSH is partially deprotonated under physiological conditions in the active site of the glutathione-S-transferase enzymes. The resulting thiolate is a very potent nucleophile which can readily attack electrophilic groups in xenobiotics.

This first step is catalyzed by glutathione-S-transferases. The formed GSH-adduct is then converted by peptidases which cut off the glycine and the glutamine. The remaining cysteine adduct is subsequently acetylated by acetyle transferases in the last step and the formed mercapturic acid derivative can be easily excreted.

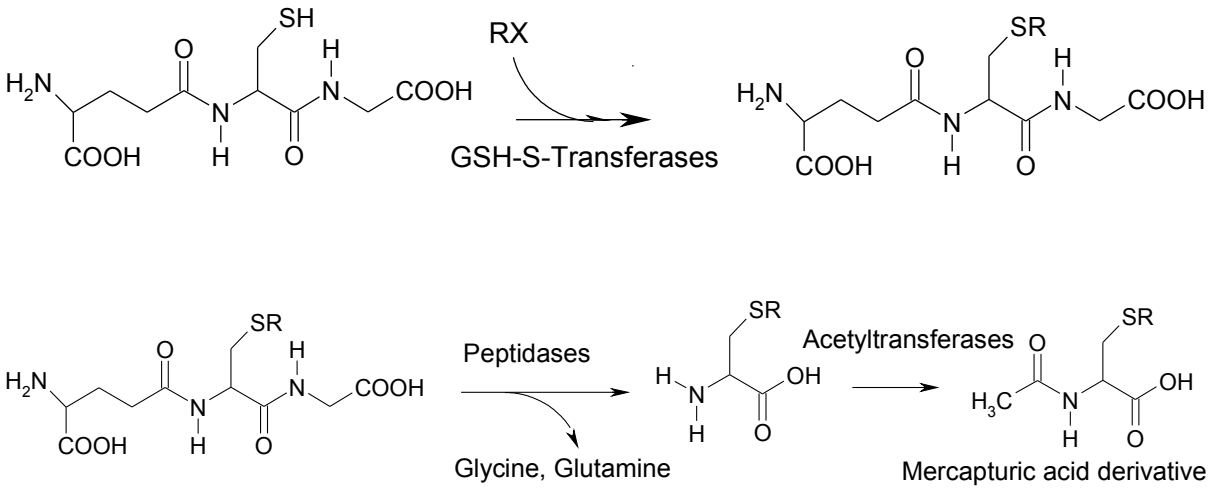

Figure 2: Detoxification of xenobiotics (RX) via coupling to GSH

In ecotoxicological studies the coupling of environmental pollutants to GSH is an important parameter to estimate, characterize and compare the reactivities of different substances. Therefore the reactivity of the isothiazolinone biocides towards GSH will be investigated.

Isothiazolone biocides as an example for highly reactive xenobiotics

One of the major interests of our department is the ability to deduce risks and modes of toxic action of chemicals from structure-activity-relationships. To reach this goal it is necessary to study and elucidate the modes of toxic action of known highly reactive substances at cellular and subcellular levels.

Currently, the substance class of the isothiazolone biocides is being investigated in our lab. Isothiazolone biocides consist of an aromatic isothazolin-3-one ring system with variable substituents at the nitrogen and carbon atoms in the ring. Figure 3 shows two out of the three biocides that we will investigate. Please draw the structure of 5-chloro-N-methylisothiazoline-3-one (CIT).

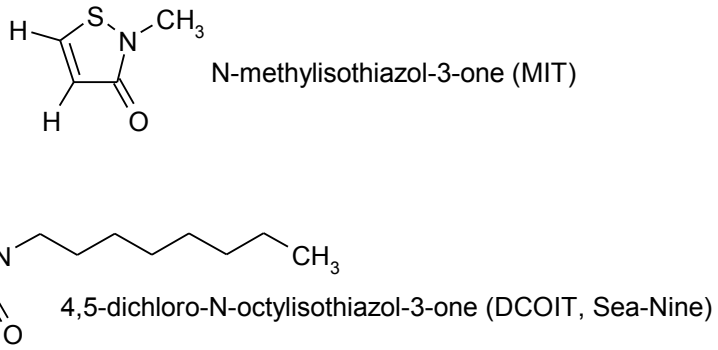

Figure 3: Chemical names and structures of two of the isothiazolone biocides

The high reactivity of these biocides is based on the very low electron density in the aromatic ring system which renders it highly susceptible to nucleophilic attack at the sulfur atom. Especially thiol groups from cysteine residues in proteins can act as electron rich nucleophiles

forming disulfides with the isothiazolones in a nucleophilic addition reaction. Such alterations lead to denaturation and inactivation of crucial enzymes and other proteins in cells providing the antimicrobial effect of the biocides.

Due to this reactivity and especially due to the different substitution patterns and chain lengths the isothiazolone biocides are very useful for studying structure-activity-relationships with respect to modes of toxic action.

To give you an idea about the reactivity and possible structure-activity-relationships of the isothiazolone biocides this experiment will address the following two key questions:

1. Do the biocides react with the GSH as it is theoretically assumed?
2. If yes, are there differences in reaction kinetics among the biocides and how can they be explained?

Some theoretical considerations

Before getting into the experimental details it is helpful to think about the following theoretical questions and remarks:

- Please color code the isothiazolones and the GSH molecule (take care of ionizable groups!). Is GSH a "normal" petide? What about the molecular interaction potentials available in the substances?

- Look at the lipophilicity of the isothiazolones. Can you suggest a ranking concerning uptake into cells and possible uptake mechanisms?

- Please formulate the detoxification process of the DCOIT compound via the coupling to GSH. Compare the molecular interaction potentials of the single biocide at the beginning of that process and for the mercapturic acid adduct at the end of the reaction chain. What is the detoxification strategy behind such a so called "phase II" metabolism? Can you give an example for another phase II detoxification reaction using the same strategy? (Please explain your statements with color coding the molecules of interest!)

Experimental section

Buffers and solutions

Before starting the experiments the following buffers and solutions need to be prepared. For the buffers the complete recipes are given.

<u>10 mM Na-phosphate buffer pH 7,0:</u>

12,2 mL 0,2 M Na_2HPO_4 solution
7,8 mL 0,2 M NaH_2PO_4 solution
ad. 400 mL with H_2O and calibrate pH to 7,0 with 0,1 M HCl

1mM Na-phosphate buffer pH 3,0:

27 mg NaH_2PO_4
29 µL 1,0 M H_3PO_4 solution
ad. 200 mL with H_2O and calibrate pH to 3,0 with 0,1 H_3PO_4 solution

10 mM GSH solution in 10 mM Na-phosphate buffer pH 7 (store on ice!)

All other solutions which are indicated in the following sections and the above mentioned ingredients for the buffers are already prepared and will be handed out to you at the beginning of the experiments.

Reaction of the isothiazolones with GSH

To check whether the isothiazolones are UV-detectable please dilute your biocide stock solution to get 1 mL of a 200 µM solution in Na-phosphate buffer 10 mM pH 7,0. With this solution a UV/VIS spectrum is scanned in the range from 200 nm up to 500 nm using pure Na-phosphate buffer as a blank. What is your suggestion for the appropriate wavelength for monitoring the reaction?

The following reaction mixtures are prepared in 1 mL quartz cuvettes with 1 cm optical path length one at a time. Directly after adding the biocide solution the absorption of the biocide at the chosen wavelength is monitored over time.

Mixture A: 1000 – x µL Na-phosphate buffer 10 mM pH 7,0 + x µL biocide solution

Mixture B: 900 - x µL Na-phosphate buffer 10 mM pH 7,0 + x µL biocide solution + 100 µL 10 mM GSH solution

Mixture C: 900 - x µL Na-phosphate buffer 1 mM pH 3,0 + x µL biocide solution + 100 µL 10 mM GSH solution

Here, x is the number of µL of the biocide stock solution containing 0.1 µmol biocide. The biocide solutions passed out to you have the following concentrations:

MIT:	116.8 mM
CIT:	18.3 mM
DCOIT:	4.95 mM

Save the data to disc as an ASCII file, import it into a software package capable of curve fitting, and find the kinetic constant k for the equation

$$A_{(t)} = A_\infty + (A_0 - A_\infty)\mathrm{e}^{-k\,t}$$

where $A_{(t)}$ ist the absorption at the chosen wavelength over time t, A_∞ is the absorption that is asymptotically reached at the end of the experiment, A_0 ist the absorption at the start of the experiment.

Try to explain the relative trend of the reaction speed for the three different biocides.