Oxidation versus Addition Reactions of Glutathione during the Interactions with Quinoid Thioethers of 4-(Dimethylamino)phenol

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4-(Dimethylamino)phenol (DMAP) is a potent cyanide antidote which forms many equivalents of ferrihemoglobin in vivo and in vitro. During this process formation of phenoxyl radicals was observed which are reduced by ferrohemoglobin, thereby sustaining a catalytic cycle of ferrihemoglobin formation, or which disproportionate to give the quinone imine of DMAP. In the presence of thiols, e.g., glutathione (GSH), formation of 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP), 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-bis-GS-DMAP), and 4-(dimethylamino)-2,3,6-tris(glutathion-*S*-yl)phenol (2,3,6-tris-GS-DMAP) was observed. While the trisubstituted glutathione conjugate is a stable end product, 2-GS-DMAP and 2,6-bis-GS-DMAP were still reactive and produced ferrihemoglobin. It is concluded that formation of polysubstituted DMAP thioethers is a result of sequential oxidation/addition reactions with quinoid intermediates. Formation of glutathione disulfide (GSSG) was minimal during the interaction of oxidized DMAP or 2-GS-DMAP with glutathione but became significant when oxidized 2,6-bis-GS-DMAP reacted with GSH. Thus it is conceivable that the bulky glutathione substituents in 2,6-bis-GS-DMAP render the addition of a third GSH molecule to the quinone imine derivative more difficult, and other reactions may get a chance. The reaction mechanism of GSSG formation has not been fully resolved, but a radical pathway mechanism involving thiyl radicals is proposed. Oxidation and addition reactions were also observed in the absence of oxygen when ferrihemoglobin served as oxidant. In the presence of oxygen, however, GSSG formation was increased, partly due to hydrogen peroxide formation, partly due to an additional trapping reaction of the glutathione disulfide radical anion. It appears that product orientation during the sequential oxidation/addition reactions is largely directed by steric factors of the bulky glutathione residues.

Introduction

Searching for suitable antidotes against cyanide poisoning, Kiese and co-workers detected the outstanding ferrihemoglobin-forming activity of 4-(dimethylamino)-phenol (DMAP)[2] (*1*), which has been marketed for this purpose as a drug in Germany. This compound produces many equivalents of ferrihemoglobin, both in vivo and in vitro. The rapid hemoglobin oxidation, however, is terminated in a few minutes, thus preventing the patient from deleterious methemoglobinemia.

Studies on the mechanisms underlying this hit-and-run action revealed that DMAP catalytically transfers electrons from ferrohemoglobin to oxygen (*2*). Formation of phenoxyl radicals of DMAP was observed (*3*), while free superoxide radicals and hydrogen peroxide were obviously not involved when DMAP reacted with purified human oxyhemoglobin (*4*).

It has been suggested (*5*) that the polarized hemoglobin–oxygen complex is able to abstract an electron from DMAP to form a short-lived ferrihemoglobin–H_2O_2 complex resembling a "compound I complex" of catalase and peroxidases (*6–8*). Simultaneously, DMAP yields a radical. The ferrihemoglobin–H_2O_2 complex rapidly abstracts a hydrogen atom from another DMAP molecule to yield a second radical and a ferryl complex in resonance with a mesomeric ferric oxide complex with a delocalized radical in the porphyrin ring or in adjacent amino acid residues like tyrosine (*8, 9*). Finally, this "compound II"-like complex is reduced by a third DMAP molecule to yield ferrihemoglobin, water and, a third DMAP radical. The three phenoxyl radicals that are generated during oxygen reduction oxidize ferrohemoglobin and are reduced to parent DMAP, thereby closing the catalytic cycle (*3*). Catalase was without any effect on the kinetics of ferrihemoglobin formation (*4*), suggesting that the "compound I"-type complex reacts much faster with DMAP compared to the decay of the complex with liberation of free H_2O_2. Hence, both oxygen atoms in oxyhemoglobin are neatly reduced to the oxidation state of water. Figure 1 illustrates the proposed (*5*) reaction sequence (reactions 1–4).

In red cells the catalytic cycle soon fades because the quinone imine of DMAP that results from the disproportionation of the phenoxyl radical reacts avidly with cellular nucleophiles, particularly with sulfhydryl groups. Of these, GSH (*10*) and the SH groups of proteins, e.g.,

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[2] Abbreviations: DMAP, 4-(dimethylamino)phenol; 2-GS-DMAP, 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol; 2,6-bis-GS-DMAP, 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol; 2,3,6-tris-GS-DMAP, 4-(dimethylamino)-2,3,6-tris(glutathion-*S*-yl)phenol; NAPAP, acetaminophen; NAPQI, acetamidoquinone imine; NEM, *N*-ethylmaleimide; Hb-NES, *S*-(*N*-ethylsuccinimido)hemoglobin; Hb-NES-Fe^{3+}, *S*-(*N*-ethylsuccinimido)ferrihemoglobin.

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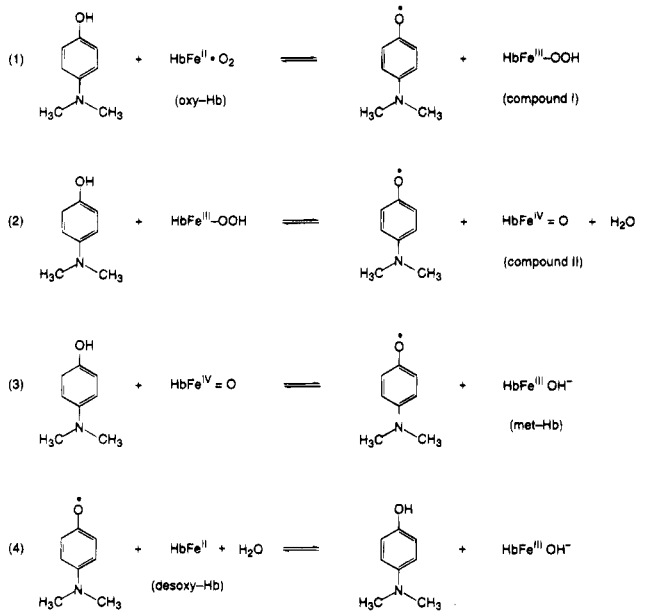

Figure 1. Proposed mechanism of the catalytic ferrihemoglobin formation by DMAP.

hemoglobin (*11*), are the most important ones. While these latter reactions are useful in preventing deleterious methemoglobinemia, they are harmful for other organs like the kidney.

Aminophenols and congeners are nephrotoxic agents which after activation to electrophilic intermediates react with cellular nucleophiles. In addition, redox cycling of the radical intermediates may contribute to cellular toxicity. Comparing the toxicities of 4-aminophenol, 4-(methylamino)phenol, and DMAP in isolated perfused rat kidneys, Elbers (*12*) found minimal toxic doses of >100, 19, and 2 μmol/g, respectively. These differences in cellular toxicity corresponded well with the ferrihemoglobin-forming activities of the above-mentioned homologous compounds. Hence, the concept arose that cytotoxicity of *p*-aminophenols might be directly correlated with steady-state levels of their higher oxidation states. Whether radical intermediates or quinoid compounds were responsible for these effects was not entirely clear.

While a lot of information on quinone chemistry and toxicity is available (for reviews cf. refs 13–16), our knowledge on quinone imine toxicity is less certain, with the exception of the quinone imine (NAPQI) of acetaminophen (NAPAP). Overdosage with this safe analgesic/antipyretic leads to fulminant liver necrosis (for review see ref 17). Such a cellular lesion can be mimicked with small amounts of NAPQI (*18*). Since dimethyl analogues substituted either vicinal to the oxygen or to the amido function of NAPAP and NAPQI showed different cytotoxicities, arylation of cellular nucleophiles by the quinone imine was supposed to be the more serious cellular event compared to redox cycling (*19, 20*). Similar observations were made when red cell membranes were exposed to NAPQI and its two dimethyl analogues (*21*). Nevertheless, the dimethyl analogue that primarily acted as an oxidant exhibited also a toxic potential which became obvious when protective mechanisms had been compromised (*20*).

When DMAP was exposed to one-electron abstraction in a H_2O_2/horseradish peroxidase system, massive NADH oxidation and GSSG formation were observed (*22*). Since GSSG formation was diminished in favor of glutathione conjugates (estimated arbitrarily from the loss in GSH + GSSG) when the peroxidase activity was increased, it suggested to the authors that the aminophenoxyl radical did not react with GSH or NADH, presumably because their one-electron oxidation potential might be too low. Pure thermodynamic considerations of the likelihood of a distinct reaction pathway, however, may lead to misconceptions. Rather, kinetic aspects of the microscopic reactions involved may explain reactions that are thermodynamically unfavored (*23*). Hence, a low redox potential of a radical *per se* does not automatically exclude direct reactions with a thiol.

Recent experiments with the phenylenediamine analogue of DMAP, namely, *N,N*-dimethyl-*p*-phenylenediamine, indicated that GSH hardly reacted with the radical. Rather, its disproportionation product, *N,N*-dimethyl-*p*-quinone iminium cation, was the ultimate electrophile that reacted under thioether formation while GSSG production was negligible (*24*). Likewise, GSSG formation is not observed when 1,4-benzoquinone or 1,4-benzoquinone imine reacts with GSH (*25, 26*), while NAPQI reacts with GSH under formation of a glutathione conjugate, the reduction product NAPAP, and the oxidation product GSSG in the proportions 2:1:1 (*18, 27*). When human red cells were exposed to DMAP, significant GSSG formation was observed (*28*), but it appeared that GSSG emerged only after DMAP had already been consumed.

When we succeeded in isolation of various polysubstituted glutathione adducts of DMAP by improved HPLC techniques, we became aware of a puzzling pattern of widely different reactivities (*29, 30*). It appeared that redox behavior and reaction pathways changed in the course of oxyhemoglobin-catalyzed derivatization of DMAP in the presence of GSH. Thus we decided to systematically investigate oxidation versus arylation reactions of the compounds and to correlate the reaction pathways with physicochemical properties of the isolated thioethers. The following articles (*56, 57*) will deal with the reactivity of the DMAP thioethers and with the role reactive oxygen species are playing when DMAP thioethers react with oxyhemoglobin.

Materials and Methods

Chemicals. 4-(Dimethylamino)phenol hydrochloride (DMAP) was synthesized by Farbwerke Hoechst (Frankfurt, FRG), glutathione was purchased from Boehringer (Mannheim, FRG), and *N*-ethylmaleimide (NEM) was purchased from Sigma-Chemie (Deisenhofen, FRG). Glutathione S-conjugates of DMAP were prepared as already described (*30, 31*).

N,N-Dimethyl-2,6-bis(glutathion-*S*-yl)-1,4-quinone imine was prepared by oxidation of 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-bis-GS-DMAP) (20 mM) with PbO_2 in 0.2 M sulfuric acid. The crude product was purified by HPLC. After evaporation of MeOH from the eluent, the quinone imine (1 mM) was immediately used for reactions with GSH. It should be noted that the quinone imine was unusually stable and did not deteriorate within 15 min at room temperature. In 50 mM formic acid, the compound showed two extinction maxima at 418 nm ($\epsilon = 8.7 \times 10^3$ M^{-1} cm^{-1}) and 545 nm ($\epsilon = 3.4 \times 10^3$ M^{-1} cm^{-1}).

All other reagents (purest grade available) were products from E. Merck (Darmstadt, FRG).

Purified Human Hemoglobin. The preparation, virtually free from catalase, superoxide dismutase, and glutathione peroxidase was prepared by gel filtration and ion exchange chromatography as described (*4*). Hemoglobin with the SH groups blocked (Hb-NES) was prepared by incubating oxyhe-

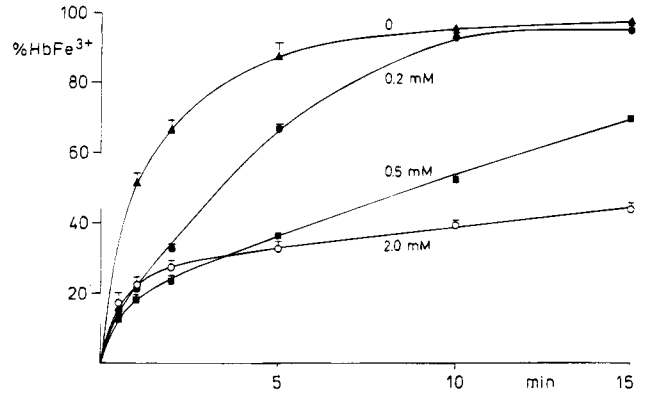

Figure 2. Influence of glutathione on the kinetics of ferrihemoglobin formation by DMAP. Hb-NES (0.6 mM) was incubated with DMAP (0.1 mM) in the presence of various concentrations of glutathione (0–2.0 mM, as indicated), in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air (n = 3 ($\bar{x}$ ± SEM)).

moglobin with 1.1 equiv of NEM (referred to as SH groups) followed by dialysis against 0.2 M sodium phosphate buffer, containing 0.1 mM EDTA (pH 7.4) at 4 °C overnight. SH-alkylated ferrihemoglobin (Hb-NES-Fe^{3+}) was prepared by oxidation of Hb-NES with 1.1 equiv of potassium ferricyanide (referred to Fe^{2+}) followed by dialysis as described before. Anaerobic reduction of ferrihemoglobin by DMAP and its derivatives was carried out under carbon monoxide. Before starting the reaction, the ferrihemoglobin containing solution was gently flushed with carbon monoxide for 1 h and kept under that atmosphere. Anaerobiosis (<0.1 vol % O_2) was checked with a Clark-type O_2 electrode from Bachofer (Reutlingen, FRG).

Methods. Ferrihemoglobin and CO–hemoglobin were estimated according to published methods (*32*).

Glutathione (GSH) and glutathione disulfide (GSSG) were determined with Ellman's reagent (*33*).

The HPLC system consisted of a Model 600/200 pump, a Model 250 B gradient former, a Model SP 4 UV detector from Gynkotek (Germering, FRG), and a μBondapak C 18 reversed phase column (3.9 × 300 mm) from Millipore-Waters (Eschborn, FRG). Elution was performed with methanol (up to 20%)/formic acid (50 mM) gradients, flow rate 1.5 mL/min, and detection at 254 nm. Using a linear methanol/formic acid (50 mM) gradient (0–20% MeOH in 20 min), the following retention times were found: DMAP, 6 min; 2-GS-DMAP, 9.8 min; 2,3,6-tris-GS-DMAP, 11.5 min; 2,6-bis-GS-DMAP, 13 min. In this system 2,6-bis-GS-DMAP could not be separated from its corresponding quinone imine. Improvement was achieved with Lichrosphere 60 RP Select B (4 × 250 mm; E. Merck) with a methanol/ammonium formate (50 mM, pH 3.6) gradient (0–10% MeOH in 10 min, up to 20% MeOH in additional 5 min). *N,N*-Dimethyl-2,6-bis(glutathione-*S*-yl)-1,4-quinone imine and 2,6-bis-GS-DMAP were eluted after 7 and 9.5 min, respectively (1.2 mL/min). After precipitation with perchloric acid (0.5 M final concentration), DMAP and its thioethers were quantified by peak integration, using authentic standards.

UV/vis spectra were recorded by a Model UV 265 spectrophotometer from Shimadzu (Duisburg, FRG).

Results

Influence of Glutathione on the Kinetics of Ferrihemoglobin Formation by DMAP. All experiments were carried out with NEM-treated hemoglobin (Hb-NES) to avoid side reactions of quinoid intermediates with the SH groups in hemoglobin. Ferrihemoglobin formation by DMAP (0.1 mM) was differently influenced by glutathione: Low (0.2 mM) and intermediate (0.5 mM) concentrations of glutathione retarded the rate, while high concentrations (2.0 mM) diminished the extent of ferrihemoglobin formation (Figure 2). This result suggested glutathione-dependent formation of products of different reactivity. Hence it was of interest to investigate the metabolic fate of DMAP and glutathione.

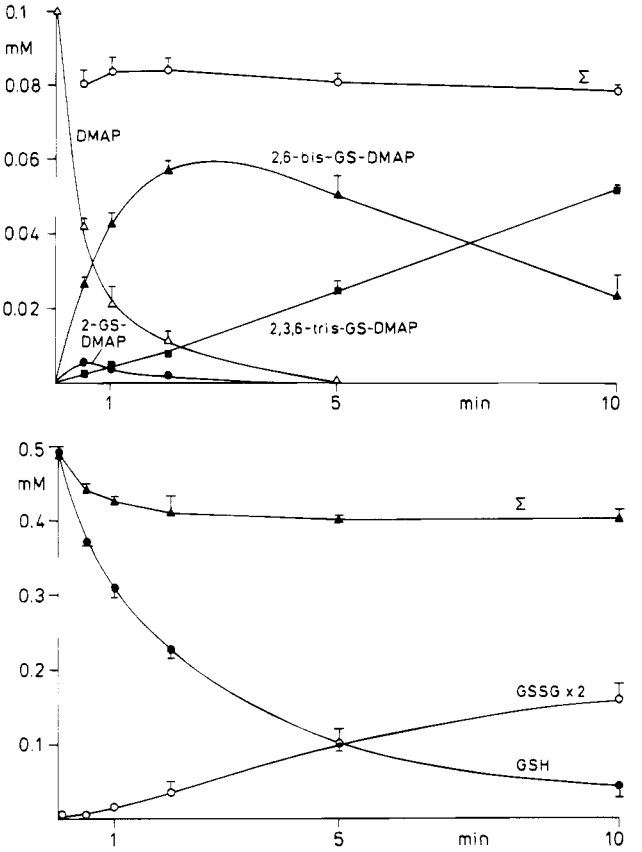

Figure 3. Kinetics of product formation during the reaction of DMAP and glutathione in the presence of oxyhemoglobin. DMAP (0.1 mM) was incubated with Hb-NES (0.6 mM) and glutathione (0.5 mM) in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air (n = 3 ($\bar{x}$ ± SEM)). *Upper panel:* Formation of DMAP thioethers and the sum of DMAP equivalents. *Lower panel:* Decrease of GSH, increase of GSSG, and the sum of GSH equivalents, including the thioethers.

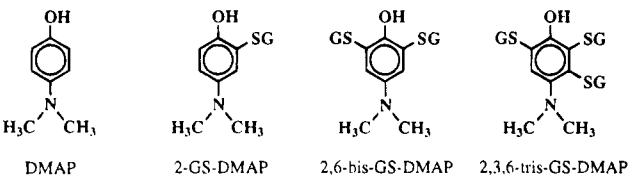

Figure 4. Structures of DMAP and its glutathione S-conjugates.

Reactions of DMAP with Glutathione in the Presence of Hb-NES. DMAP (0.1 mM) was allowed to react with glutathione (0.5 mM) in the presence of Hb-NES (0.6 mM), in 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C under air. As shown in Figure 3, upper panel, DMAP rapidly disappeared in solutions of Hb-NES and glutathione with formation of three glutathione adducts (for formulae see Figure 4) which were identified as 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP), 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-bis-GS-DMAP), and 4-(dimethylamino)-2,3,6-tris(glutathion-*S*-yl)phenol (2,3,6-tris-GS-DMAP). The time course of the reaction showed intermediate formation of 2-GS-DMAP and 2,6-bis-GS-DMAP, while 2,3,6-tris-GS-DMAP rose continuously. As shown in the lower panel of Figure 3, glutathione was only partly oxidized to GSSG; the major part was consumed for thioether formation. Glutathione oxidation was minimal during

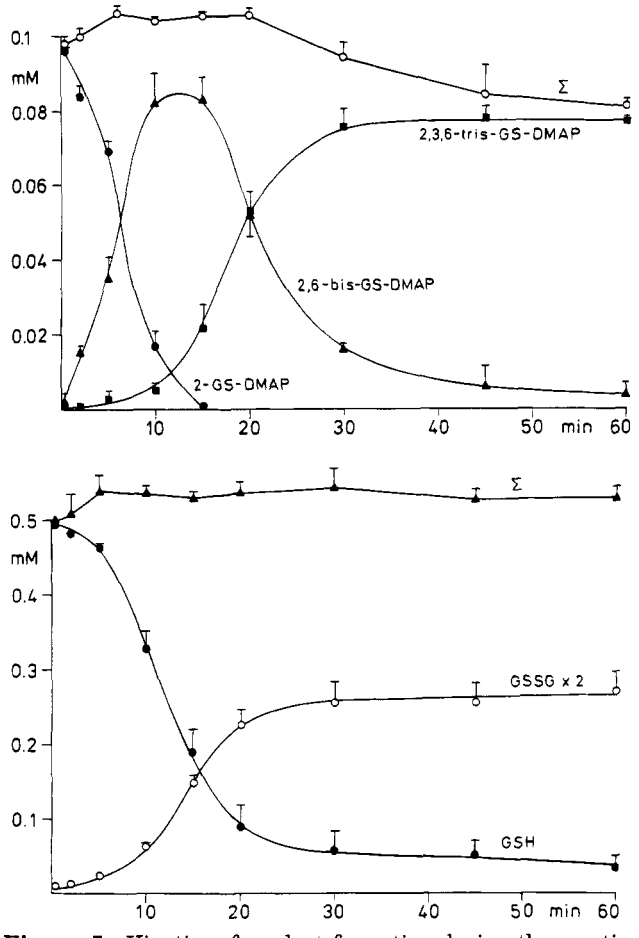

Figure 5. Kinetics of product formation during the reaction of 2-GS-DMAP and glutathione in the presence of oxyhemoglobin. 2-GS-DMAP (0.1 mM) was incubated with Hb-NES (0.6 mM) and glutathione (0.5 mM) in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air ($n = 3$ ($\bar{x} \pm$ SEM)). *Upper panel:* Formation of 2,6-bis-GS-DMAP, 2,3,6-tris-GS-DMAP, and the sum of DMAP equivalents. *Lower panel:* Decrease of GSH, increase of GSSG, and the sum of GSH equivalents, including the thioethers.

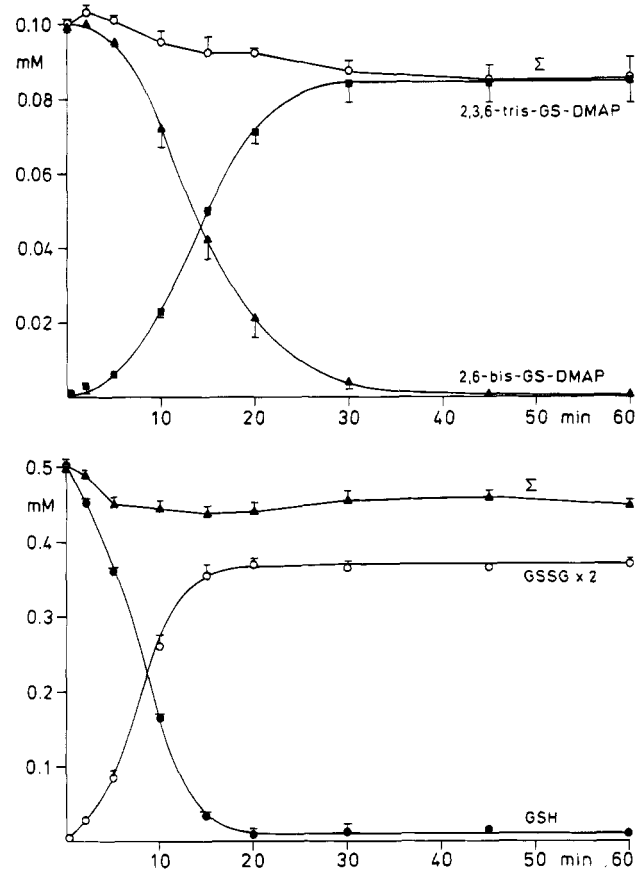

Figure 6. Kinetics of product formation during the reaction of 2,6-bis-GS-DMAP and glutathione in the presence of oxyhemoglobin. 2,6-Bis-GS-DMAP (0.1 mM) was incubated with Hb-NES (0.6 mM) and glutathione (0.5 mM) in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air ($n = 3$ ($\bar{x} \pm$ SEM)). *Upper panel:* Decrease of 2,6-bis-GS-DMAP, formation of 2,3,6-tris-GS-DMAP, and the sum of DMAP equivalents. *Lower panel:* Decrease of GSH, increase of GSSG, and the sum of GSH equivalents, including the thioethers.

the initial phase of the reaction but increased when 2,3,6-tris-GS-DMAP was formed. Therefore, we decided to investigate the reactions of each thioether separately.

Reactions of 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) with Glutathione in the Presence of Hb-NES. After a short lag phase, 2-GS-DMAP rapidly disappeared with transient formation of 2,6-bis-GS-DMAP that finally formed 2,3,6-tris-GS-DMAP (Figure 5, upper panel). Again, GSSG formation was only significant when 2,3,6-tris-GS-DMAP appeared (Figure 5, lower panel).

Reactions of 4-(Dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-Bis-GS-DMAP) with Glutathione in the Presence of Hb-NES. After a lag phase, 2,6-bis-GS-DMAP quantitatively reacted to 2,3,6-tris-GS-DMAP in the presence of glutathione and Hb-NES (Figure 6). GSSG formation paralleled the increase of 2,3,6-tris-GS-DMAP.

Since previous experiments have already indicated some interactions of reactive oxygen intermediates (*10*), we investigated the above reactions under anaerobic conditions with ferrihemoglobin (Hb-NES-Fe^{3+}) as oxidant. The gas phase consisted of carbon monoxide to shift the equilibrium toward ferrohemoglobin.

Reactions of 4-(Dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-Bis-GS-DMAP) with Glutathione in the Presence of Ferrihemoglobin under Carbon Monoxide. 2,6-Bis-GS-DMAP (0.1 mM) was incubated with Hb-NES-Fe^{3+} (0.6 mM) and glutathione (0.5 mM) in 0.2 M phosphate buffer (pH 7.4) at 37 °C under carbon monoxide. The rapid ferrihemoglobin reduction correlated with the decrease of 2,6-bis-GS-DMAP (Figure 7). All 2,6-bis-GS-DMAP was transformed into 2,3,6-tris-GS-DMAP, while 0.1 mM total glutathione was consumed. GSSG was already formed in the initial stage. The continuing slow GSSG formation correlated with slow ferrihemoglobin reduction that was also observed without any thioether (not shown).

When *N*,*N*-dimethyl-2,6-bis(glutathion-*S*-yl)-1,4-quinone imine (0.1 mM) was allowed to react with GSH (0.5 mM) in 0.2 M sodium phosphate (pH 7.4) at 37 °C under argon, the solution was immediately bleached. GSSG increased by 0.033 mM with formation of 0.04 mM 2,6-bis-GS-DMAP and 0.06 mM 2,3,6-tris-GS-DMAP. The reaction was very rapid and went to completion in less than 2 min.

Reactions of 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) with Glutathione in the Presence of Ferrihemoglobin under Carbon Monoxide. 2-GS-DMAP rapidly disappeared with transient occurrence of 2,6-bis-GS-DMAP and final conversion into 2,3,6-tris-GS-DMAP. At that time 0.4 mM ferrihemoglobin had been reduced and 0.2 mM glutathione consumed. GSSG formation was not observed during 2,6-

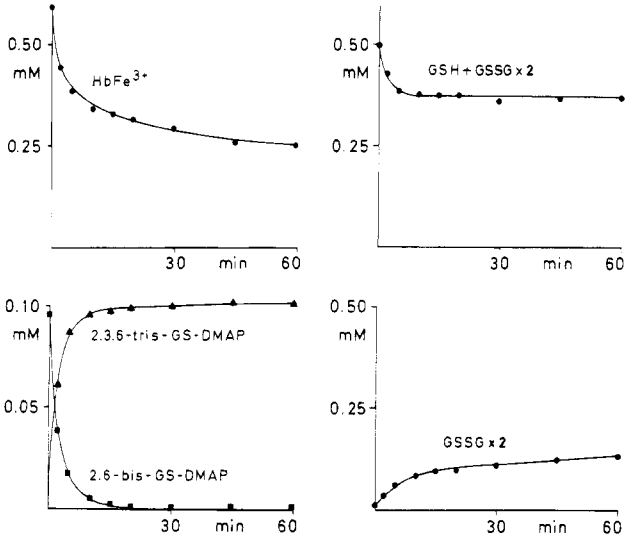

Figure 7. Reactions of 2,6-bis-GS-DMAP with ferrihemoglobin in the presence of glutathione under carbon monoxide. 2,6-Bis-GS-DMAP (0.1 mM) was allowed to react with glutathione (0.5 mM) in the presence of Hb-NES-Fe^{3+} (0.6 mM), in 0.2 M phosphate buffer (pH 7.4), 37 °C, unter carbon monoxide. *Left:* Decrease of ferrihemoglobin and thioether formation. *Right:* Decrease of total glutathione (GSH and GSSG) and formation of GSSG.

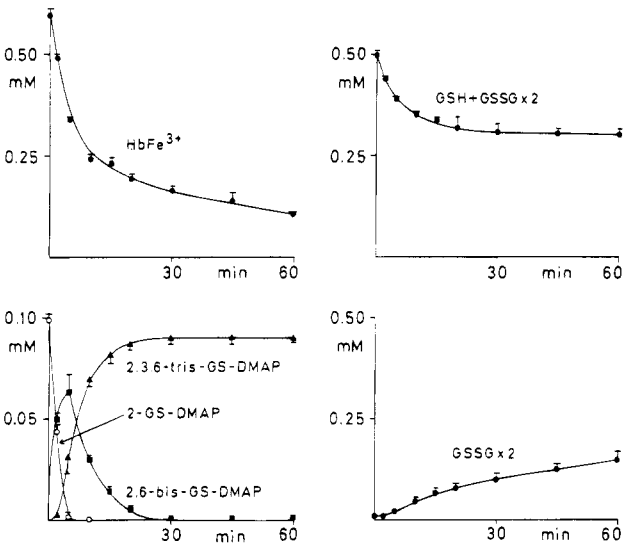

Figure 8. Reactions of 2-GS-DMAP with ferrihemoglobin in the presence of glutathione under carbon monoxide. 2-GS-DMAP (0.1 mM) was allowed to react with glutathione (0.5 mM) in the presence of Hb-NES-Fe^{3+} (0.6 mM), in 0.2 M phosphate buffer (pH 7.4), 37 °C, unter carbon monoxide ($n = 3$ ($\bar{x} \pm$ SEM)). *Left:* Decrease of ferrihemoglobin and thioether formation. *Right:* Decrease of total glutathione (GSH and GSSG) and formation of GSSG.

bis-GS-DMAP formation, but occurred when 2,6-bis-GS-DMAP reacted to 2,3,6-tris-GS-DMAP (Figure 8).

Reactions of DMAP with Glutathione in the Presence of Ferrihemoglobin under Carbon Monoxide. Ferrihemoglobin oxidized DMAP more slowly than 2-GS-DMAP and 2,6-bis-GS-DMAP. Transient 2-GS-DMAP was immediately transformed into 2,6-bis-GS-DMAP that was hardly converted into 2,3,6-tris-GS-DMAP (Figure 9). From the 30th min onward, the sum of the known thioethers got more and more incomplete and HPLC showed unidentified peaks.

Apparently, the small remaining amounts of GSH were no longer able to trap the oxidation product of 2,6-bis-GS-DMAP under formation of 2,3,6-tris-GS-DMAP.

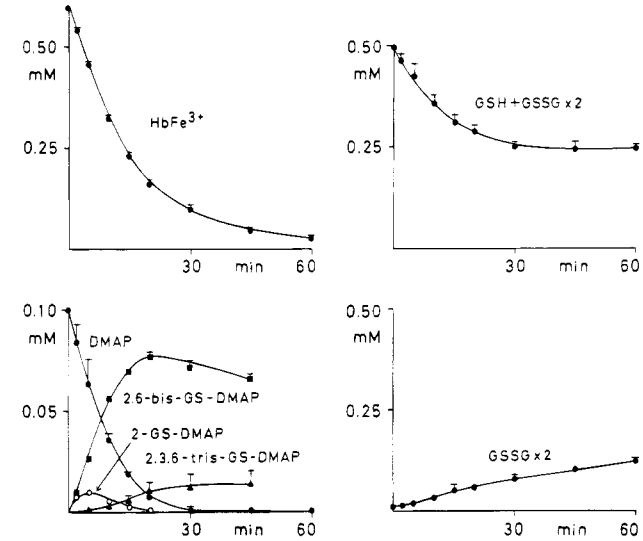

Figure 9. Reactions of DMAP with ferrihemoglobin in the presence of glutathione under carbon monoxide. DMAP (0.1 mM) was allowed to react with glutathione (0.5 mM) in the presence of Hb-NES-Fe^{3+} (0.6 mM), in 0.2 M phosphate buffer (pH 7.4) 37 °C, unter carbon monoxide ($n = 3$ ($\bar{x} \pm$ SEM)). *Left:* Decrease of ferrihemoglobin and thioether formation. *Right:* Decrease of total glutathione (GSH and GSSG) and formation of GSSG.

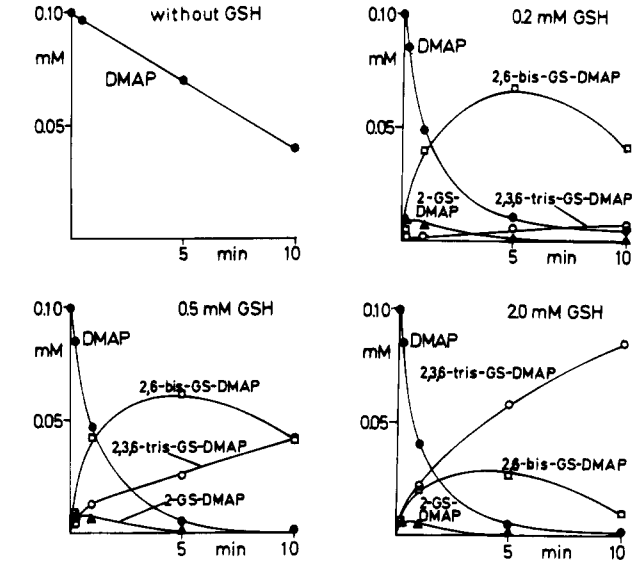

Figure 10. Influence of the glutathione concentration on the thioether formation. DMAP (0.1 mM) was incubated with Hb-NES (0.6 mM) in the presence of various concentrations of glutathione (0–2.0 mM, as indicated), in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air.

Hence, the GSH concentration should crucially influence the pattern of reaction products.

Such a phenomenon may have played a role in the different ferrihemoglobin formation kinetics as observed in the experiments of Figure 2. Thus it was of interest to determine the reaction products at various glutathione concentrations.

Influence of the Glutathione Concentration on Thioether Formation. DMAP (0.1 mM) was allowed to react with different concentrations of glutathione (0–2.0 mM) in the presence of Hb-NES (0.6 mM), in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air. Figure 10 shows that in the presence of glutathione DMAP disappeared more rapidly, but the decrease of DMAP and the intermediate 2-GS-DMAP were hardly influenced by the glutathione concentration. Increasing glutathione con-

centrations, however, markedly favored formation of 2,3,6-tris-GS-DMAP at the expense of 2,6-bis-GS-DMAP.

Discussion

The above experiments have shown that oxyhemoglobin catalyzes reactions of DMAP with glutathione yielding 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP), 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-bis-GS-DMAP), and 4-(dimethylamino)-2,3,6-tris(glutathion-*S*-yl)phenol (2,3,6-tris-GS-DMAP). High glutathione concentrations resulted in a lower extent of 2,6-bis-GS-DMAP formation and favored rapid increase of 2,3,6-tris-GS-DMAP. The biphasic course of ferrihemoglobin formation at intermediate GSH concentrations (Figure 2) is caused by the rapid displacement of DMAP by its less reactive thioethers 2-GS-DMAP and 2,6-bis-GS-DMAP, while the lower extent of ferrihemoglobin by high glutathione concentrations may result from the rapid disappearence of the reactive intermediates in favor of 2,3,6-tris-GS-DMAP that is quite inactive (*30*).

The mechanism of thioether formation during oxidative activation of hydroquinone and aminophenols is most easily explained by a reductive Michael 1,4-addition of glutathione to the quinoid oxidation products (*13, 26, 34*). However, oxyhemoglobin-catalyzed oxidation of DMAP, and most probably of other aminophenols, is a one-electron reaction yielding phenoxy radicals (*3*). Since N- and O-centered free radicals of aromatic compounds tend to disproportionate at high rates (*24, 35–38*) it is difficult to decide whether redox and addition reactions originate directly from the radical or from the higher oxidation state, i.e., from quinones and quinone imines. Previous investigations with the DMAP analogue *N,N*-dimethyl-*p*-phenylenediamine have unequivocally shown that reactions with glutathione occurred predominantly with the quinoid disproportionation product and not with the radical (*24*). The same result was obtained with the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine radical cation (*31*). Moreover, two phenoxyl radicals of DMAP were shown to be bleached by one glutathione molecule, indicating again that the quinoid disproportionation product is the actual reactant (*3*). Hence, it appears reasonable to assume that formation of polysubstituted DMAP thioethers is a result of sequential oxidation/addition reactions with quinoid intermediates (Figure 11).

GSSG formation was minimal during the interaction of oxidized DMAP with glutathione and increased only at the final stage of polysubstitution. Evidently, addition reactions are favored in the case of oxidized DMAP and 2-GS-DMAP. However, when oxidized 2,6-bis-GS-DMAP reacted with GSH, a GSSG-producing redox reaction was also observed. Since H_2O_2 is involved in ferrihemoglobin formation by 2,6-bis-GS-DMAP (*10*), GSSG may be formed by the reaction of GSH with H_2O_2 (*3*). In fact, catalase (13 000 units/mL) retarded GSSG formation but did not prevent it. After 60 min reaction, the same amount of GSSG was formed as in the absence of catalase (*39*). To prevent any interaction of reactive oxygen species, the reactions of oxidized thioethers were also carried out under anaerobic conditions, with ferrihemoglobin as oxidant. Again, GSSG formation was observed and was only significant during the reaction of oxidized 2,6-bis-GS-DMAP with GSH.

The ratio of glutathione addition to reduction amounted to about 3:2. A similar ratio has been found when the quinone imine of *N*-acetyl-4-aminophenol reacted with

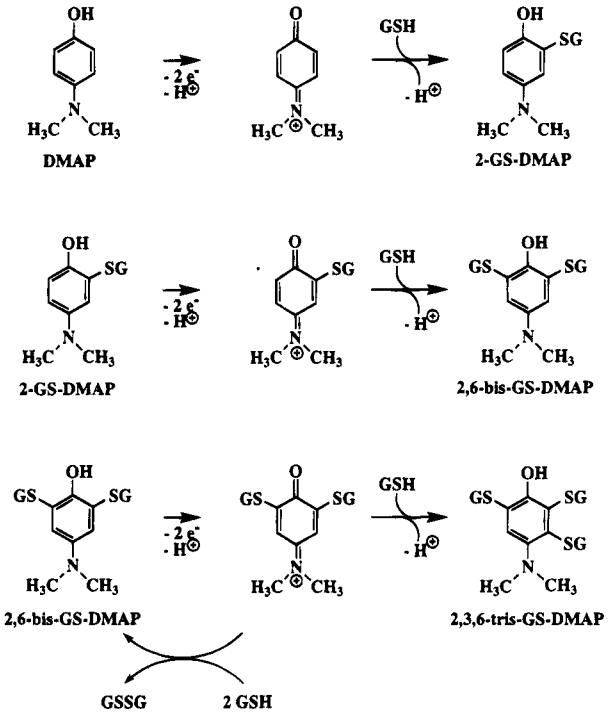

Figure 11. Sequential oxidation/addition reactions during thioether formation of DMAP.

glutathione (*19, 40, 41*), but not in the case of benzoquinone or the quinone imine of 4-aminophenol (*26*). Introduction of methyl substituents at C-3 and C-5 of the quinone imine of *N*-acetyl-4-aminophenol abolished the reduction by GSH but not its addition, whereas methyl substitution at C-2 and C-6 abolished addition but not reduction (*19*). This phenomenon was attributed to steric effects (*19, 42*). In addition, increasing the number of methyl substituents is known to retard the reaction rate of benzoquinone with glutathione (*43*). Thus it is conceivable that the bulky glutathione substituents in 2,6-bis-GS-DMAP render the addition of a third glutathione molecule across the 2,3 carbon–carbon double bond more difficult, and other reactions may get a chance. In line with this view of steric hindrance is the observation that formation of a 2,3 bis-substituted thioether of 4-aminophenol occurred with the small molecule 2-hydroxyethyl mercaptan but not with glutathione (*25*).

The reaction mechanisms involved in GSSG formation deserve separate comment. Oxidation of GSH by the quinone imine of *N*-acetyl-4-aminophenol leads to radical formation including thiyl radicals which may form GSSG (*19, 44*). Similarly, the quinone imine and the radicals of DMAP and its thioethers might oxidize GSH to thiyl radicals. The latter reaction 1 (Figure 12), however, is not favored thermodynamically: The redox potential of the phenoxyl radical of DMAP is 0.174 V at pH 13.5 (*45*) and about 0.33 V at pH 7.4 (pK_a of the phenolate form 10.0 (*30*)). In analogy with 2,6-bis-GS-1,4-hydroquinone (*26*), one can assume that the redox potential of 2,6-bis-GS-DMAP is by 70 mV lower than that of the parent compound. Considering a redox potential of $E(RS^{\bullet}/RS^{-})$ = 0.75 V (*23*) and a pK_a value of GS^{-}/GSH = 8.6 (*33*), an actual redox potential of 0.82 V is calculated for the glutathionyl radical at pH 7.4. Ignoring other possible prototropic equilibria, the equilibrium constant K_1 of reaction 1 is therefore 3×10^{-10}. Nevertheless, reactions which remove $GS^{\bullet}$ from the equilibrium will drive the reaction over to the right.

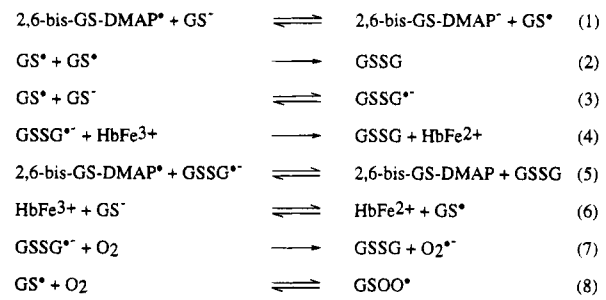

Figure 12. Tentative scheme of the radical pathway of GSSG formation during the reactions with the phenoxyl radical of 4-(dimethylamino)-2,6-bis(glutathion-*S*-yl)phenol (2,6-bis-GS-DMAP•).

Thiyl radicals may react in different ways: Dimerization of two thiyl radicals (reaction 2) yielding GSSG is highly improbable. Under our experimental conditions (0.5 mM GSH) GS• should be below 10^{-14} M. Despite the high rate constant of dimerization ($k_2 = 3.4 \times 10^9$ M^{-1} s^{-1}, pH 3.9 (*46*)), the bimolecular reaction leading to GSSG would proceed at a rate below 10^{-18} M s^{-1}.

More likely is the reaction of GS• with GS^- (reaction 3), which under our experimental conditions would approach a rate of 10^{-5} M s^{-1} ($k = 8 \times 10^8$ M^{-1} s^{-1} (*47*)). The resulting $GSSG^{\bullet-}$ ($E_0 = -1.6$ V (*48*)) is a strong reductant that is easily oxidized by $HbFe^{3+}$ (reaction 4)[3] or by the phenoxyl radical of 2,6-bis-GS-DMAP (reaction 5). These coupled reactions thus may pull equilibrium 1 over to the right side. The same would apply to the thermodynamically unfavored $HbFe^{3+}$ reduction by glutathione (reaction 6). It should be mentioned that all these reactions are even more favored by trapping the resulting $HbFe^{2+}$ with carbon monoxide.

At present, we do not know the one-electron redox potentials of the quinone imines of DMAP. Since DMAP is easily oxidized to the phenoxyl radical by benzoquinone (*3*), we can expect that the redox potential is below 0.078 V as determined for benzoquinone/semiquinone (*51*). Hence, the thermodynamic equilibrium analogous to reaction 1 is even more unfavored. Nevertheless, GSSG formation was observed to a similar extent when the isolated quinone imine of 2,6-bis-GS-DMAP was allowed to react with GSH.

Conceivably, GSSG formation might also result from ipso addition by attack of GS^- across the quinone imine double bond as repeatedly proposed by several authors for NAPQI derivatives (*20, 27, 52, 53*). Formation and thiolytical cleavage of an ipso adduct of 2,6-bis-GS-DMAP, however, should be markedly hindered both at position 1 (hindrance by two glutathione moieties) and at position 4 (dimethylamino group). Thus, the elucidation of the mechanism(s) underlying GSSG formation still awaits further experimental work.

Under oxygen, GSSG formation was considerably higher than under anaerobic conditions. During the oxidation of *N*-acetyl-4-aminophenol in the presence of glutathione, Ross et al. (*54*) also observed an increase of GSSG formation in the presence of oxygen. This phenomenon might be explained by an additional trapping reaction of the $GSSG^{\bullet-}$ radical by O_2 with formation of $O_2^{\bullet-}$ (reaction 7). This reaction is very rapid ($k = 1.6 \times 10^9$ M^{-1} s^{-1} (*47*)) and is pulled over to the right side by the subsequent disproportionation of the superoxide radicals. Finally, thiyl radicals react with O_2 to form peroxosulfenyl radicals (reaction 8), as described by Jayson et al. (*55*) for 2-hydroxyethyl mercaptan. The fate of the reactive peroxosulfenyl radical is still a matter of debate and should not be discussed here (cf. Wardman (*23*)). The sometimes incomplete balance of the identified glutathione species (cf. Figure 3 and 6) could well be due to formation of oxo derivatives of glutathione.

In addition, GSSG formation might also result from the interaction of H_2O_2 that is formed when 2,6-bis-GS-DMAP reacts with oxyhemoglobin (*10*). The uncatalyzed reaction of GSH with H_2O_2 is very sluggish, but as proposed by a reviewer (we thank him for his valuable comments!), H_2O_2 might combine with ferrihemoglobin to produce a "compound II"-like complex which—in analogy to reaction 3 (Figure 1)—would oxidize GSH very readily.

In conclusion, our results stress once more that oxidation and arylation reactions of aminophenols and its thioethers originate mainly from the quinoid and not from the radical intermediates. The product orientation during the sequential oxidation/addition reactions appears to be largely directed by steric factors of the bulky glutathione residues. GSSG formation is obviously of minor importance and gains only weight when addition reactions are markedly slowed down. It remains to be established whether influences of glutathione addition on the redox potential of the thioethers (*26*) also contribute to the preference over distinct reaction pathways.

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[3] The midpotential of the couple $HbFe^{3+}/HbFe^{2+}$ is +130 mV at pH 7.4 and 30 °C (*49*). This value was obtained in the absence of oxygen. Under air, normal human ferrohemoglobin is oxygenated by about 98% (0.1 M phosphate, pH 7.4, 37 °C (*50*)). Under these conditions the apparent midpotential increases by +100 mV.

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