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The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity

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Biochemical and Biophysical Research Communications 2005;338:923-929

Abstract

Background

Quercetin is one of the most prominent dietary antioxidants. During its antioxidant activity, quercetin becomes oxidized into its *o*-quinone/quinone methide QQ. QQ is toxic since it instantaneously reacts with thiols of e.g. proteins. In cells, QQ will initially form an adduct with glutathione (GSH), giving glutathionyl-quercetin adducts, denoted as GSQ. Little is known about the fate of GSQ once formed in a biological system. Therefore, the aim of the present study is to investigate the stability of GSQ and to examine the exchange of GSH in GSQ for other thiols.

Methods

All incubations were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

We have found that GSQ is not stable; it dissociated continuously into GSH and QQ with a half life of 2 minutes. Surprisingly, GSQ incubated with 2-mercapto-ethanol (MSH), a far less reactive thiol, resulted in the conversion of GSQ into the MSH-adduct MSQ. A similar conversion of GSQ into relatively stable protein thiol-quercetin adducts is expected. With the dithiol dihydrolipoic acid ($L(SH)_2$), quercetin was formed out of GSQ.

Conclusions

The results of the present study indicate that GSQ might act as transport and storage of QQ. In that way, the initially highly focussed toxicity of QQ is dispersed by the formation of GSQ that finally spreads QQ-induced toxicity, probably even over cells.

Introduction

Quercetin is the most studied flavonoid and is ubiquitous in our diet, especially in vegetables, tea, fruit and wine (1,2). Moreover, quercetin is a major constituent of countless food supplements and other nutraceuticals. Quercetin has gained special attention since it is an antioxidant which very efficiently scavenges highly reactive biological species such as peroxynitrite (3,4) and the hydroxyl radical (5,6).

Oxidative stress is defined as an imbalance between the formation of highly reactive species and the protection against these species by antioxidants. This condition is implicated in the etiology of various diseases including chronic obstructive pulmonary disease (COPD) (7,8), sarcoidosis (9), fibrosis (10), diabetes (11,12) and cancer (13,14). An increased intake of dietary antioxidants is expected to reduce oxidative stress. Quercetin seems to be an excellent candidate to empower the endogenous antioxidant defence system.

During the scavenging of highly reactive species, an antioxidant is converted into an oxidation product. The oxidation product often is relatively stable but it usually retains a part of the reactivity of the species it has scavenged (15). Oxidation of quercetin results in the formation of an *o*-quinone/quinone methide, denoted as QQ (16,17). QQ is highly reactive towards thiols groups and is therefore toxic (18). Gluthathione (GSH) is the primary target of QQ in cells (17). GSH rapidly reacts with QQ, resulting in the formation of two glutathionyl-quercetin adducts, i.e. 6-glutathionyl-quercetin (6-GSQ) and 8-glutathionyl-quercetin (8-GSQ) (16,17,19), together denoted as GSQ. Little is known about the fate of these adducts once formed in a biological system. This prompted us to study the stability of GSQ and to examine the exchange of GSH in GSQ for other thiols.

Materials and methods

Materials

Quercetin, reduced GSH, tyrosinase, 2-mercapto-ethanol (MSH), dihydroic lipoic acid ($L(SH)_2$) and 1-chloro-2,4-dinitrobenzene (CDNB) were all purchased from Sigma (St. Louis, MO, USA). Sep-Pak C18 cartridges were obtained from Waters (Ireland).

Methods

All incubations were performed at 37ºC in a 143 mM phosphatebuffer pH 7.4 and were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Formation of QQ

Due to the relative instability of QQ, it had to be generated *in situ* for each experiment. The oxidation of quercetin leading to the formation of its *o*-quinone methide (QQ) was performed by adding 100 U/ml tyrosinase to 100 µM quercetin. After 30 seconds, the solution was filtered using a Sep-Pak C18 cartridge in order to stop the reaction by removing the enzyme. The concentration of QQ was calculated as previously described (17). QQ generated in this way contained not oxidized quercetin. In the reactions of QQ studied, quercetin did not interfere.

Synthesis of GSQ, MSQ and QOH

GSQ, MSQ and QOH also had to be synthesized *in situ* due to limited stability. Synthesis of GSQ was performed by adding 10 U/ml tyrosinase to 100 µM quercetin in the presence of 120 µM GSH. After approximately 80% of the quercetin was oxidized, based on spectrophotometrical analysis (the exact degree of quercetin oxidation was determined using HPLC), the solution was directly filtered using a Sep-Pak C18 cartridge in order to stop the reaction and purify the GSQ formed during this reaction. The GSQ concentration was quantified as previously described (17). The formed GSQ contained a substantial amount of quercetin and the excess of GSH. The GSH present in GSQ might have an effect on the rate constant obtained with relatively low concentrations of MSH and $L(SH)_2$. To reduce possible interference, these experiments were performed with concentrations of MSH and $L(SH)_2$ that were much higher than the concentrations of GSH present in GSQ.

As model compound for protein thiols 2-mercapto-ethanol (MSH) was used. The adduct of MSH with quercetin (MSQ) is easier to detect than adducts of proteins such as albumin with quercetin. The synthesis of MSQ was performed similar to the strategy used for the synthesis of GSQ. 10 U/ml tyrosinase was added to 100 µM quercetin in the presence of 120 µM MSH. Purification and calibration are comparable to that described for GSQ.

QOH was synthesized according to Boots *et al.* (20).

HPLC analysis

For qualitative and quantitative HPLC analysis of the three synthesized compounds, a reversed phase Supelcosil LC318 column (25 cm x 4.6 mm) was used. For GSQ, the column was eluted with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile for 5 minutes, followed by a gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes. For MSQ, the column was eluted with water containing 0.1% (v/v) trifluoroacetic acid and 7.5% acetonitrile for 5 minutes, followed by a gradient to 15% acetonitrile from 5 to 22 minutes and to 30% acetonitrile from 22 to 38 minutes. For QOH, the column was eluted with 20% acetonitrile and 80% 0.1% (v/v) trifluoroacetic acid. Typical chromatograms of GSQ, MSQ and QOH are shown in Figure 5.1.

HPLC analysis of the incubation mixtures, unless otherwise noted, was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). The column was eluted isocratically with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile during 5 minutes, followed by a linear gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes.

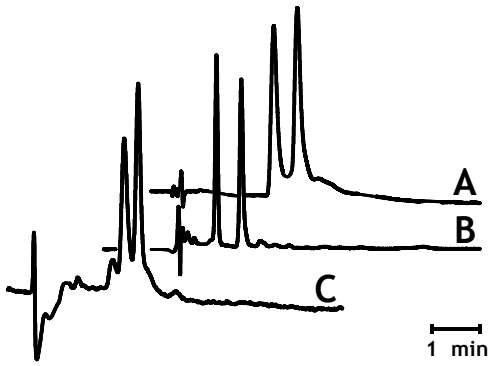

Figure 5.1 The product formed by the reaction of QQ with either GSH (trace A), H_2O (trace B) or MSH (trace C).
The HPLC analysis shows the different isomers of the adducts formed, i.e. 6-GSQ and 8-GSQ with GSH, 6-QOH and 8-QOH with H_2O and 6-MSQ and 8-MSQ with MSH. A typical example is shown.

Thiol reactivity

The reactivity of GSH, MSH and $L(SH)_2$ with CDNB was determined by mixing 1 mM thiol with 1 mM CDNB. This reaction was monitored for 10 minutes. The increase in absorption at 340 nm was used to calculate the second order rate constant k_{CDNB} for each thiol.

Statistics

All experiments were performed, at least, in triplicate. Data are given as mean ± SEM or as a typical example.

Results

Incubation of GSQ with mercapto-ethanol (MSH) results in the formation of a MSH-QQ adduct, i.e. MSQ. In an excess of MSH, the reaction follows the kinetics of a (pseudo) first order reaction (Figure 2A and 2B).

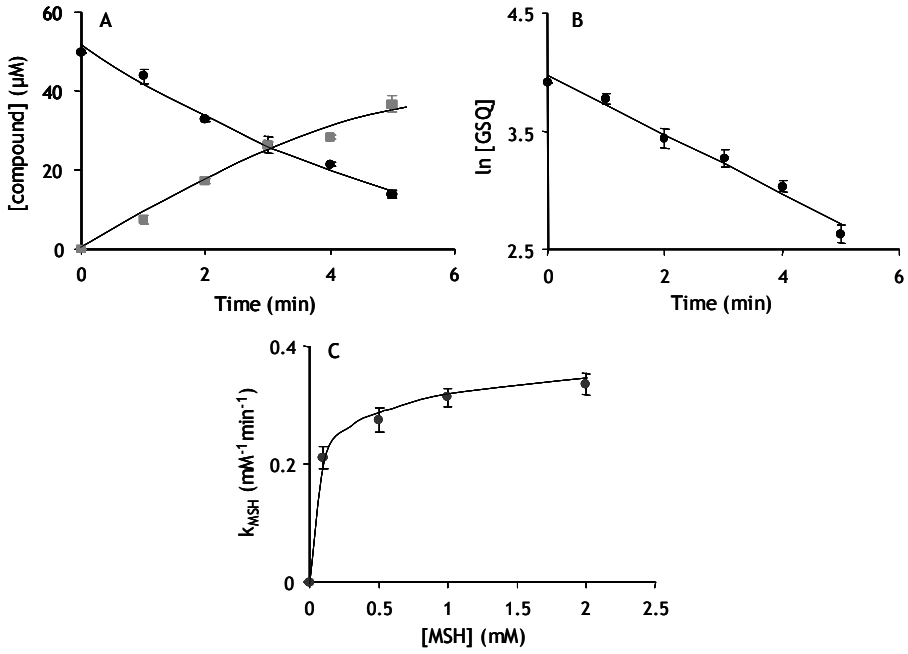

Figure 5.2 Reaction kinetics of the incubation of GSQ with MSH. Panel A shows the formation of MSQ (open squares) out of GSQ (closed dots) in time during the incubation of GSQ (initial concentration of 50 μM) with MSH (100 μM). In panel B the natural logarithm of the GSQ concentration is plotted in time. In panel C k_{MSH} is plotted as function of the MSH concentration. Experiments are performed at least in triplicate and data are shown as mean ± SEM.

The (apparent) first order reaction constant (k_{MSH}) increases upon increasing the concentration of MSH, but surprisingly the rate does not linearly depend on the concentration of MSH (Figure 5.2B). At high concentrations of MSH, the rate constant k_{MSH} approaches 0.4 min^{-1} (Figure 5.2C).

Incubation of GSQ with the dithiol lipoic acid ($L(SH)_2$) results in the formation of quercetin. Again, in an excess of $L(SH)_2$ the reaction follows the kinetics of a (pseudo) first order reaction (Figure 5.3A and 5.3B). Similarly to MSH, the rate with $L(SH)_2$ does not linearly depend on the concentration of the thiol and the rate constant $k_{L(SH)2}$ approaches 0.4 min^{-1} at high concentrations of $L(SH)_2$ (Figure 5.3C).

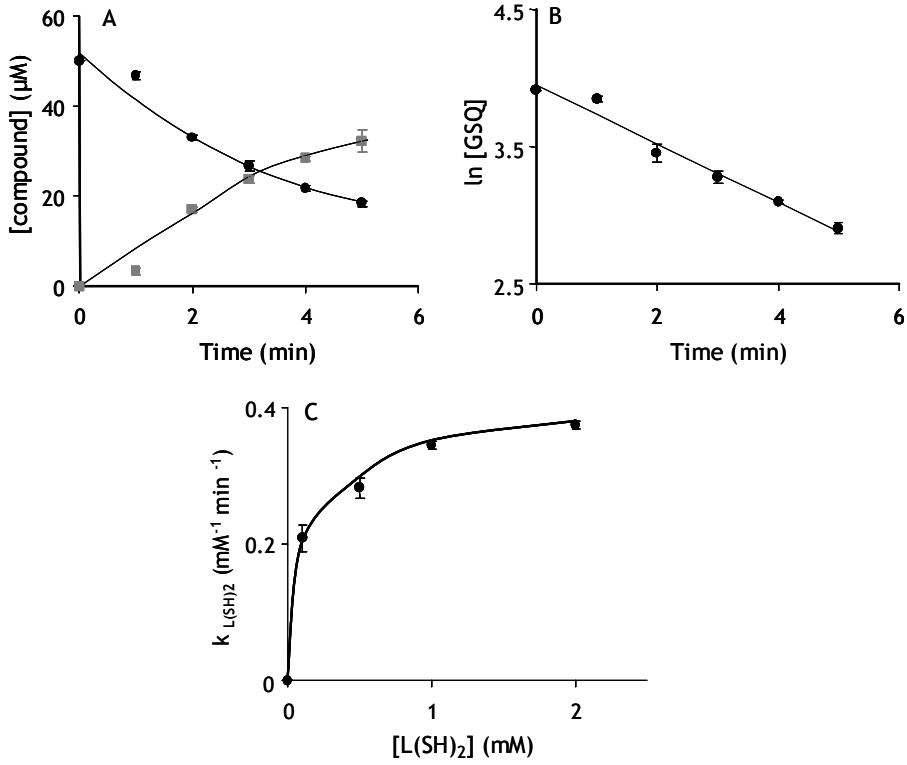

Figure 5.3 Reaction kinetics of the incubation of GSQ with $L(SH)_2$. Panel A shows the formation of quercetin (open squares) out of GSQ (closed dots) in time during the incubation of GSQ (initial concentration of 50 μM) with $L(SH)_2$ (100 μM). In panel B the natural logarithm of the GSQ concentration is plotted in time. In panel C $k_{L(SH)2}$ is plotted as function of the $L(SH)_2$ concentration. Experiments are performed at least in triplicate and data are shown as mean ± SEM.

When QQ is formed in the presence of both GSH and MSH, initially GSQ is formed (Figure 5.4). After a longer time of incubation, GSQ is gradually converted into MSQ. When QQ is formed in the presence of both GSH and $L(SH)_2$ again initially GSQ is formed. After a longer time of incubation, GSQ gradually disappears and quercetin reappears (data not shown).

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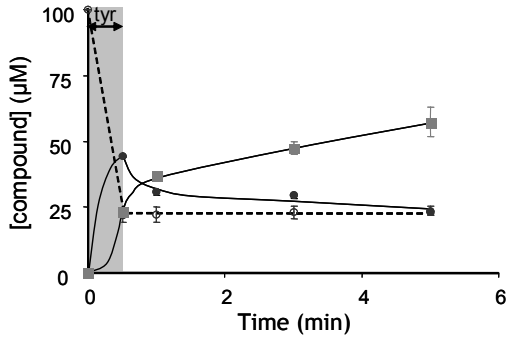


Figure 5.4 The fate of QQ in the presence of equimolar concentrations of both GSH and MSH. The initial concentration of GSH and MSH is 500 µM. QQ is formed *in situ* by adding 100 U/ml tyrosinase (tyr) to 100 µM quercetin (open dots, dotted line). After 30 seconds, this reaction is stopped by filtering the solution, thereby removing the enzyme and stopping the QQ formation.
Initially, GSQ (closed dots) is formed. In time GSQ is readily converted into MSQ (open squares). All experiments are performed at least in triplicate and data are expressed as mean ± SEM.

The reactivity of the thiols used in the present study was determined by monitoring the reaction of the thiols with CDNB. The rate constant of the

second order reaction with GSH (0.020 $mM^{-1} \cdot min^{-1}$) was twice that with MSH (0.0073 $mM^{-1} \cdot min^{-1}$) and $L(SH)_2$ (0.011 $mM^{-1} \cdot min^{-1}$) (Figure 5.5).

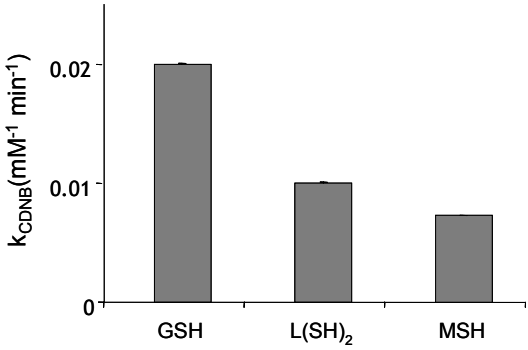


Figure 5.5 Second order rate constant of the reaction of GSH, MSH and $L(SH)_2$ with CDNB. Data are expressed as mean ± SEM (n=3).

Discussion

QQ formed during the oxidation of quercetin is highly reactive towards thiol groups (17). *In vivo*, the most abundant thiol is glutathione (GSH). Furthermore, GSH is a better nucleophile than most other cellular thiols and its nucleophilicity is often further enhanced by the catalytic activity of various GSH-S-transferases. Therefore it is expected that initially QQ will react with GSH to form GSQ. The formation of the glutathionyl-adducts 6-GSQ and 8-GSQ from QQ under various conditions has been reported (16,17,19).

In the present study the fate of GSQ is examined. Our results show that GSQ is not stable; in both glutathionyl adducts of quercetin GSH is readily exchanged for another thiol such as MSH or $L(SH)_2$.

The reaction of MSH with GSQ results in the formation of an MSH-QQ adduct, i.e. MSQ. Similar to GSQ there are two mercapto-ethanyl-adducts of quercetin, tentatively identified as 6-MSQ and 8-MSQ. Both forms of MSQ are formed in approximately equimolar concentrations and in all experiments the ratio of both adducts was constant. For sake of clarity MSQ is used to denote the combination of 6-MSQ and 8-MSQ. At a high concentration of MSH, the rate of MSQ formation is independent of the MSH concentration. This suggests a reaction scheme for the MSQ formation as depicted in Figure 5.6A. The binding of GSH to QQ is reversible and at high concentrations of MSH, the dissociation of GSQ is the rate limiting step in the MSQ formation. The first order rate constant of the GSQ dissociation (k_{GSQ}) is 0.4 min^{-1} at pH 7.4 and 37ºC. Under these conditions the half life of GSQ is approximately 2 minutes.

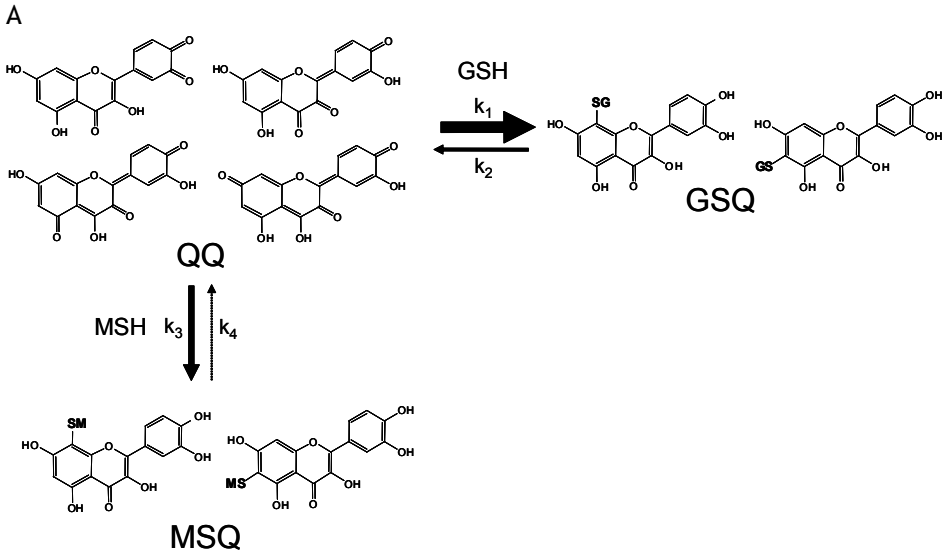
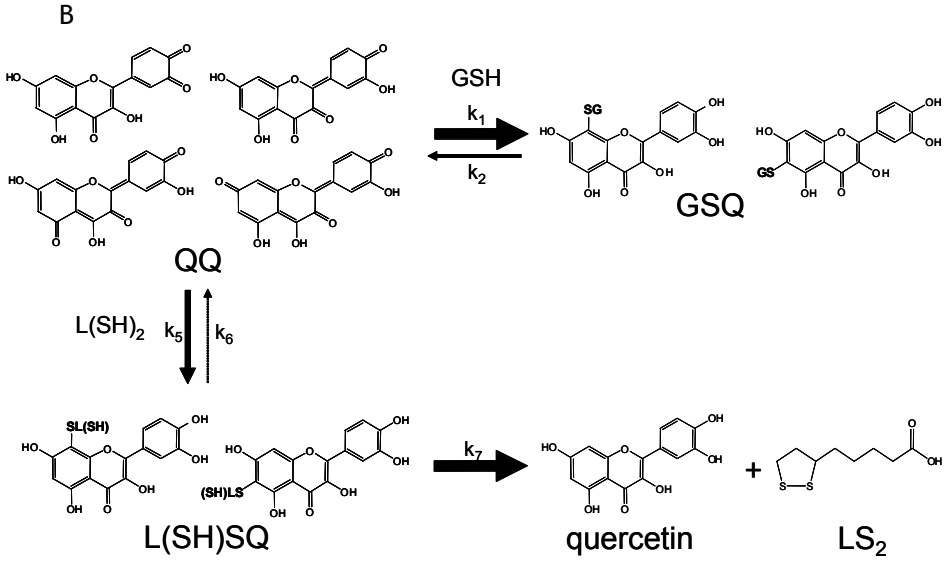


Figure 5.6 Proposed schemes for the reaction of GSQ with MSH (scheme A) or $L(SH)_2$ (panel B). As depicted in both schemes, GSQ will continuously dissociate into GSH and QQ (k_2=k_{GSQ}). When the GSH concentration is high enough, QQ will again react with GSH to form GSQ at a relatively high rate (k_1). However, when the GSH concentration is relatively low, the dissociated QQ will react with other thiols, although k_3 is usually lower than k_1. When QQ reacts with MSH (scheme A) the quercetin-thiol-adduct MSQ is formed. MSQ dissociates at a low rate (k_4) and may therefore accumulate.

The conversion of GSQ into MSQ is in line with the previously reported conversion of isolated pure 6-GSQ into an equimolar mixture of 6-GSQ and 8-GSQ (16). It was suggested that this conversion was due to an intramolecular isomerization. The results of the present study indicate that this is rather due to the dissociation of GSQ into QQ and GSH, followed by the recombination of GSH with QQ either at the 6 or 8 position. The half life of either isomer, deduced from the data of Boersma *et al.*, is approximately 2 minutes, identical to the half life of GSQ found in this study.

With $L(SH)_2$ a comparable scheme as for MSH can be constructed (Figure 6B). Here, again the dissociation of GSQ is the rate limiting step (k_{GSQ}=0.4 min^{-1}) at high concentrations of the thiol. The difference between $L(SH)_2$ and MSH is that with MSH a thiol-adduct of QQ is formed while with $L(SH)_2$ quercetin is formed. It is proposed that with $L(SH)_2$ at first also a thiol-QQ adduct is formed, i.e. the L(SH)SQ adduct. For the L(SH)SQ adduct four isomers are probably formed, namely 6(6S-lipoic acid) quercetin, 6(8S-lipoic acid) quercetin, 8(6S-lipoic acid) quercetin and 8(8S-lipoic acid) quercetin.



When QQ reacts with $L(SH)_2$ (scheme B), quercetin is formed. It is suggested that the formation of quercetin is preceded by the formation of a quercetin-thiol-adduct, i.e. L(SH)SQ-adduct. This adduct has 4 isomers, but for sake of clarity only two isomers are presented in the scheme. Due to the presence of a second intramolecular thiol group, the L(SH)SQ-adducts are rapidly converted into quercetin and lipoic acid (LS_2) (k_7).
The reaction constant rates k_{MSH} (k_3) and $k_{L(SH)2}$ (k_5) approach k_2 at high concentrations of respectively MSH and $L(SH)_2$.

Due to the presence of an intramolecular thiol group, these adducts are readily converted into quercetin and lipoic acid (LS_2). With monothiols, the reaction of a second thiol with the thiol-QQ adduct to form quercetin and a disulfide proceeds very slowly. Incubation of GSQ with high concentrations of either MSH or GSH for 15 minutes did indeed not result in the formation of detectable amounts of quercetin (data not shown). A similar superiority of $L(SH)_2$ compared to monothiols with regard to the reduction of thiol adducts has previously been described (21).

The reactivity of the thiol group of GSH at a pH of 7.4, determined with CDNB, is twice as high as that of MSH and $L(SH)_2$. This is in line with the high initial formation of GSQ versus MSQ when QQ is generated in the presence of equimolar concentrations of both thiols. After a longer time of incubation, GSQ is converted into MSQ, despite the higher reactivity of GSH.

This can be explained by the fact that GS^- is a better leaving group compared to MS^-. The leaving group quality is correlated with the pK_a of the protonated form of the leaving group; the lower the pK_a, the better the leaving group quality. The pK_a of GSH is 8.6 whereas the pK_a of MSH is 10.6

(22), indicating that GS^- is indeed the better leaving group and QQ is trapped as MSQ in the end, despite the higher reactivity of GSH.

The reactivity of the thiol group in nucleophilic reactions also depends on the pK_a. In most reactions, including the Michael addition studied in the present study, the thiolate anion is the most reactive form of a thiol. The thiolate of MSH, i.e. MS^-, has a higher nucleophility and will thus be more reactive than the thiolate of GSH, i.e. GS^-, deduced from the higher pK_a of MSH. Since the pH of 7.4 is well below the pK_a of both compounds, only a small fraction of the thiols will be deproteinated. It can be calculated that for GSH the deproteinated fraction is higher (6%) than for MSH (0.1%). Figure 5.5 shows that at a pH of 7.4 GSH is more reactive than MSH with CDNB. This implicates that the difference in fraction of the thiol that is deproteinated has a higher contribution to the overall reactivity of the thiol than the intrinsic reactivity of the thiolate. In glutathione-S transferases the pK_a of GSH is reduced, partially explaining the catalytic mechanism of these enzymes.

Based on these results it can be concluded that at first QQ will form an adduct with thiols such as GSH, i.e. thiols with a relatively low pKa and high reactivity. In the end this adduct will be converted into an adduct of QQ with thiols that are in first instance less reactive, for example protein thiols. The latter QQ-adduct is far more stable than GSQ. Incubation of MSQ with an excess of GSH or $L(SH)_2$ did not show any detectable conversion of MSQ into GSQ or quercetin (data not shown). This indicates a high stability of MSQ and protein-quercetin adducts with half lives of over one hour.

Previously, we have shown that H_2O can also react with QQ to form QOH, i.e. 6-hydroxy-quercetin and 8-hydroxy-quercetin (20). The pK_a of H_2O is 14, indicating the relatively poor leaving group quality of the OH group in QOH. It is therefore expected that QOH is even more stable than MSQ. Based on the high stability of QOH, it could be argued that QQ would finally end up as QOH. QOH does not react with thiols (20). However, QOH is not formed out of QQ in aqueous solutions when thiols are present. Apparently, the nucleophilicity of H_2O is too low to form QOH in the presence of thiols. This suggests that there is an optimum in nucleophilicity of a compound in forming an adduct with QQ. What kind of QQ-adduct will be present depends on (i) the reactivity of the nucleophiles present (ii) the quality of the leaving group of the adducts (iii) the relative concentrations of the various nucleophiles and (iv) the time span of the incubation period. The reactivity of the nucleophile and the leaving group quality of the nucleophile of a thiol at physiological pH are respectively negatively and positively correlated with the pK_a value, as explained above.

QQ is extremely reactive, e.g. it reacts with thiols instantaneously. It will react at the site it is formed. As stated above, in cells GSQ is expected to

be the initial product of QQ. GSQ is far less reactive than QQ as indicated by their half lives of respectively 2 minutes and a fraction of a second. The half life of 2 minutes indicates that GSQ will diffuse over a relatively great distance.

The relatively long life time of GSQ indicates that this adduct can also be transported by an active metabolically dependent process, the ATP-dependent glutathione S-conjugate export pump (23-25). In this way QQ induced toxicity is even further spread *in vivo* by transporting QQ to sites different from those of its generation. The glutathione-S-transferases, that reduce the pK_a value and thus increase the leaving group quality of GSH, may promote QQ induced toxicity by catalysing the dissociation of GSQ into GS^- and QQ as previously suggested for other GSH adducts (26). Therefore, the glutathione-S-transferases might play a dual role by enhancing the formation as well as the dissociation of GSQ.

The non-enzymatic dissociation of GSQ into GSH and QQ will occur continuously. As long as the GSH concentration is high enough, QQ will react with GSH to form GSQ again. In this way GSH will still protect against QQ. However, when the GSH concentration is low, the dissociated QQ will react with other thiol groups, such as protein thiols (Figure 5.7). Protein thiols usually have a higher pK_a value than GSH. Therefore, the formed quercetin-protein-adducts are expected to be far more stable than GSQ. This will result for example in the non-competitive blocking of essential enzymes with critical thiol groups, a major mechanism for cell toxicity (18). It is therefore concluded that the "protection" of GSH against thiol arylation by QQ is ambiguous. Initially, GSH protects against QQ by scavenging QQ at the site of formation, whereas on the long run GSH will spread QQ toxicity.

Efficient protection against QQ toxicity is offered by dithiols that easily form intramolecular disulfides such as $L(SH)_2$. These dithiols convert QQ into quercetin. The synergistic interplay between $L(SH)_2$ and quercetin is expected to form an efficient antioxidant network, since regeneration of quercetin by $L(SH)_2$ makes the flavonoid available to the antioxidant network again. In this way the positive effect of quercetin during elevated oxidative stress might be boosted.

In conclusion it can be stated that GSH protects against QQ by formation of GSQ. However, GSQ is not stable, it dissociates into GS^- and QQ with a dissociation rate constant of 0.4 min^{-1}. The dissociated QQ may react with for example protein thiols, resulting in a much more stable quercetin-protein adduct. On the long run these more stable adducts might accumulate. Therefore, GSQ formation will not offer complete protection against QQ, it may also serve as transport or storage of QQ. This demonstrates that glutathione conjugation, generally regarded as a detoxification mechanism, may also spread toxicity.

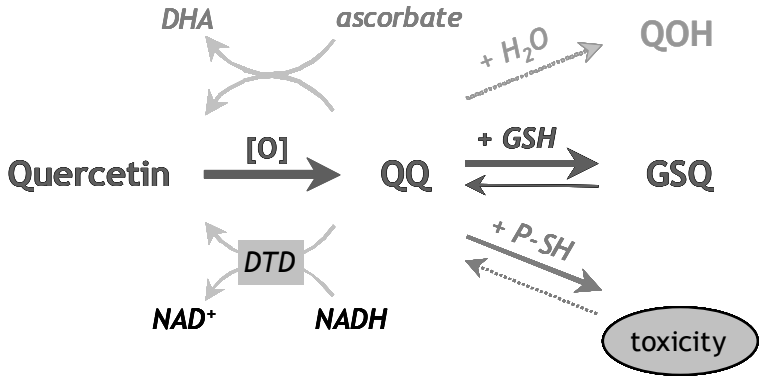


Figure 5.7 Possible reactions of the oxidation product of quercetin (adapted from Boots *et al.*, 2003).
When quercetin is oxidized, an *o*-quinone/quinonmethide (referred to as QQ) is formed. QQ is highly reactive, it will react at the site it is formed. The most likely primary target of QQ is glutathione (GSH). At low GSH concentrations, QQ reacts with protein-thiols (P-SH) to form protein-quercetin adducts. The reaction of QQ with H_2O is too slow to occur *in vivo*. Regeneration of QQ by either ascorbate or DT-diaphorase (DTD) is also negligible compared to the thiol-adduct formation.
DHA= dehydroascorbate; NAD^+= nicotinamide adenine dinucleotide; NADH= reduced NAD^+.

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