Reactivity of Glutathione Adducts of 4-(Dimethylamino)phenol. Formation of a Highly Reactive Cyclization Product

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During ferrihemoglobin formation, 4-(dimethylamino)phenol (DMAP), a potent cyanide antidote, forms a quinoid compound that is prone to sequential oxidation/addition reactions. In human red cells and hemoglobin solutions fortified with glutathione, a transient adduct has been isolated and identified as 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP). This compound still formed ferrihemoglobin but differed from parent DMAP in that the reaction rate was roughly proportional to the oxygen concentration and exhibited a lag phase, pointing to a reactive autoxidation product. The compound was isolated and tentatively identified as an intramolecular cyclization product of 2-GS-DMAP. Formation of this product includes three reaction steps: (1) formation of a quinoid intermediate, (2) addition of the α-amino nitrogen atom of the glutamate residue to the aromatic ring, and (3) autoxidation of the cyclization product to give a highly reactive *o*-quinone imine. The isolated compound existed in two isomeric states (^{1}H-NMR) which upon reduction could be separated by HPLC. The isolated reduced isomers mutually converted into each other. A model compound which was synthesized to mimic the most important structural features, 4-(dimethylamino)-6-[*S*-(2′-hydroxyethyl)-thio]-*N*-(2″-phenylethyl)-1,2-quinone imine, had a very similar visible spectum and exhibited an even higher ferrihemoglobin activity than the cyclization product. A similar phenomenon of intramolecular cyclization of a thioether of DMAP had been obseved earlier: DMAP covalently bound to the SH groups of the β-chains in hemoglobin formed a cross-link with the C-terminal histidine residue in the presence of oxygen but not in its absence. It can be anticipated that similar reactions may also occur with other quinoid compounds, resulting in altered protein function and possible antigenicity. The results indicate once more that thioether formation should not be regarded as obligatory detoxication reaction.

Introduction

4-(Dimethylamino)phenol (DMAP)[2] is a suitable cyanide antidote that rapidly forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen (*1, 2*). Deleterious methemoglobinemia is prevented by side reactions of oxidized DMAP, i.e., the quinone imine, particularly with glutathione (GSH). In human red cells, both in vitro and in vivo, formation of a transient bis-glutathione and a stable tris-glutathione adduct was observed (*3–5*); the latter is actively transported across the red cell membrane (*6*) and excreted as premercapturic acid with urine in dogs and humans (*4, 5*).

Interestingly, the bis-glutathione adduct produces still ferrihemoglobin and is quite autoxidizable while the tris-glutathione adduct is inactive (*3*). This behavior contrasts with analogous thioethers from hydroquinone and 4-aminophenol (*7, 8*). Recently, we isolated and identified five different glutathione adducts and investigated the structural requirements for their ferrihemoglobin-forming activity. It was found that glutathione substitution vicinal to the dimethylamino group abolishes ferrihemoglobin-forming activity and autoxidation of the thioethers, while the adducts (mono and bis) substituted vicinal to the phenolic OH group were remarkably active (*9*). Interestingly, ferrihemoglobin formation by these two thioethers showed a lag phase and was markedly enhanced at 100% oxygen. This behavior was not observed with the parent 4-(dimethylamino)phenol.

The preceding paper dealt with differences in reaction pathways when the oxidized thioethers reacted with glutathione (*10*). In this article we report on formation of a highly reactive intermediate that emerges upon autoxidation of 4-dimethylamino-2-(glutathion-*S*-yl)phenol. This derivative is probably an intramolecular cyclization product after addition of the N-terminal α-amino group of the glutathione moiety to the aromatic ring. Since a similar reaction type has been previously found to occur also after covalent binding of DMAP to the SH groups of hemoglobin (*11*), we find it worthwhile to draw attention to such a new toxic mechanism that results after thioether formation of autoxidizable hydroquinone derivatives.

Materials and Methods

Chemicals. 4-(Dimethylamino)phenol hydrochloride (DMAP) and the radioactive compound (*phenyl*-U-^{14}C)DMAP, specific activity 9 mCi/mmol, were synthesized by Farbwerke Hoechst

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[1] Part of Thesis.

[2] Abbreviations: DMAP, 4-(dimethylamino)phenol; 2-GS-DMAP, 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol; 2-Nac-GS-DMAP, 4-(dimethylamino)-2-(*N*-acetylglutathion-*S*-yl)phenol; HEM, 2-hydroxyethyl mercaptan; 2-HEM-DMAP, 4-(dimethylamino)-2-[*S*-(2′-hydroxyethyl)-thio]phenol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; NEM, *N*-ethylmaleimide; Hb-NES, *S*-(*N*-ethylsuccinimido)hemoglobin.

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(Frankfurt, FRG). Catalase (from bovine liver, 65 000 units/mL) and glutathione were purchased from Boehringer (Mannheim, FRG), 2-phenylethylamine and ninhydrin spray reagent for chromatography were from Merck (Darmstadt, FRG), sodium borohydride and *N,N,N′,N′*-tetramethyl-4-phenylenediamine were from Aldrich (Steinheim, FRG), 2-hydroxyethyl mercaptan was from Fluka (Neu-Ulm, FRG), and superoxide dismutase (from bovine erythrocytes, 5000 units/mg) and *N*-ethylmaleimide (NEM) were from Sigma-Chemie (Deisenhofen, FRG).

All other reagents (purest grade available) were products from 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 (*12*). Hemoglobin with the SH groups blocked (Hb-NES) was prepared by incubating oxyhemoglobin with 1.1 equiv of NEM (referred to SH groups) followed by dialysis against 0.2 M sodium phosphate buffer, containing 0.1 mM EDTA (pH 7.4), at 4 °C overnight.

Syntheses. 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) was synthesized from *N,N,N′,N′*-tetramethyl-4-phenylenediamine as already described (*13*). FAB-MS indicated the expected mass (442 amu), and ^{1}H-NMR spectroscopy confirmed the structure (*9*). The UV spectrum in 50 mM formic acid exhibited two maxima at 289 and 253 nm ($\epsilon_{289nm} = 3.5 \times 10^3$ M^{-1} cm^{-1}). The pK_a values were 5.6 (-N(CH$_3$)$_2$) and 9.4 (-OH), respectively. The compound gave a positive ninhydrin reaction.

4-(Dimethylamino)-2-(N-acetylglutathion-S-yl)phenol (2-Nac-GS-DMAP) was prepared by incubation of 2.3 μmol of 2-GS-DMAP in glacial acetic acid (0.5 mL) with acetic anhydride (0.5 mL) at 37 °C for 30 min. After extensive evaporation of the solvents the product was purified on μBondapak C 18 (7.8 × 300 mm) with a MeOH/formic acid (50 mM) gradient. The MeOH content was linearily increased from 0% to 10% within 10 min followed by an increase to 20% within additional 5 min. 2-Nac-GS-DMAP was eluted after 13 min at a flow rate of 3.5 mL/min. The compound had the same UV spectrum as 2-GS-DMAP and similar pK_a values of the (dimethylamino)phenol moiety, but gave no positive ninhydrin reaction.

4-(Dimethylamino)-2-[*S*-(2′-hydroxyethyl)thio]phenol (2-HEM-DMAP) was synthesized from *N,N,N′,N′*-tetramethyl-4-phenylenediamine (TMPD) as described for 2-GS-DMAP. Instead of glutathione, 250 μmol of 2-hydroxyethyl mercaptan was used. The extraction of TMPD with ether was omitted, because 2-HEM-TMPD would be extracted, too. 2-HEM-DMAP was purified isocratically on μBondapak C 18 (7.8 × 300 mm) with 10% MeOH/90% formic acid (50 mM). 2-HEM-DMAP was eluted after 4 min at a flow rate of 3.5 mL/min. The ^{1}H-NMR spectrum showed the aromatic protons at 7.25 (H$_3$), 7.07 (H$_5$), and 7.00 ppm (H$_6$) at neutral pH with unresolved couplings. In DCl, the protons were shifted to 7.68 (H$_3$; $^4J = 2.8$), 7.46 (H$_5$; $^3J = 8.8$; $^4J = 2.8$), and 7.14 ppm (H$_6$; $^3J = 8.8$), indicating substitution at position 2. The -N(CH$_3$) group gave resonances at 2.90 ppm which were shifted to 3.30 ppm on acidification. The methylene protons showed signals at 3.15 (-SCH$_2$-) and 3.80 ppm (-CH$_2$OH). The UV maxima (50 mM formic acid) were found at 289 and 251 nm.

4-(Dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine was prepared by reaction of 2 mmol of 2-phenylethylamine hydrochloride with 20 μmol of 2-HEM-DMAP in 20 mL of 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C in the presence of 1300 units/mL catalase. Inclusion of catalase increased the yield of the desired product, presumably because addition of H_2O_2 to the quinoid moiety was abolished. After bubbling the solution with oxygen for 15 min, the pH was adjusted to 4 and the product concentrated on three Sep-Pak cartridges connected in series. Elution was performed with formic acid (50 mM)/MeOH (up to 20%). The eluate was concentrated under reduced pressure and isocratically purified on μBondapak C 18 (7.8 × 300 mm) with 30% MeOH/70% formic acid (50 mM). The compound was eluted after 8 min at a flow rate of 3.5 mL/min.

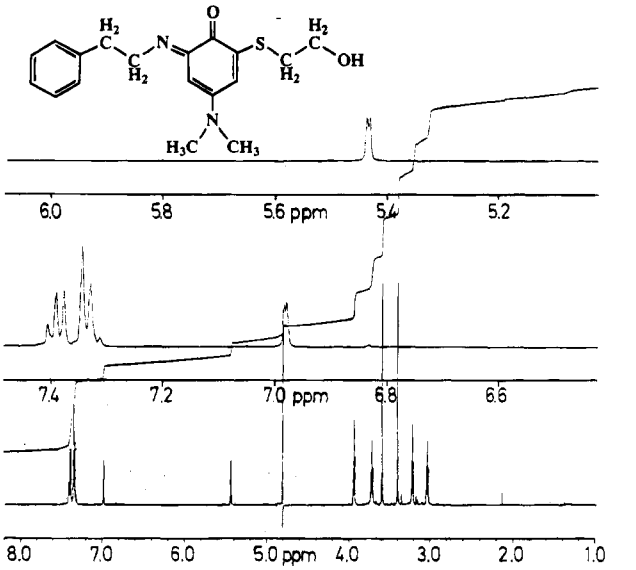

Figure 1. ^{1}H-NMR spectrum of 4-(dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine, recorded in D$_2$O/DCl (pH 2) using H$_2$O (set to 4.8 ppm) as internal standard.

Figure 1 shows the ^{1}H-NMR spectrum in D$_2$O/DCl (pH 2). Besides the cluster of the phenyl protons two signals appeared at 7.00 (1) and 5.42 ppm (1) which showed ^4J coupling of 3.2 Hz. The dimethylamino group gave two different signals at 3.4 and 3.6 ppm, respectively. The four methylene protons showed resonances at 3.05, 3.72, 3.25, and 3.92 ppm, respectively. The UV/vis spectrum (50 mM formic acid) exhibited two maxima at 590 and 416 nm. Upon reduction with borohydride the green color disappeared, which reappeared upon autoxidation. This behavior confirmed the quinone imine structure. Because of the remarkable stability of the quinone imine toward hydrolysis we favored the compound to be an *o*-quinone imine.

Metabolite B was obtained when 50 μmol of 2-GS-DMAP in 50 mL of 5 mM sodium phosphate buffer, pH 7.4 (corrected with NH$_3$), was allowed to oxidize under pure oxygen at 37 °C in the presence of 1300 units/mL catalase. After 20 min, the solution was adjusted to pH 3 (5 N HCl) and cooled. The product was purified from byproducts by ion exchange chromatography on Sephadex SP C 25 (2.5 × 10 cm) with a linear gradient of formate buffers (20−100 mM), pH 3.5. The combined fractions were adjusted to pH 2.7 (1 M HCl) and lyophilized.

HPLC. The 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 (analytical: 3.9 × 300 mm, semipreparative: 7.8 × 300 mm) from Millipore-Waters (Eschborn, FRG). Elution was performed with MeOH/formic acid gradients at a flow rate of 1.5 mL/min for the analytical and 3.5 mL/min for the semipreparative column, respectively, with detection at 254 nm. Applying a linear gradient (0−10% MeOH in 10 min, up to 20% MeOH in an additional 5 min), the following retention times were found (analytical column): 2-GS-DMAP, 6.5 min; 2-GS-1,4-hydroquinone, 7 min; 2-GS-1,4-benzoquinone, 11 min; metabolite B, 13 min. With a steeper gradient (0−20% MeOH in 10 min) the reduced isomers of metabolite B were eluted after 7 and 8 min, respectively.

Spectroscopy. UV/vis spectra were recorded by a Model UV 265 spectrophotometer from Shimadzu (Duisburg, FRG). pK_a values were determined spectroscopically in the presence of 2 mM sodium disulfite under argon to avoid autoxidation. The UV spectra showed isosbestic points which allowed estimation of the pK_a values according to the Henderson−Hasselbalch equation (*14*).

Fast atom bombardment mass spectrometry (FAB-MS) was performed in a glycerol matrix with a Finnigan MAT 312 mass spectrometer (Bremen, FRG). ^{1}H-NMR spectra of samples in

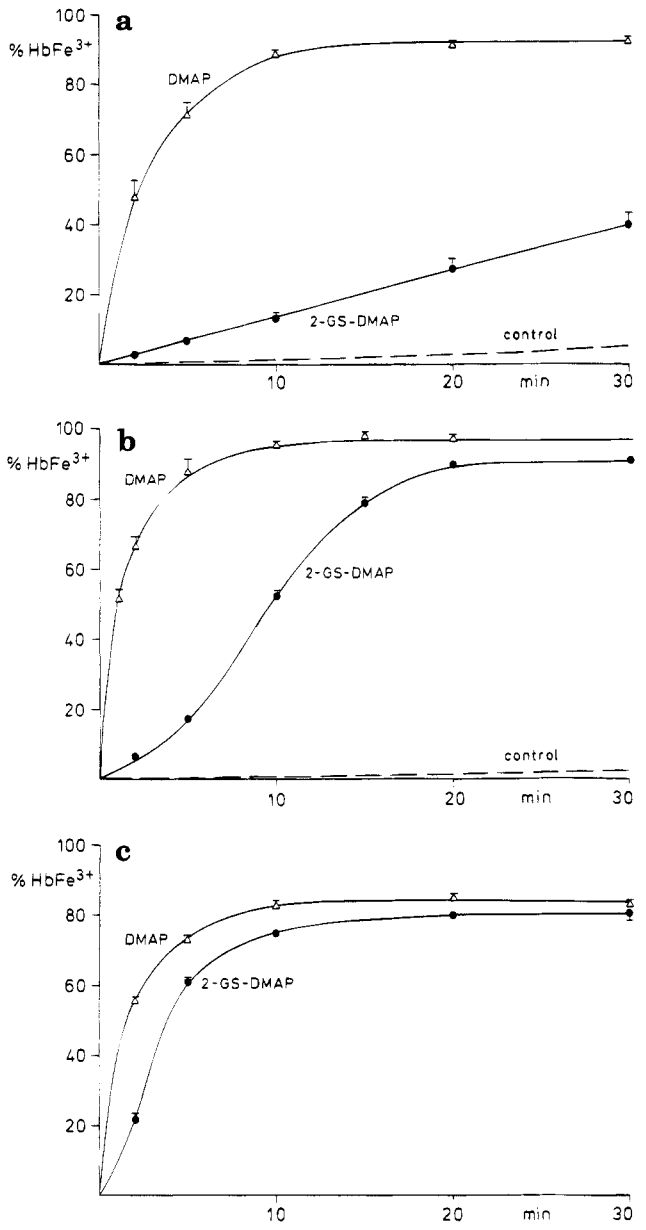

Figure 2. Ferrihemoglobin formation by DMAP and 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP). The compounds (0.1 mM) were incubated with Hb-NES (0.6 mM) in 0.2 M phosphate buffer (pH 7.4) at 37 °C under 2% oxygen (a, upper panel), under air (b, middle panel), or under 100% oxygen (c, lower panel).

D_2O/DCl using H_2O (set to 4.8 ppm) as internal standard were performed with a Bruker AM 400-MHz NMR spectrometer (Rheinstetten-Forchheim, FRG) at room temperature.

Radioactivity was measured in Bray's solution (*15*) with a Rackbeta 1217 scintillation counter (LKB, Wallac, Turku, Finland).

The determination of the N-terminal amino acid of glutathione was performed as described (*16, 17*).

Results

Reaction of 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) with Hemoglobin. All experiments were carried out with NEM-treated hemoglobin (Hb-NES) to avoid side reactions of quinoid intermediates with the SH groups in hemoglobin.

In contrast to DMAP, ferrihemoglobin formation by 2-GS-DMAP occurred after a marked lag phase. Maximal rate of ferrihemoglobin formation was lower with 2-GS-DMAP compared to DMAP. In both cases almost all hemoglobin was oxidized by catalytic amounts of DMAP and its glutathione conjugate (Figure 2b). The observed lag phase was suggested to result from a gradually built-up intermediate that might be caused by autoxidation of 2-GS-DMAP. Therefore, we studied ferrihemoglobin kinetics under various oxygen pressures.

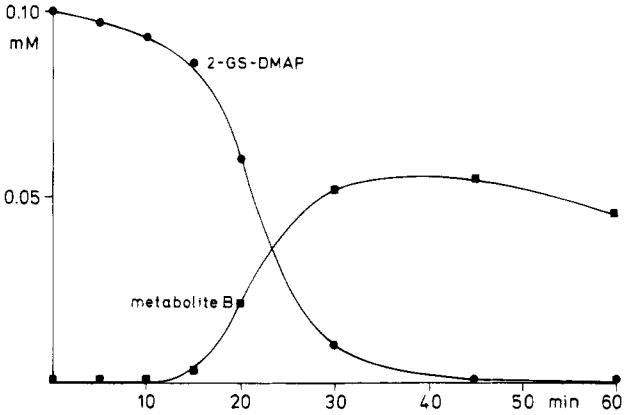

Figure 3. Decrease of 4-(dimethylamino)-2-(glutathion-*S*-yl)-phenol (2-GS-DMAP) and increase of metabolite B during the reaction of 2-GS-DMAP with Hb-NES. 2-GS-DMAP (0.1 mM) was incubated with Hb-NES (0.6 mM) in 0.2 M phosphate buffer (pH 7.4) at 37 °C.

In fact, ferrihemoglobin formation by 2-GS-DMAP was significantly diminished under reduced oxygen pressure (Figure 2a) and markedly increased under pure oxygen (Figure 2c), while ferrihemoglobin formation rates by DMAP were only moderately affected by varying the oxygen pressure (*12*).

These results pointed to an autoxidation product of 2-GS-DMAP as the ultimate reactive agent. HPLC analysis confirmed the assumption: 2-GS-DMAP disappeared more rapidly under pure oxygen while a new compound appeared correspondingly (Figure 3). The same product could also be detected when 2-GS-DMAP was allowed to autoxidize in the absence of hemoglobin. We, therefore, tried to identify this compound in incubations of autoxidized 2-GS-DMAP.

Autoxidation of 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP). Since preliminary experiments had shown that the unknown product could be obtained in higher yields in the presence of catalase, we included this enzyme. 2-GS-(*phenyl*-U-^{14}C-)DMAP (0.1 mM) was allowed to autoxidize in 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C under air in the presence of catalase (13 000 units/mL). HPLC separated two main products which were quantified by their radioactivity (Figure 4). One of these products (A) was identified as 2-(glutathion-*S*-yl)benzoquinone; the reduction with ascorbic acid resulted in 2-(glutathion-*S*-yl)hydroquinone. Both compounds had identical retention times (HPLC) and UV spectra with authentic specimens (*7*). The other product (B) had a green color with extinction maxima at 567 and 418 nm (50 mM formic acid) (Figure 5). This compound was identical with the new metabolite occurring during ferrihemoglobin formation.

Oxidation of 4-(Dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) with PbO_2. The oxidation of 2-GS-DMAP (0.2 μmol in 250 mM H_2SO_4) with PbO_2 (5 μmol) resulted in formation of a red colored product (λ_{max} = 487, 301 nm; 10 mM HCl), and no formation of the new metabolite could be observed. However, upon neutralization the red color turned into green and metabolite

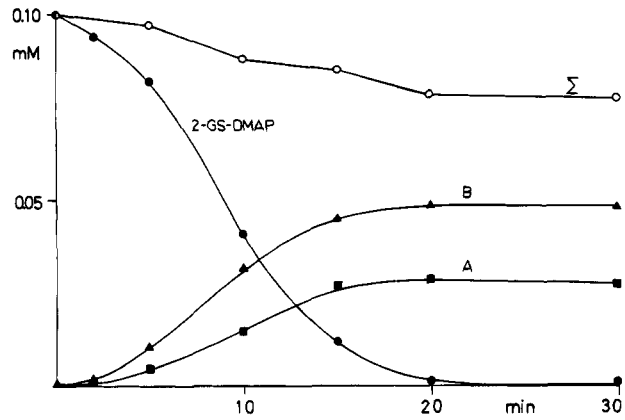

Figure 4. Autoxidation of 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol (2-GS-DMAP) and formation of the two main autoxidation products A [2-(glutathion-*S*-yl)benzoquinone] and metabolite B. 2-GS-(*phenyl*-(U-^{14}C)-DMAP (0.1 mM) was incubated in 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C in the presence of catalase (13 000 units/mL). Σ: Sum of the three compounds, based on radioactivity.

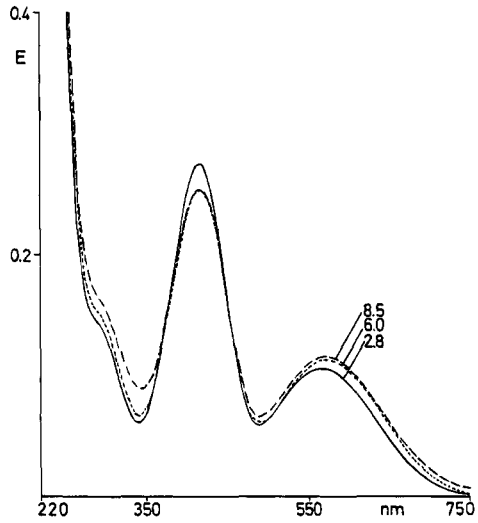

Figure 5. UV/vis spectrum of metabolite B (0.03 mM) at pH 2.8, pH 6.0, and pH 8.5 (λ_{max} = 567 and 418 nm; ϵ_{418} = 8.8 × 10^3 M^{-1} cm^{-1}, 10 mM HCl).

B was formed together with 2-(glutathion-*S*-yl)benzoquinone.

Characterization of the New Metabolite. The UV/vis spectrum of the green colored compound B (ϵ_{418nm} = 8.8 × 10^3 M^{-1} cm^{-1}; 10 mM HCl) was nearly independent of the pH (Figure 5).

FAB-MS indicated a molecular weight of 438 (2-GS-DMAP 442 amu). In contrast to 2-GS-DMAP the new compound gave no positive ninhydrin reaction, and treatment with dansyl chloride followed by hydrolysis did not produce dansyl glutamate.

Unexpectedly, ^{1}H-NMR showed four signals of the ring protons. The total intensity of the four signals corresponded to two protons only. Since such a ^{1}H-NMR spectrum was reproducibly obtained with several different batches, we suggested two isomeric forms in the ratio of 2 to 1 (Figure 6). Similarly, the signals of the glutathione moiety showed multiplicity that did not allow unambiguous identification. In addition, the signals of the two methyl groups appeared separately at 3.6 and 3.7 ppm. Reduction with borohydride decolorized compound B into a product with a mass of 440 amu (FAB-MS).

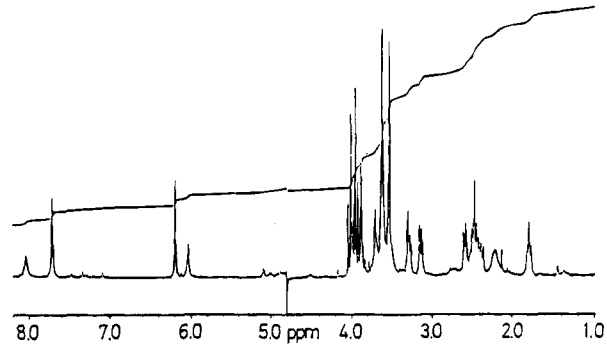

Figure 6. ^{1}H-NMR spectrum of metabolite B, recorded in D_2O/DCl (pH 4) using H_2O (set to 4.8 ppm) as internal standard.

Interestingly, HPLC of the reduced metabolite separated two compounds with virtually identical UV spectra in a ratio of 2 to 1 (ϵ_{310nm} = 2.5 × 10^3 M^{-1} cm^{-1}; 10 mM HCl).

Immediate rechromatography showed that the isolated cuts were pure. On standing at room temperature, however, both compounds showed mutual conversion which finally (45 min) approached the original 2 to 1 ratio. Such a behavior strongly pointed to isomerism. Both isomers autoxidized rapidly and formed green compounds which were indistinguishable from metabolite B by HPLC and UV/vis spectroscopy.

The whole body of these data suggested that the α-amino group of the glutathione moiety may have added to the ring at position 6 followed by autoxidation. In fact, the ring protons showed coupling constants of about 2 Hz as expected for meta-coupling (C_3 and C_5).

To corroborate this hypothesis, we N-acetylated the α-amino group of the glutamate residue of 2-GS-DMAP (2-Nac-GS-DMAP). This compound did not form any green derivative upon autoxidation. Similarly, 4-(dimethylamino)-2-[*S*-(2′-hydroxyethyl)thio]phenol did not form an analogous product. On the other hand, the model compound 4-(dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine (Figure 1) exhibited a similar UV/vis spectrum (λ_{max} = 590, 416 nm; 10 mM HCl) and redox behavior as metabolite B.

Ferrihemoglobin Formation by Metabolite B and 4-(Dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine. Both compounds formed many equivalents of ferrihemoglobin. 4-(Dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine was more reactive than metabolite B and approached almost the activity of DMAP (Figure 7). No lag phase was observed, and metabolite B formed ferrihemoglobin much faster than the parent 2-GS-DMAP (Figure 8). In contrast to 2-GS-DMAP, reduced oxygen tension (2%) did hardly affect ferrihemoglobin formation by metabolite B. Formation of metabolite B in the presence of Hb-NES was maximal at 100% O_2, delayed at 20% O_2 (Figure 3), and not observed at all under 2% O_2.

Discussion

The above results show that the monoglutathionyl derivative of DMAP is still reactive in forming ferrihemoglobin. In addition, 2-GS-DMAP autoxidizes faster than DMAP (*9*). Such a behavior has previously been described for thioethers of hydroquinone and attributed to a lowered redox potential and a decrease of the pK_a values of the phenolic OH group that facilitates autoxi-

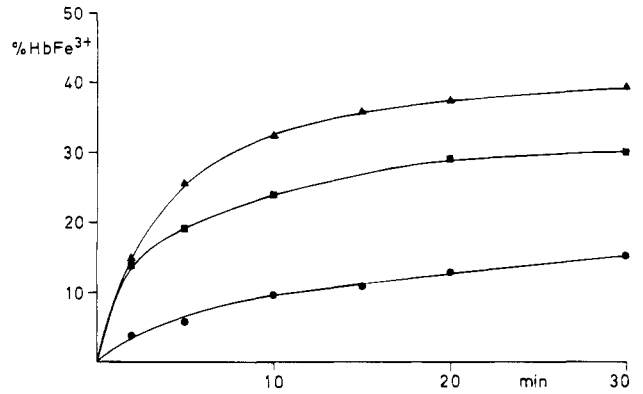

Figure 7. Ferrihemoglobin formation by 4-(dimethylamino)-phenol (▲), metabolite B (■), and 4-(dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine (●). The compounds (0.02 mM) were incubated with 3 mM Hb-NES in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air (n = 1).

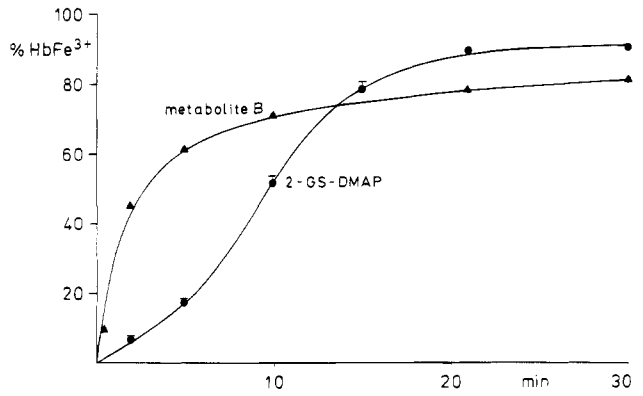

Figure 8. Ferrihemoglobin formation by 4-(dimethylamino)-2-(glutathion-*S*-yl)phenol and metabolite B. The compounds (0.1 mM) were incubated with Hb-NES (0.6 mM) in 0.2 M phosphate buffer (pH 7.4), 37 °C, under air.

dation. Similar effects were observed with the analogous aminophenols (*7*, *8*).

During oxidative activation, e.g., by autoxidation or cooxidation with oxyhemoglobin, radical intermediates are formed which are prone to rapid disproportionation reactions (*18*, *19*). The resulting quinoid products are reactive electrophiles which easily undergo reductive Michael addition reactions (*20*, *21*). Formation of polysubstituted thioethers document such sequential oxidation/addition reactions (*9*, *22*). In most cases, addition occurs preferably with the soft nucleophilic thiols, either with GSH (*23*), CoA (*24*), or macromolecular SH groups (*11*). Addition reactions with amino groups are less favored, particularly when the nitrogen atom is protonated (*23*). While coupling reactions of quinoid compounds with primary and secondary aromatic amines are facilitated (pK_a(-NH_2) < 7), aliphatic amines, e.g., amino acid residues, are much less reactive (pK_a(-NH_2) > 9). However, such reactions may be kinetically favored if an intramolecular (unimolecular) reaction is possible.

Aminochrome formation from dopamine and derivatives thereof is probably the best known examples of such an intramolecular cyclization caused by the addition of an alkylamino group to a quinoid moiety followed by immediate autoxidation (*25*, *26*). Another example of intramolecular cyclization was found when DMAP had covalently bound to the SH groups of hemoglobin. After autoxidation of the thioether, addition of the C-terminal histidine residue to the quinoid thioether occurred. The resulting cross-link impeded conformational changes of

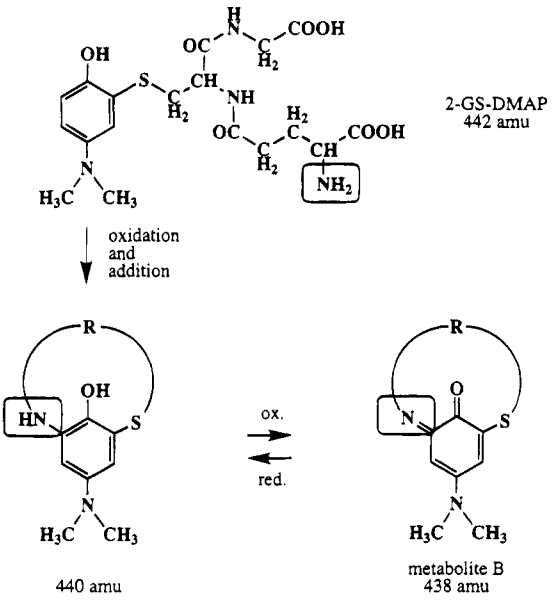

Figure 9. Reaction pathway and assumed structure of metabolite B. (The reacting glutathione nitrogen is highlighted.)

the molecule during deoxygenation and kept the pigment frozen in the quarternary *R*-state (*11*). An analogous reaction has now been detected with the glutathione derivative of DMAP.

While the parent compound 2-GS-DMAP has been unequivocally identified as **2**-GS-DMAP by ^{1}H-NMR (*9*), the structure of the new metabolite is less proved, but many facts point to an intramolecular cyclization: the molecular weight shows loss of four protons compared to the parent compound. The long-waved absorption indicates a highly conjugated system, and the N-terminal amino group of glutathione does not react with ninhydrin and dansyl chloride, contrary to 2-GS-DMAP. Further, one aromatic proton is missing (^{1}H-NMR), pointing to a substitution at the aromatic system, presumably at position 6, since the two aromatic protons (C_3 and C_5) showed poorly dissolved coupling constants of about 2 Hz, as expected for meta-coupling. In additional support of the proposed structure is the fact that similar metabolites were not found during autoxidation of 2-HEM-DMAP and 2-Nac-GS-DMAP, and during oxidation of 2-GS-DMAP by PbO_2 in acidic solution because of the protonation of the nitrogen atom.

The ^{1}H-NMR of the new metabolite exhibited four signals of the two ring protons in a ratio of 2 to 1. Although the isomers of the oxidized compound could not be separated by HPLC, the reduced form allowed separation of two isomers with almost identical UV spectra converting mutually into each other until the original peak ratio of 2 to 1 was reached.

Ultimately, the model compound 4-(dimethylamino)-6-[*S*-(2′-hydroxyethyl)thio]-*N*-(2″-phenylethyl)-1,2-quinone imine was found to have a very similar UV/vis spectrum, redox behavior, and ferrihemoglobin forming activity compared with the new metabolite. Hence, substitution at position 6 by the α-amino group of glutamate appears highly probable, in agreement with the meta-coupling signals.

Figure 9 shows the tentative structure of metabolite B and its route of formation from 2-GS-DMAP. The suggested isomerism of metabolite B and its reduced form may be explained by different conformations of the sloppy glutathione moiety as indicated by simple molecular

models: the glutathione skeleton can exist in at least two positions with different proximities to the aromatic ring, thus explaining different proton resonances of the ring and the glutathione moiety.

Conclusion

Formation of a glutathione S-conjugate of DMAP resulted in a thioether that is not only more autoxidizable than DMAP (*9*) but formed a highly reactive derivative by intramolecular cyclization. This reaction mimics the intramolecular cyclization that has previously been described when DMAP had covalently bound to hemoglobin. It can be anticipated that similar reactions may also occur with other quinoid compounds, resulting in altered protein function and possible antigenicity.

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