Structural Requirements for the Ferrihemoglobin-forming Activity of Glutathione S-Conjugates of 4-Dimethylaminophenol

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4-Dimethylaminophenol (DMAP) is a suitable cyanide antidote that rapidly forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen. Deleterious methemoglobinemia, because of the catalytic cycling, is prevented by side reactions of oxidized DMAP with thiols, particularly with glutathione (GSH). In human red cells, both in vitro and in vivo, the formation of a transient bis-glutathione and a stable tris-glutathione adduct was observed. To investigate the reactivity of GSH adducts of DMAP, we synthesized various thioethers by oxidizing DMAP with PbO₂ in 0.1 M sulfuric acid followed by reaction with GSH. The following compounds were isolated and characterized by ¹H-NMR spectroscopy and determination of the pK values: 4-dimethylamino-2-glutathione-S-yl-phenol (2-GS-DMAP), 4-dimethylamino-3-glutathione-S-yl-phenol (3-GS-DMAP), 4-dimethylamino-2,5-bis(glutathione-S-yl)-phenol (2,5-GS₂-DMAP), 4-dimethylamino-2,6-bis-glutathione-S-yl-phenol (2,6-GS₂-DMAP), and 4-dimethylamino-2,3,6-bis-glutathione-S-yl-phenol (2,3,6-GS₃-DMAP). Ferrihemoglobin-forming activity was investigated with oxyhemoglobin, alylated with 2-Acrylaminobenzaldehyde (Hb-ALD) to prevent binding of oxidized compounds to the protein SH groups. DMAP, 2,6-bis-GS-DMAP, and 2-GS-DMAP (0.1 mM each) completely oxidized Hb-ALD (0.6 mM) in a decreasing order of activity (pH 7.4, 37°C, air); the other derivatives were quite inactive. The same thioether reactivity was observed during autoxidation. Ferrihemoglobin formation by the reactive thioethers was greatly enhanced when the oxygen tension was increased from 2 to 100%. In contrast, variation of the oxygen tension had only marginal effects on the activity of DMAP. Hence, autoxidation of the thioethers must precede ferrihemoglobin formation. The wide variation in reactivity of the thioethers suggests that glutathione substitution vicinal to the dimethylamino group abolishes ferrihemoglobin-forming activity and autoxidation of the DMAP derivatives. Probably, the bulky substituent causes some distortion of the dimethylamino group, thereby inhibiting electronic conjugation of the system. — Environ Health Perspect 102(Suppl 6):133–136 (1994)

Key words: 4-dimethylaminophenol, glutathione S-conjugates, ferrihemoglobin formation, autoxidation, structure

Introduction

4-Dimethylaminophenol (DMAP) is a suitable cyanide antidote that rapidly forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen. Deleterious methemoglobinemia is prevented by side reactions of oxidized DMAP with thiols, particularly with GSH. In human red cells, both in vitro and in vivo, the formation of a transient bis-glutathione and a stable tris-glutathione adduct was observed. The former still produced ferrihemoglobin, whereas the latter was inactive (1). This behavior contrasted with analogous thioethers from 1,4-hydroquinone and 4-aminophenol. Therefore, we decided to prepare a variety of glutathionyl derivatives of DMAP to investigate their reactivity.

Materials and Methods

4-Dimethylaminophenol hydrochloride (DMAP) and the radioactive compound (U-¹⁴C-phenyl)-DMAP, specific activity 9 mCi/µmol, were synthesized by Farberwerke Hoechst (Frankfurt, Germany). Purified human hemoglobin, virtually free from catalase, superoxide dismutase, and glutathione peroxidase, was prepared by gel filtration and ion exchange chromatography. N-Ethylmaleimide (Hb-NES) was prepared by incubating oxyhemoglobin with 1.1 equivalents Hb-NES (referred to SH groups) followed by dialysis against 0.2 M sodium phosphate buffer, containing 0.1 mM EDTA, pH 7.4, overnight at 4°C. Hemoglobin and ferrihemoglobin were measured by the method reported by Kiese (3). HPLC was usually performed on µ-Bondapak C₁₈ (3.9 × 300 mm). The thioethers were separated with a formic acid (50 mM)/methanol (up to 20%) gradient, 1.5 ml/min. Radioactivity was measured in Bray’s solution (4). Gas mixtures were prepared by a mixing pump from Wösthoff (Bochum, Germany). The pK values were determined spectroscopically in the presence of 2 mM sodium sulfite under argon to avoid autoxidation. The UV spectra showed isobestic points that allowed estimation of the pK values according to the Henderson-Hasselbalch equation (5).

Synthesis of Glutathione S-Conjugates

DMAP (130 µmoles in 2 ml 0.1 M H₂SO₄) was vigorously shaken with PbO₂(420 µmoles) for 0.5 min, followed by centrifugation (10,000g, 2 min). The supernatant was mixed with GSH (250 µmoles in 1 ml H₂O). Rapid processing (20 °C) prevents hydrolysis of N,N-dimethylquinoneimine. The products were separated by gel filtration on Sephadex LH 20 (2.5 × 180 cm) with 10 mM acetic acid.

By this procedure, 4-dimethylaminophenol, 2,3,6-tris(glutathione-S-yl) (2,3,6-tris-GS-DMAP) was obtained without impurities (See Table 1 for structure). Two cuts of isomeric bis- and isomeric mono-GS-DMAP derivatives were also separated sufficiently. Further purification by ion exchange chromatography to isolate the isomers was unsuccessful. Therefore, we took advantage of the widely varying

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Table 1. \(^1\)H-NMR data of DMAP and its glutathione S-conjugates.

| None | 2     | 3     | 2,6   | 2,5   | 2,3,6 |
|------|-------|-------|-------|-------|-------|
| Signals | δ ppm (relative intensity) | | | | |
| J [Hz] |       |       |       |       |       |
| Position |       |       |       |       |       |
| 2 | δ 7.02 | – | 7.09 (1) | – | – | |
| J | – | 2.45 | | | | |
| δ 7.08 | – | 7.30 (1) | – | – | – | |
| J | – | 2.60 | | | | |
| 3 | δ 7.33 | 7.29 (1) | – | 7.20 (1) | 7.46 (1) | – | |
| J | NR | – | NR | | | | |
| δ 7.52 | 7.70 (1) | – | 7.72 (1) | 7.84 (1) | – | |
| J | 2.75 (1) | – | NR | | | | |
| 5 | δ 7.33 | 7.15 (1) | 7.38 (1) | 7.20 (2) | – | 7.43 (1) | |
| J | 8.80 | 8.80 | NR | | | | |
| δ 7.08 | 7.50 (1) | 7.70 (1) | – | 7.72 (2) | – | 7.93 (1) | |
| J | 8.80 | 9.00 | NR | | | | |
| J | 2.75 | – | NR | | | | |
| 6 | δ 7.02 | 7.01 (1) | 6.91 (1) | – | 7.07 (1) | | |
| J | 8.80 | 8.80 | 2.45 | | | | |
| δ 7.52 | 7.14 (1) | 7.12 (1) | – | 7.32 (1) | – | | |
| J | 8.80 | 9.00 | | | | | |
| J | 2.60 | | | | | | |

Recorded in D₂O and DCI (bold types), respectively, with H₂O set to 4.80 ppm as internal standard.

Table 2. Structure, yield, and pK values of DMAP and its thioclates (peak number refers to HPLC separation).

| Peak | a | b | c | d | e | f |
|------|---|---|---|---|---|---|
| Yield, % | 44.0 | 4.0 | 19.7 | 16.0 | 5.9 | 3.7 |
| Structure | | | | | | |
| pK(OH) | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 |
| pK(N(CH₃)₂) | 6.1 | 6.1 | 6.1 | 6.1 | 6.1 | 6.1 |

autodioxidability of the isomers: 2-GS-DMAP and 2,6-bis GS-DMAP were degraded by incubating the mixture at pH 7.4, 37°C under oxygen for 2 and 4 hr, respectively. The remaining 3-GS-DMAP and 2,5-bis GS-DMAP were purified from degradation products by ion exchange chromatography on Sephadex SP C₂₅ (2.5 × 10 cm) with linear gradients of formate buffers (50–150 mM), pH 3.5. The pure cuts, as revealed by HPLC, were lyophilized. 2-GS-DMAP was synthesized from \(\text{N},\text{N}',\text{N}''\)-tetramethyl-4-phenylenediamine as previously described (6). 2,6-bis GS-DMAP was prepared in a more selective oxidation procedure using Hb-NES, DMAP (10 μmol), GSH (50 μmol), and Hb-NES (1 g) in 100 ml 0.02 M phosphate buffer, pH 7.4, were incubated at 37°C under air for 3 min, followed by addition of 8.5 ml perchloric acid (70% v/v). After centrifugation, the supernatant was adjusted to pH 2 with K₂PO₄, KClO₄ spun off, and lyophilized. Chromatography on Sephadex LH 20 (as before) separated 2,3,6-tris GS-DMAP (2.1 μmol) and pure 2,6-bis GS-DMAP (3.4 μmol). The structure of the isolated conjugates as shown in Table 1 was confirmed by \(^1\)H-NMR spectroscopy (Table 2).

Results and Discussion

Formation of Glutathione S-Conjugates of DMAP

To quantify the reaction products, U-\(^1\)C-phenyl-DMAP was oxidized and reacted with GSH as described (smaller scale) and analyzed by HPLC. Besides DMAP, five compounds were formed. Figure 1 shows the product pattern after separation by HPLC. The distribution of radioactivity is shown in the lower part. The product pattern is indicative of sequential oxidation/addition reactions. When isolated 2-GS-DMAP was oxidized and reacted with GSH, 2,6-bis GS-DMAP together with 2,3,6-tris GS-DMAP were formed. Such a reaction sequence was also formed when oxymyoglobin served as oxidant (7). The pK values of the isolated derivatives as determined by spectroscopic titration (Table 2) confirm the structure: ortho...
substitution to the phenolic group lowers the pK value by about 0.7 pH units, meta substitution by 0.3 pH units. Polysubstitution showed additive effects. In contrast, effects of substitution on the pK values of the dimethylamino group were puzzling: meta substitution lowered the pK by 0.5 pH units, ortho substitution only by 0.4 pH units. With homologous thioethers of 4-aminophenol ortho substitution resulted in a shift of ~1.3 pH units (8). These data indicate that the electronic conjugation of the nitrogen lone-pair electrons with the aromatic ring may be impaired by the bulky glutathione substituent.

**Autoxidation of DMAP and Its Glutathione S-Conjugates**

In the absence of oxygen, DMAP in 0.2 M phosphate buffer, pH 7.4, at 37°C, was found to be stable for hours (9). Under air, half the DMAP had been autoxidized within 30 min. While 2-GS-DMAP and 2,6-bis GS-DMAP autoxidized more rapidly than DMAP, the other derivatives were quite stable (Figure 2). These data suggest that derivatives with ortho substituents to the dimethylamino group are no longer susceptible to autoxidation. Interestingly, autoxidation kinetics showed a lag phase, indicating that reaction products may accelerate the autoxidation process (10).

**Ferrihemoglobin Formation by DMAP and Its Glutathione S-Conjugates**

The ferrihemoglobin-forming activity was investigated with Hb-NES to prevent binding of the compounds to the protein SH groups. DMAP, 2,6-bis GS-DMAP, and 2-GS-DMAP (0.1 mM each) completely oxidized Hb-NES (0.6 mM) in a decreasing order of activity; the other derivatives were rather inactive (Figure 3). This reactivity correlated with the proneness to autoxidation. In contrast to DMAP, ferrihemoglobin formation by the reactive thioethers exhibited an induction period.

**Influence of Oxygen Pressure on Ferrihemoglobin Formation by DMAP and Its Reactive Glutathione S-Conjugates**

DMAP and the thioethers (0.1 mM each) were incubated with Hb-NES (0.6 mM), pH 7.4, at 37°C under reduced oxygen pressure (2% O2). Ferrihemoglobin formation by DMAP was hardly affected, whereas ferrihemoglobin formation by 2-GS-DMAP and 2,6-bis GS-DMAP was markedly diminished (Figure 4). High oxygen pressure (100% O2) had only minor influence on ferrihemoglobin formation by DMAP but accelerated greatly ferrihemoglobin formation by 2-GS-DMAP and 2,6-bis GS-DMAP (Figure 5).

**Conclusion**

Of the various glutathione S-conjugates of DMAP, either prepared chemically or formed within red cells, two were found to be rather reactive. 2-GS-DMAP and 2,6-bis GS-DMAP autoxidized more rapidly than DMAP and formed ferrihemoglobin at an extent similar to DMAP. In contrast to the parent compound, the rate of ferrihemoglobin formation by the thioethers exhibited a lag phase and a marked influence on the oxygen tension, indicating that autoxidation products of the thioethers may be the ultimate oxidizing agents. The inactivity of glutathione S-conjugates substituted vicinal to the dimethylamino group points to steric effects of the bulky substituent leading to some distortion of the dimethylamino group, thereby inhibiting electronic conjugation of the system.

The results illustrate once more that thioether formation of reactive arylamines should not be regarded as an exclusive detoxication reaction.
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