

A Mechanistic Investigation into the Irreversible Protein Binding and Antigenicity of *p*-Phenylenediamine

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Exposure to the skin sensitizer *p*-phenylenediamine (PPD) is associated with allergic contact dermatitis; however, the ability of PPD to modify protein has not been fully investigated. The aims of this study were to characterize the reactions of PPD and the structurally related chemical 2,5-dimethyl-1,4-benzoquinonediimine with model nucleophiles, a synthetic peptide (DS3) containing each of the naturally occurring amino acids and His-tagged glutathione-*S*-transferase π (GSTP), and to explore the effect of dimethyl substitution on PPD-specific T-cell responses using lymphocytes from allergic patients. The reductive soft nucleophiles *N*-acetyl cysteine and glutathione prevented PPD self-conjugation reactions and Bandrowski's base formation, but no adducts were detected. *N*-Acetyl lysine, a hard nucleophile, did not alter the rate of PPD degradation or form PPD adducts. With PPD and 2,5-dimethyl-1,4-benzoquinonediimine, only cysteine was targeted in the DS3 peptide. PPD and 2,5-dimethyl-1,4-benzoquinonediimine were also found to selectively modify the reactive Cys 47 residue of GSTP, which has a pK_a of 3.5–4.2 and therefore exists in a largely protonated form. Glutathione formed mixed disulfides with the DS3 peptide, reducing levels of PPD binding. Lymphocytes from PPD allergic patients proliferated in the presence of PPD but not with 2,5-dimethyl-1,4-benzoquinonediimine. These results reveal that PPD and 2,5-dimethyl-1,4-benzoquinonediimine bind selectively to specific cysteine residues in peptides and proteins. Lymphocytes from PPD allergic patients were capable of discriminating between the different haptenic structures, suggesting that the hapten, but not the peptide moiety associated with MHC, is an important determinant for T-cell recognition.

Introduction

p-Phenylenediamine (PPD)¹ is one of a large group of chemicals used in products such as printing ink, photo development solutions, plastics, and rubber. More commonly, PPD is used as an intermediate in permanent hair dye formulations and recently in henna dyes (1, 2). PPD is classed as a strong sensitizer, based on murine local lymph node assay data (3, 4), and on human exposure causes T-cell-mediated severe allergic contact dermatitis (ACD) (1, 3, 5–7).

Although a variety of factors including skin penetration, cell stress, cytotoxicity, and dendritic and T-cell activation determine the sensitizing potential of low molecular weight chemicals such as PPD, the formation of a protein adduct is the primary chemical factor required to initiate the response (8, 9). Subsequent uptake and processing of modified protein by antigen presenting cells results in immune priming and programming. The electrophilicity and therefore protein reactivity of a chemical is thought to relate closely to its ability to promote an immune reaction (10, 11), and indeed, peptide reactivity models are currently considered useful screening approaches for skin sensitization testing (12–18). Despite this, little is known about the nature of compound-specific protein modifications and how

this relates to the initiation of an immune response. The scarcity of data in this respect is largely due to the number of potential target proteins for reactive chemicals [over 3000 have been separated from skin cells (19, 20)], the abundance of amino acid binding sites on any given protein candidate, and the accessibility of binding sites for reactive chemicals. It is possible that sensitizing potential relates to a compound's chemical reactivity toward a few critical amino acids. In this respect, directly reactive *p*-benzoquinonediimines—first oxidation derivatives of allergenic *p*-amino aromatic compounds—have been shown to react with nucleophilic amino acids through a set of complex mechanisms (21, 22), indicating a potential route to protein haptentation. Eilstein et al. (23) have shown the selectivity of 2,5-dimethyl-1,4-benzoquinonediimine's covalent binding to lysine in a model peptide containing other potentially reactive amino acids (except cysteine). 2,5-Dimethyl-1,4-benzoquinonediimine reacts with lysine via Schiff base formation and an oxidoreduction process, giving rise to an aldehyde intermediate and peptide cyclization.

PPD does not react directly with protein. However, in solution and on contact with skin, it is susceptible to sequential oxidation and self-conjugation reactions, resulting in the formation of an unstable primary quinonediimine intermediate, products of dimerization and trimerization, and a rearrangement product of the trimer known as Bandrowski's base (BB) (24–26). Recently, we have shown that protein irreversibly modified with oxidation products of PPD provides a chemical signal to stimulate T-cells from allergic patients (6). The objective of this study was to

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¹ PPD, *p*-phenylenediamine; BB, Bandrowski's base; GSH, glutathione; ACD, allergic contact dermatitis; GSTP, glutathione-*S*-transferase π .

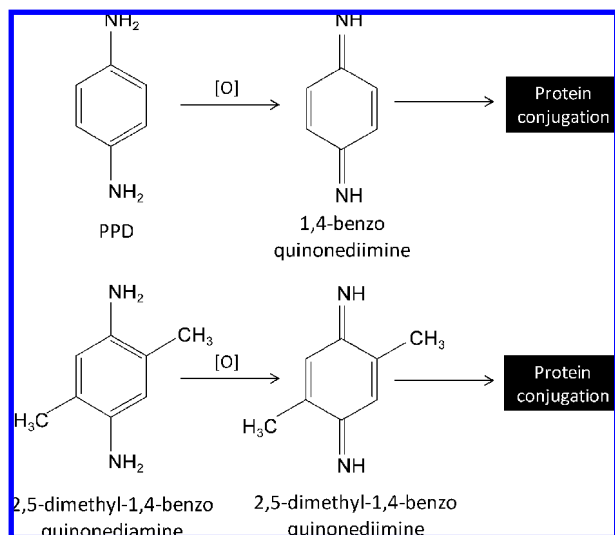


Figure 1. Structures of (a) PPD and (b) 2,5-dimethyl-1,4-benzoquinonediimine.

utilize mass spectrometry to define the amino acid binding site(s) and nature of the covalent interaction when PPD and structurally related 2,5-dimethyl-1,4-benzoquinonediimine (Figure 1) were incubated with nucleophilic amino acids, a model peptide, and the cellular protein glutathione-*S*-transferase π (GSTP). Further experiments were conducted using lymphocytes from PPD allergic patients to explore whether the peptide carrier or the hapten itself is the important determinant for lymphocyte reactivity.

Experimental Procedures

Safety Precaution. Aromatic amines and oxidation derivatives are sensitizing substances. Therefore, they must be handled carefully and skin contact should be avoided.

Chemicals. PPD, 2,5-dimethyl-1,4-benzoquinonediimine, *N*-acetyl cysteine, *N*-acetyl lysine, and 4-aminobenzoic acid were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom). [^{14} C]PPD (specific activity 60 mCi/mmol) was synthesized by GE Healthcare (Amersham, Buckinghamshire, United Kingdom). DS3 peptide (VLSPADKTNWGHEYRMFCQIG) was kindly donated by Unilever.

Generation of His-Tagged GSTP. GSTP constitutes an ideal protein for studies of adduct formation by low molecular weight chemicals for the following reasons: It is relatively small (23.5 kDa); therefore, its analysis is straightforward; it contains only four cysteine residues so that modification at sulphhydryl groups results in few changes to the mass spectral profile; and mass spectrometric methods for the analysis are available and have been described recently (27).

The cDNA for His-tagged human GSTP was cloned into the pET-15b vector (Novagen, Madison, WI) and expressed as previously described (28). Briefly, transformed BL21 cells were grown under carbenicillin selection and induced by the addition of 2 mM IPTG, and the proteins were released by sonication using a Soniprep 150 (MSE, London, United Kingdom). The supernatant was recovered following centrifugation (14000g) at 4 °C for 1 h. His-Select nickel affinity gel (Sigma) was used to isolate His-GSTP from bacterial lysate. The gel was washed with five changes of phosphate buffer, and 100 μ L of beads was incubated with 2 mg of lysate for 10 min at room temperature with agitation. The beads were washed with 5 $\times$ 1 mL phosphate buffer. On-bead BCA assays (Pierce, Rockford, IL) to determine protein concentration were performed as described (29).

Characterization of Reactions of *N*-Acetyl Cysteine, *N*-Acetyl Lysine, and Glutathione (GSH) with PPD. [^{14} C]PPD (50 μ M [0.2 μ Ci]) was incubated in phosphate buffer, pH 7.4, in the presence

or absence of *N*-acetyl cysteine, *N*-acetyl lysine, or GSH (1 mM; volume 1 mL) at 37 °C for 16 h. At 0, 2, and 16 h, 100 μ L aliquots were taken and analyzed by radiometric liquid chromatography–mass spectrometry. The eluent was delivered by Jasco PU980 pumps (Great Dunmow, Essex, United Kingdom). Analytes in the eluate were monitored with a Jasco UV-975 spectrophotometer (λ = 254 nm). Radiolabeled analytes were quantified using a Radiomatic Flo-One A-250 flow detector (Perkin-Elmer, Pangbourne, Berkshire, United Kingdom). The eluate was mixed with Ultima-Flo AP scintillant at a rate of 1 mL/min. The split flow of eluate to the mass spectrometer was $\sim$ 50 μ L/min. Mass spectra were acquired between m/z 50 and 1050 with a Quattro II instrument (Waters Corp., Manchester, United Kingdom) at 1 scan/5 s. The source temperature was 80 °C, the capillary voltage was 3.9×10^3 V, and the standard cone voltage was 30 V. Data were processed via MassLynx 3.5 software (Waters Corp.). Aliquots of the incubations were eluted without treatment from a Zorbax SB-C18 column (250 mm $\times$ 46 mm i.d.; Phenomenex, Macclesfield, Cheshire, United Kingdom) at room temperature with a gradient of methanol (5% for 5 min; 5–60% over 15 min) in 10 mM ammonium acetate, pH 6.9; the flow rate was 0.9 mL/min.

Characterization of Specific Amino Acid Modifications of the Peptide DS3 by PPD and Related Compounds. DS3 peptide solutions were prepared to a final concentration of 80 μ M in ammonium acetate (50 mM). PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid (800 μ M) were prepared in a solution of acetonitrile/ammonium acetate (50 mM; 50:50 v/v) and mixed with the DS3 peptide (peptide:chemical ratio 1:10). The control DS3 peptide was prepared without chemical in a mixture of acetonitrile and ammonium acetate [50 mM; 25:75 (v/v)]. Samples were incubated in the dark at room temperature for 24 h. Prior to analysis, unbound material was removed using SepPak C18 cartridges (Millipore; Watford, Hertfordshire, United Kingdom). Samples were reconstituted in 50 mM ammonium acetate containing trypsin (20:1 ratio trypsin to DS3) and digested overnight before being reconstituted in 5 μ L aqueous acetonitrile (5% v/v)/trifluoroacetic acid (0.1% v/v).

Characterization of the Time-Dependent Irreversible Binding of PPD to the Peptide DS3. DS3 peptide (40 μ M) was incubated with PPD (400 μ M; 1% DMSO) for 72 h in phosphate buffer (pH 7.4; 100 μ L). After 2, 16, 48, and 72 h, a 10 μ L aliquot was taken, and unbound material was removed using C18 ZipTip columns according to the manufacturer's instructions (Millipore) and reconstituted in an aqueous solution containing acetonitrile (5% v/v)/trifluoroacetic acid (0.1% v/v); 10 μ L was the final volume.

Aliquots of sample (0.5 μ L) were spotted onto a target plate for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) together with an equal volume of α -cyano-4-hydroxycinnamic acid (10 mg/mL; LaserBio Laboratories, Sophia-Antipolis, France) in aqueous acetonitrile (50%)/trifluoroacetic acid (0.1%). Peptide mass fingerprints were acquired on a Voyager DE Pro MALDI instrument (Applied Biosystems, Foster City, CA) in positive ion reflector mode.

To test the reversibility of PPD binding, GSH was included in certain experiments. The peptide DS3 (40 μ M) was incubated for 16 h alone, in the presence of PPD (400 μ M) only, GSH (1 mM) only, and PPD (400 μ M) and GSH (1 mM) simultaneously. Peptide samples were analyzed on a Voyager De Pro MALDI Instrument as described previously.

Characterization of Specific Amino Acid Modification of GSTP by PPD and Related Compounds. His-GSTP captured on nickel beads was eluted with an excess of histidine (200 mM). To evaluate whether PPD binds irreversibly to the protein, [^{14} C]PPD (400 μ M [1 μ Ci]) was incubated with His-GSTP (1 mg) in phosphate buffer for 16 h. On completion of the incubation period, unbound PPD was removed by exhaustive solvent extraction, and protein was precipitated by the addition of acetonitrile (100 μ L). An aliquot containing 20 μ g of total protein was taken and added to an equal volume of reducing sample buffer. Samples were then heated at 95 °C for 10 min, and the proteins were separated by electrophoresis on SDS-polyacrylamide gels using the discontinuous buffer system

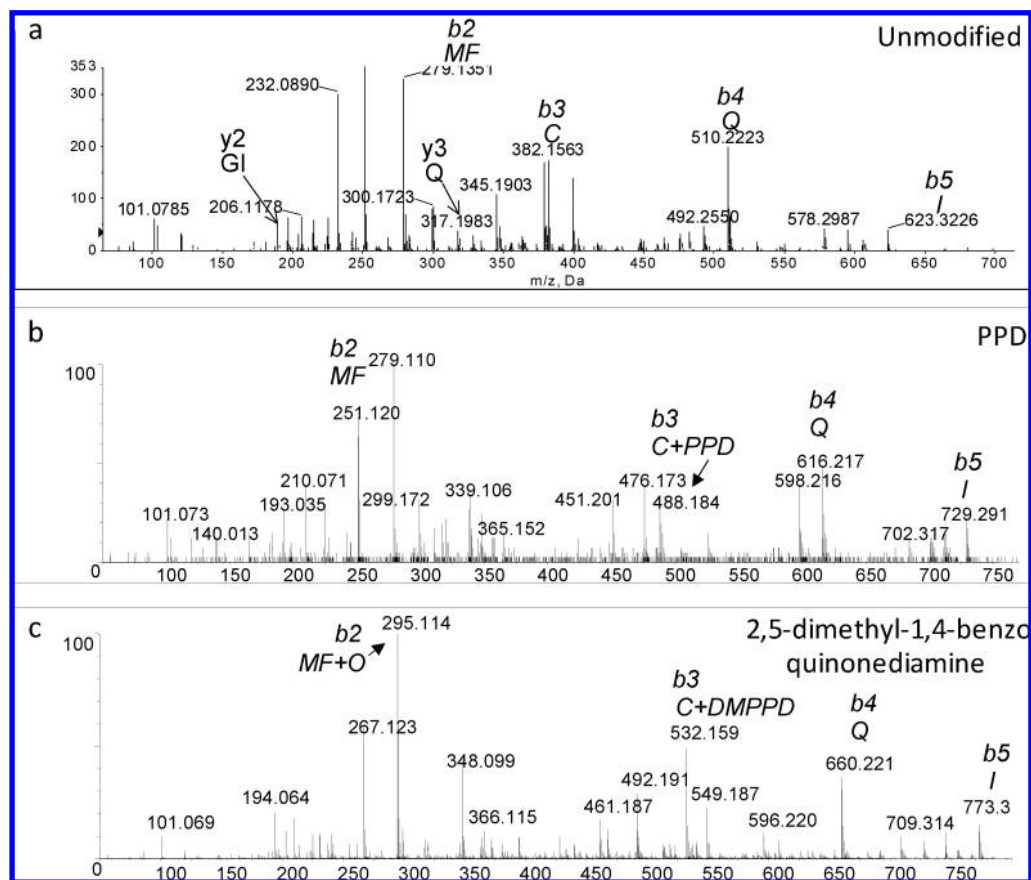


Figure 2. LC-MS/MS sequence analysis of DS3 peptide MFCQIG: (a) unmodified $m/z = 349.5$, MW 697.3; (b) modified $m/z = 402.6$, MW 803.3; $\Delta = 106$ Cys shows PPD modification; (c) modified $m/z = 424.5$, MW 847.3; $\Delta = 134$ Cys shows 2,5-dimethyl-1,4-benzoquinonediimine modification; $+\Delta = 16$ for oxidation of methionine.

described by (30). Bands were then excised and digested. Briefly, the gel was cut into 0.5 cm³ sections and digested with Soluene-350 (PerkinElmer, Beaconsfield, Buckinghamshire, United Kingdom) at 50 °C for 1 h before the addition of 30 μ L of glacial acetic acid. The level of [¹⁴C]PPD incorporated into each band was then determined by scintillation counting.

Specific amino acid modification of His-GSTP by PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid was analyzed following incubation of nickel bead-bound His-GSTP suspended in phosphate buffer with either the compounds (400 μ M; DMSO [1%]) or DMSO alone. Samples were incubated for 16 h at room temperature before the beads were recovered by centrifugation and washed (5 $\times$ 1 mL changes of phosphate buffer and 2 $\times$ 500 μ L of 50 mM ammonium bicarbonate buffer). Modified protein was then digested on-bead by suspension in 30 μ L of ammonium bicarbonate buffer with 20 ng of trypsin and incubation for 16 h at 37 °C. The samples were desalted using C18 ZipTip cartridges (Millipore) and reconstituted in 5 μ L of aqueous acetonitrile (5%)/trifluoroacetic acid (0.1%) for mass spectrometric analysis.

Mass Spectrometry. For LC-MS/MS analysis of DS3 peptide and the tryptic peptides of DS3 and His-GSTP, aliquots of 1 μ L were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems) by automated in-line liquid chromatography (integrated LC-Packings System), 5 mm C18 nanorecolumn and 75 μ m $\times$ 15 cm C18 PepMap column (Dionex, Sunnyvale, CA) via a nanoelectrospray source head and 10 μ m inner diameter PicoTip (New Objective, Woburn, MA). A gradient from 5% acetonitrile/0.05% TFA (v/v) to 48% acetonitrile/0.05% TFA (v/v) in 60 min was applied at a flow rate of 300 nL/min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (IDA) (Analyst, Applied Biosystems). Database searching was performed using ProteinPilot version 2 (Applied Biosystems) against the latest version of the SwissProt database, with the confidence level set at

90% and with relevant chemical modifications included as high probability user-defined modification on cysteine.

Determination of Lymphocyte Proliferation. Proliferation of lymphocytes from PPD allergic patients ($n = 10$) was measured using the lymphocyte transformation test, as described previously (6). Approval for the study was obtained from the Liverpool local research ethics committee; informed written consent was obtained from all donors. Patients were classed as allergic to PPD based on a positive patch test and previous reaction to hair dye. Patients were all female, and their ages ranged from 33 to 77. Lymphocytes were cultured with PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid (final concentrations ranged from 0.1 to 20 μ M) for 6 days (37 °C; 5% CO₂); the absence of the chemicals was taken as a negative control. PPD (0–10 μ M) has previously been shown to have no effect on cell viability through toxicity. A 20 μ M concentration was included, as this is the initial concentration where concentration-related toxicity is detectable. [³H]Thymidine was added for the last 16 h of the incubation period to monitor antigen-specific lymphocyte stimulation. Results are expressed as a stimulation index.

Statistical Analysis. Data were analyzed for normality using the Shapiro–Wilks test. Because the data were found to be non-normally distributed, the Mann–Whitney test was used for comparison of control and test values; $P < 0.05$ was accepted as significant.

Results

Reactivity of PPD toward *N*-Acetyl Cysteine, *N*-Acetyl Lysine, and GSH. The stability of PPD in aqueous solution, pH 7.4, was investigated using parallel radiometric HPLC and LS-MS analyses. Initial experiments revealed that PPD ($[M + 1]^+$ at m/z 109; R_t , 7.5 min) was converted to BB (m/z , 319; R_t ,

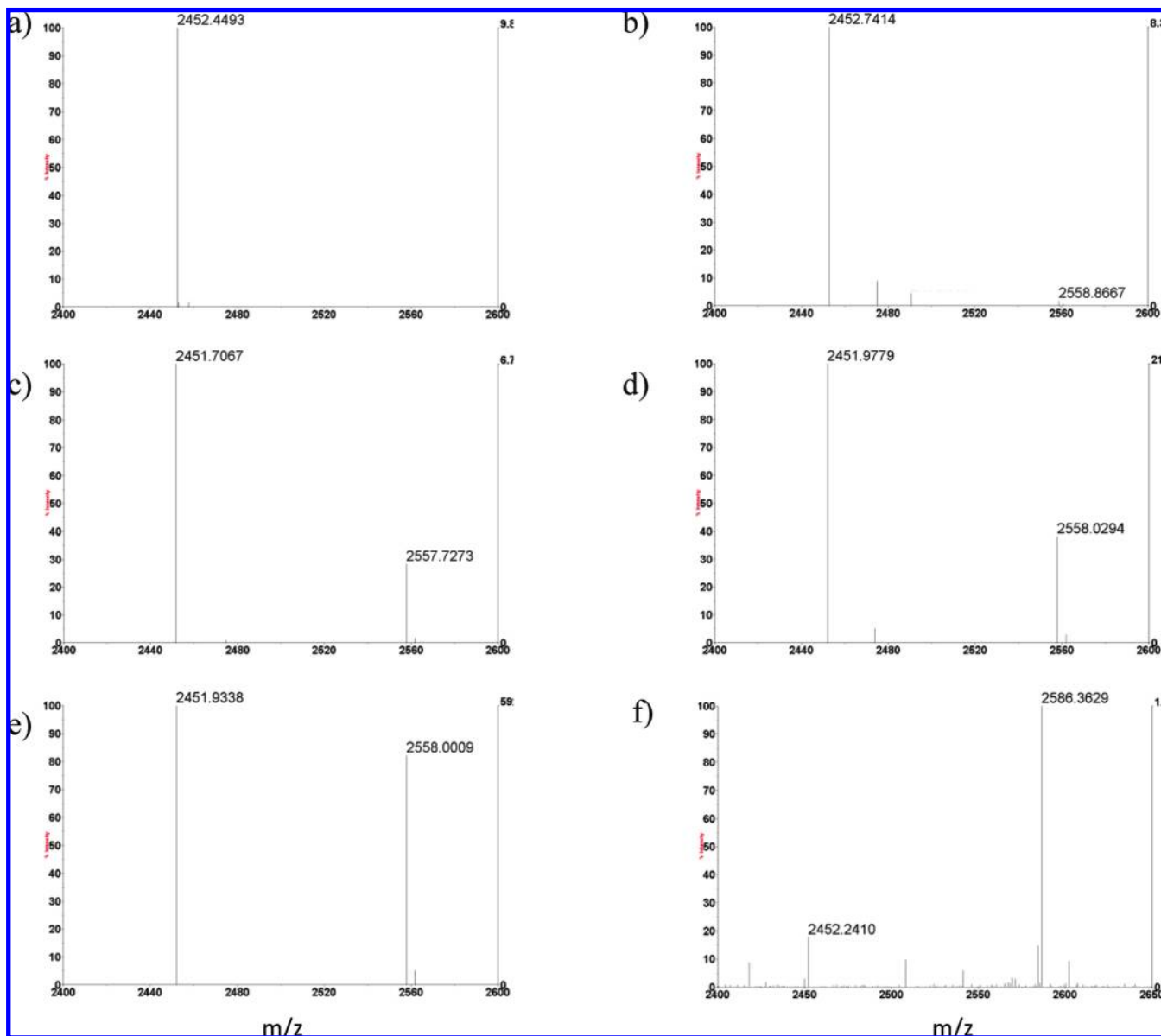


Figure 3. Time course of PPD binding to the DS3 peptide analyzed by MALDI-MS. (a) Control; (b) PPD + DS3, 2 h; (c) PPD + DS3, 24 h; (d) PPD + DS3, 48 h; and (e) PPD + DS3, 72 h. Unmodified peptide $[M + H]^+ = 2452.5$; modified peptide $[M + H]^+ = 2558.9$; $\Delta = 106$. (f) Representative spectra for 2,5-dimethyl-1,4-benzoquinonediimine + DS3 at 16 h. Unmodified peptide $[M + H]^+ = 2452.6$; modified peptide $[M + H]^+ = 2586.4$; $\Delta = 134$ (+16 for oxidation of methionine).

22.5 min) within 16 h at 37 °C. The addition of *N*-acetyl cysteine or GSH, but not *N*-acetyl lysine, was found to completely inhibit the formation of BB. It is noteworthy that PPD adducts were not detected following addition of *N*-acetyl cysteine, *N*-acetyl lysine, or GSH (results not shown).

PPD and 2,5-Dimethyl-1,4-benzoquinonediimine Specifically Modify the Cysteine Residue in the Peptide DS3. DS3 peptide was incubated with PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid for 16 h at a molar ratio of chemical to peptide of 10:1.

Modification of DS3 was observed after incubation with PPD, the m/z of the monoprotonated peptide increasing from m/z 2451.2 (2+, 1226.6; 3+, 818.1; and 4+, 613.8) to m/z 2557.2 (2+, 1279.6; 3+, 853.4; and 4+, 640.3), corresponding to an increase of 106 amu. This suggested the addition of the benzoquinonediimine intermediate of PPD. Modification of the cysteine-containing tryptic peptide of DS3 (MFCQIG) was also observed, with the m/z of the monoprotonated peptide increasing from m/z 697.3 to 803.3 (2+, 402.6), corresponding to an

increase of 106 amu. Collision-induced dissociation (CID) of the m/z 402.6 parent ion revealed that the adduct was associated with the cysteine residue (Figure 2).

Similar modification of the cysteine of the DS3 peptide was observed after incubation with 2,5-dimethyl-1,4-benzoquinonediimine. The m/z of the peptide $[M + H]^+$ ion increased from 2451.2 to 2601.2 (2+, m/z 1301.6; 3+, m/z 868.1; and 4+, m/z 651.3), corresponding to an increase of 152 amu. Modification of the cysteine-containing tryptic peptide of DS3 (MFCQIG) was again observed, with the m/z of the peptide $[M + H]^+$ ion increasing from 697.3 to 849.3 (2+, m/z 425.7). CID of the m/z 425.7 ion revealed a 134 amu adduct associated with the cysteine residue and oxidation of the methionine residue (Figure 2). Modification of DS3 was not observed in incubations containing 4-aminobenzoic acid.

Time-Dependent Binding of PPD to the Peptide DS3. DS3 peptide was incubated with PPD for up to 72 h, with aliquots being taken for analysis by MALDI-MS. The MALDI

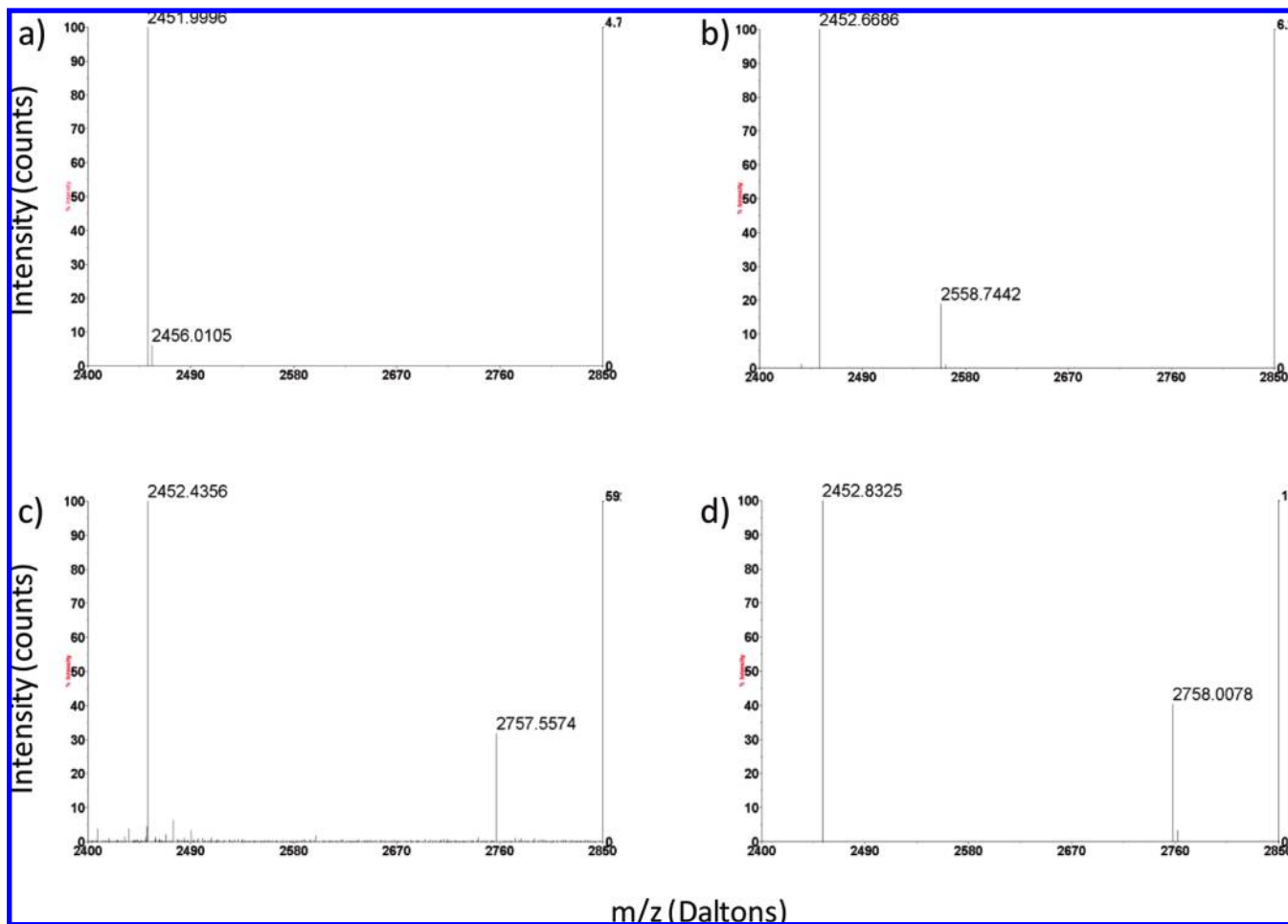


Figure 4. Analysis of PPD binding to the DS3 peptide in the presence and absence of GSH by MALDI-MS. (a) Control, (b) DS3 + PPD, (c) DS3 + GSH, and (d) DS3 + PPD + GSH. Unmodified peptide $[M + H]^+ = 2452$; PPD-modified peptide $MR = 2558$; $\Delta = 106$; GSH-modified peptide $MR = 2757$; $\Delta = 305$.

spectrum of the control DS3 peptide showed a single abundant ion at m/z 2452.5, corresponding to the monoprotonated form of the intact peptide (Figure 3a). Incubation of the peptide with PPD for 2 h resulted in the appearance of a new ion at m/z 2558.8, representing an increase in mass of 106 amu (Figure 3b) and corresponding to the addition of a single molecule of PPD diimine. The relative intensity of this new peak increased over the 72 h incubation period (Figure 3c–e). Incubation of the peptide with 2,5-dimethyl-1,4-benzoquinonediimine for 16 h resulted in a new ion at m/z 2586.8 corresponding to the addition of a single molecule of 2,5-dimethyl-1,4-benzoquinonediimine (Figure 3f). No new peaks were observed following incubation of DS3 peptide with 4-aminobenzoic acid.

Effect of GSH on PPD Binding. GSH was added to a subset of experiments to assess the reversibility of PPD binding. DS3 peptide was incubated in the absence of PPD, with PPD alone, with GSH alone, and with PPD plus GSH for 16 h. As before, MALDI analysis of the control DS3 peptide revealed a single abundant ion at m/z 2452.5 corresponding to the intact peptide (Figure 4a), and the PPD diimine addition product was detected at m/z 2558.7 (Figure 4b). Incubation of the peptide with GSH resulted in the appearance of a new ion at m/z 2757.6, representing an increase in mass of 305 amu (Figure 4c) and corresponding to the addition of a single residue of GSH. In the presence of both GSH and PPD, only the unmodified peptide (m/z 2452.5) and the GSH-modified peptide (m/z 2758.0) were observed (Figure 4d).

PPD Binds Irreversibly to the Model Protein GSTP. Denaturing gel electrophoresis of His-GSTP exposed to radiolabeled PPD followed by band excision and scintillation counting revealed that PPD bound irreversibly to His-GSTP (Figure 5a).

Selective Modification of GSTP at Cys47 by PPD and 2,5-Dimethyl-1,4-benzoquinonediimine. His-GSTP was incubated for 16 h with PPD and related compounds at a molar ratio of chemical to protein of 10:1. The protein was precipitated to remove unbound material before tryptic digestion and LC-MS/MS analysis. Sequence coverage of between 82 and 89% was achieved.

Modification of the Cys47-containing peptides 45–54 (ASCLYGQLPK) was observed after incubation with PPD for 16 h. The m/z of the peptide $[M + H]^+$ ion increased from 1078.6 (2+, m/z 540.3) to 1184.6 (2+, m/z 593.3), corresponding to an increase of 106 amu. These data suggest that the primary PPD oxidation intermediate benzoquinone-diimine binds covalently to His-GSTP. Fragmentation of the m/z 593.3 ion revealed the absence of the y8 ion at m/z 921, corresponding to unmodified Cys47 and the presence of the b3 ion at m/z 368 corresponding to Cys47 plus 106 amu (Figure 5b–d). No other residues were found to be modified in His-GSTP, including Cys residues at positions 14, 101, and 169. Incubation of His-GSTP with 2,5-dimethyl-1,4-benzoquinonediimine also resulted in modification of the Cys47-containing peptide (residues 45–54). The m/z of the

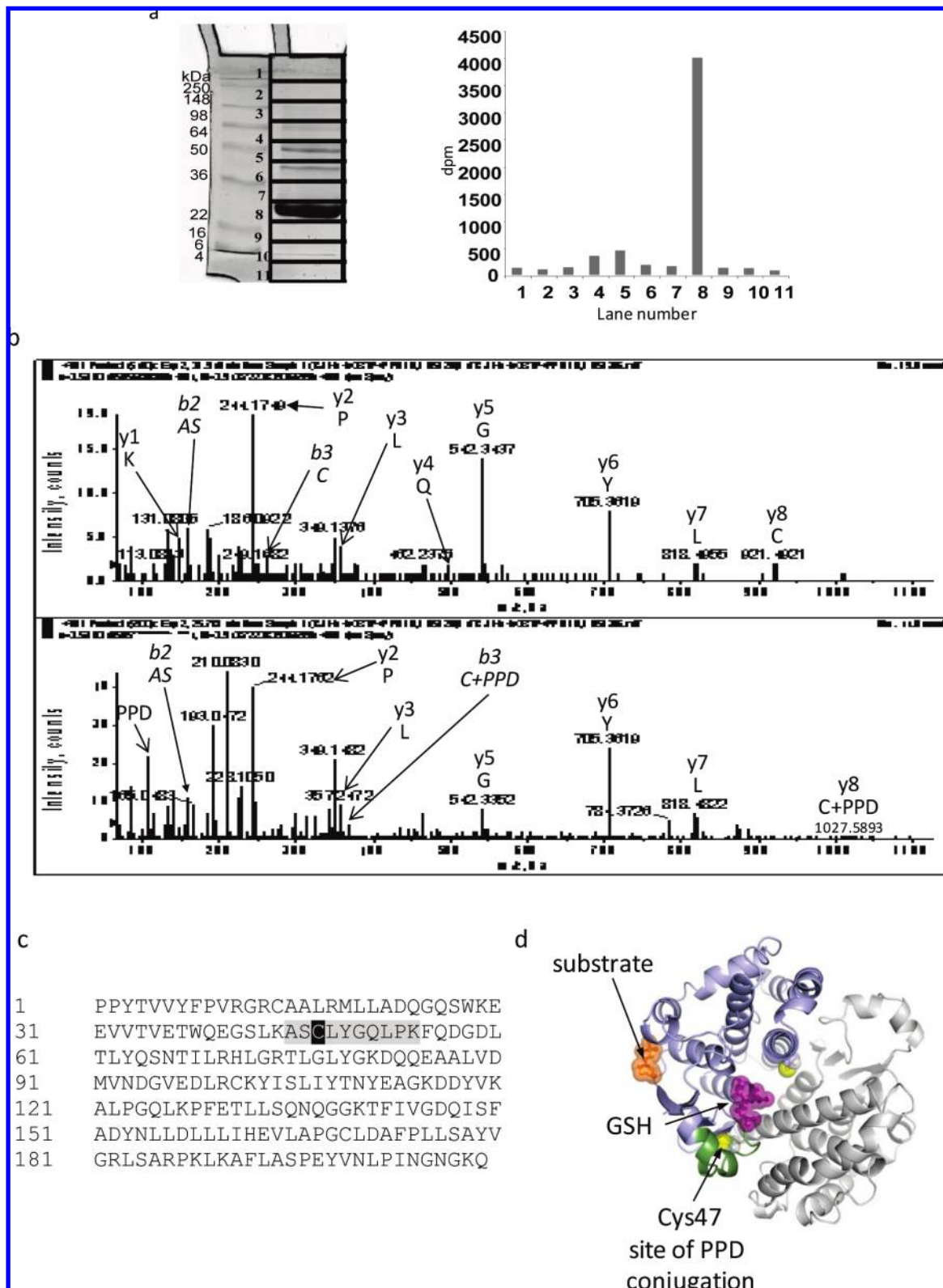


Figure 5. Exposure of GSTP to PPD and 2,5-dimethyl-1,4-benzoquinonediaimine leads to adduct formation through modification of cysteine residues. (a) GSTP was incubated with [^{14}C]PPD and separated by SDS-PAGE (10 μg /per track). Bands were excised, and incorporated radioactivity was determined by scintillation counting. DPM values correspond to each excised section of the gel. (b) LC-MS/MS sequence analysis of human GSTP peptide ASCLYGQLPK (i) unmodified $m/z = 540.3$, MW 1078.6; (ii) modified $m/z = 593.3$, MW 1184.6; $\Delta = 106$ Cys34 shows PPD modification. (c) Amino acid sequence of human GSTP1-1. Tryptic peptides 45–54 and Cys47 are indicated. (d) Molecular model of the dimer of human GSTP1, with the sites of substrate and GSH binding indicated. Cys47 is located close to the GSH binding site. The model is based on the structure 6GSS contained in the Protein Data Bank (36) and has been modified using PyMol (31).

peptide $[\text{M} + \text{H}]^+$ ion increased from m/z 1078.6 (2+, 540.3) to 1212.6 (2+, 607.3), consistent with modification by the chemical, and the sequence of the peptide could be inter-

preted. However, no adduct was detected in the MS/MS spectrum, possibly due to instability of the adduct in the collision chamber of the MS (data not shown). No adducts

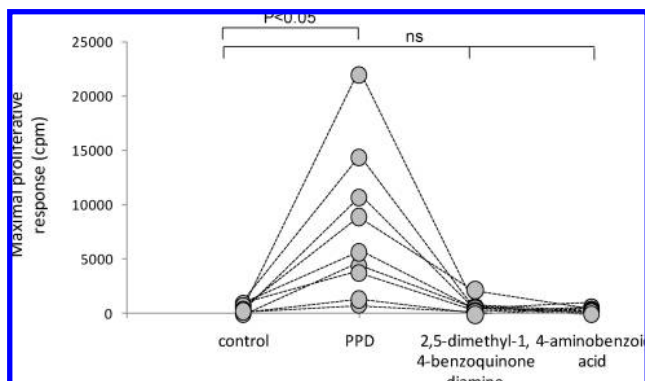


Figure 6. Proliferation of lymphocytes from 10 allergic patients stimulated with PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid. Proliferation was measured by [^3H] thymidine incorporation for the last 16 h of the experiment. Results are presented as a stimulation index (cpm in test incubations with antigen/cpm in control incubations with solvent alone). Data points indicate the maximum proliferative response of lymphocytes from each individual in response to treatment. Concentration-dependent lymphocyte proliferation is described in the Results. Statistical analysis was performed by comparing incubations in the presence and absence of drug ($P < 0.05$ was accepted as significant).

were observed following incubation of His-GSTP with 4-aminobenzoic acid.

PPD, but Not 2,5-Dimethyl-1,4-benzoquinonediimine or 4-Aminobenzoic Acid, Stimulates Proliferation of Lymphocytes from Allergic Patients. Lymphocytes from all 10 PPD-allergic patients, but not nonallergic controls, proliferated in vitro in the presence of PPD (0, 709 ± 208 cpm; $0.1 \mu\text{M}$, 1276 ± 505 cpm; $0.5 \mu\text{M}$, 2537 ± 2935 cpm; $1 \mu\text{M}$, 3880 ± 3502 cpm; $2 \mu\text{M}$, 6388 ± 3851 cpm; $5 \mu\text{M}$, 3855 ± 1975 cpm; $10 \mu\text{M}$, 982 ± 811 cpm; and $20 \mu\text{M}$, 224 ± 40 cpm). 2,5-Dimethyl-1,4-benzoquinonediimine evoked a weak response from the lymphocytes of one patient. 4-Aminobenzoic acid did not stimulate lymphocyte proliferation. Figure 6 shows the maximum lymphocyte proliferative response obtained with PPD, 2,5-dimethyl-1,4-benzoquinonediimine, and 4-aminobenzoic acid in cells from each allergic patient.

Discussion

Exposure to PPD can result in severe ACD, which is a major cause of increasing occupational morbidity and disability (1). Stimulation of an antigen-specific T-cell response is thought to be decisive in determining whether PPD exposure results in the development of contact dermatitis. In this respect, we have previously shown that lymphocyte stimulation with PPD bound irreversibly to protein, but not BB, is an important determinant of individual susceptibility (6). This finding corroborates Landsteiner and Jacobs' theory of hapten recognition of low molecular weight compounds by the immune system and underlines the importance of delineating the site and nature of PPD-specific protein interaction(s) and whether particular modifications stimulate a T-cell response.

PPD is readily converted into 1,4-benzoquinonediimine in solution, which is susceptible to hydrolysis, yielding *p*-benzoquinone and self-conjugation that generates BB and several as yet ill-defined products. Because of the complexity of PPD's oxidative chemistry, the nature of the compounds interaction with protein has not been investigated. The most closely related studies derive from the group of Lepoittevin and co-workers; NMR spectrometric analyses of reactions between the exploratory compound 2,5-dimethyl-1,4-benzoquinonediimine and the nucleophilic amino acids show a classical Michael addition

reaction with *N*-acetyl cysteine, whereas a complex series of reaction mechanisms yielded multiple structures with the hard electrophile *N*-acetyl lysine (21, 22). More recently, 2,5-dimethyl-1,4-benzoquinonediimine was found to react covalently with lysine in a cysteine-free peptide via Schiff base formation followed by an oxidoreduction process, giving rise to an aldehyde intermediate and peptide cyclization (23). The authors concluded that lysine should be considered an important amino acid for conjugate formation by allergenic *p*-benzoquinonediimines. However, the ratio of binding to specific amino acids in a cysteine-containing peptide, the nature of the covalent binding interaction with protein, and the effect of autoxidation on adduct formation were not evaluated. Thus, using PPD and 2,5-dimethyl-1,4-benzoquinonediimine, we have adopted a mass spectrometric approach to address these issues.

Unlike the directly reactive 2,5-dimethyl-1,4-benzoquinonediimine, PPD did not bind covalently to the soft nucleophiles *N*-acetyl cysteine or GSH. However, both nucleophiles decreased the rate of PPD degradation and BB formation. These results demonstrate that reduction of 1,4-benzoquinonediimine is favored over direct conjugation and adduct formation. The high concentration of nucleophile used, which mimics the intracellular environment, is known to favor reduction over conjugation reactions, since the rate of reaction is dependent on concentration of the substrate (31). Furthermore, the thiol/thiolate ion ratio of GSH and *N*-acetyl cysteine at physiological pH [approximately 63:1 for GSH (pK_a 9.2) and 125:1 for *N*-acetyl cysteine (pK_a 9.5)] may explain the absence of detectable adduct formation since sulfhydryl groups in a thiolated form are more nucleophilic and react faster (32). When PPD was exposed to an excess of *N*-acetyl lysine, the rate of PPD degradation was not altered, and no adducts were detected.

Aleksic et al. (14) have recently shown that PPD interacts with small peptides containing one of the nucleophilic amino acids cysteine, lysine, histidine, or arginine. The interaction of PPD with nucleophilic amino acids in a more complex chemical environment has not been studied in detail. Using the synthetic peptide DS3, PPD was found to bind exclusively to cysteine, despite all of the nucleophilic amino acid residues being represented in the peptide sequence. LC-MS/MS analysis of the DS3 peptide with PPD revealed a mass shift of 106 amu in modified samples as compared to control, suggesting a direct nucleophilic/electrophilic reaction between PPD benzoquinonediimine and sulfur of the cysteine residue (Figure 2). 2,5-Dimethyl-1,4-benzoquinonediimine also bound selectively to the cysteine residue of the DS3 peptide with a mass addition of 134 amu detected. These experiments, conducted at physiological pH 7.4, demonstrate that the context of the amino acid residue in a peptide has an important effect on the binding profile of a chemical and suggest that selective cysteine binding could be a key determinant in the induction of ACD.

GSTP was used to further explore the covalent interaction of PPD and 2,5-dimethyl-1,4-benzoquinonediimine with polypeptides. The reactivity of cysteine residues in proteins is determined partly by their ability to dissociate to the thiolate anion. Reactivity is also dependent on local steric factors and the accessibility of the residues to compounds penetrating the protein structure. The four cysteine residues of GSTP (Cys 14, 47, 101, and 169) differ in their reactivity. A recent study using the reactive metabolite of paracetamol *N*-acetyl-*p*-benzoquinonediimine characterized site-specific modification of two cysteine residues (Cys 47 and 101) that exist in relatively accessible positions (27). In these investigations, the Cys47-containing tryptic peptide (ASCLYGQLPK) of His-GSTP was found to

Scheme 1. Summary of the Binding Interaction of PPD and 2,5-Dimethyl-1,4-benzoquinonediimine with Nucleophilic Amino Acids, Peptides, and Protein

	<u>Electrophile</u>		
	PPD	2,5-dimethyl-1,4-benzoquinonediimine	2,5-dimethyl-1,4-benzoquinonediimine Eilstein et al. (2006,2007)
<u>Nucleophile</u>	1,4-benzoquinonediimine	2,5-dimethyl-1,4-benzoquinonediimine	
N-acetyl cysteine	Reduction	Not performed	conjugation
N-acetyl lysine	No reaction	Not performed	conjugation
DS3 peptide	Cysteine selective conjugation	Cysteine selective conjugation	not performed not performed
GSTP	Cysteine selective conjugation	Cysteine selective conjugation	not performed not performed
Adduct structure			
Lymphocyte stimulation	YES	NO	

be selectively modified by PPD and 2,5-dimethyl-1,4-benzoquinonediimine, consistent with the relative reactivity of the cysteine residues in this protein. Cys47 has a low pK_a value [3.5–4; the pK_a of most cysteine residues in protein is around 8.5 (33); pK_a of GSTP Cys14, 10.2; Cys101, 12.7; and Cys169, 9.1 (34)], making it highly nucleophilic and subject to modification by all soft electrophiles independent of chemical structure (27, 28). The unusual nucleophilicity of the residue is due to its deprotonation by the nearby lysine 54 residue (35). The selective binding of PPD to a single cysteine binding site in an isolated protein such as GSTP suggests that PPD protein binding in vivo might also display a high degree of intramolecular selectivity. The lysine residues of GSTP were not modified by either PPD or 2,5-dimethyl-1,4-benzoquinonediimine.

The reversibility of the PPD–peptide reaction was tested using GSH as it can prevent oxidation of PPD (25). Inclusion of GSH reduced the binding of PPD to the cysteine residue of the DS3 peptide. Originally, it was presumed that PPD would favor a conjugative reaction with the sulphydryl group of GSH. However, no evidence of a PPD–GSH conjugate was found. GSH effected reformation of PPD from the quinonediimine intermediate, presumably resulting in an equilibrium reaction between the two. This would explain why the binding of PPD decreases in the presence of GSH but is never fully inhibited and how it prevents BB being formed. A direct reaction between the GSH and the cysteine residue of DS3 generating a peptide/GSH mixed disulfide was also observed, suggesting that GSH outcompetes PPD for binding to the cysteine residue, thereby decreasing the availability of binding sites for PPD.

To explore whether the protein carrier or the hapten itself represents the major antigenic determinant for lymphocyte reactivity, PPD and 2,5-dimethyl-1,4-benzoquinonediimine were tested for their ability to stimulate proliferation of lymphocytes from allergic patients. A vigorous PPD-specific proliferative response was observed with lymphocytes from all patients. In contrast, 2,5-dimethyl-1,4-benzoquinonediimine, which binds covalently to the same cysteine residues in DS3 peptide and GSTP as PPD, via a similar mechanism, stimulated low levels of proliferation in only one patient. These data indicate that the

lymphocyte response in PPD allergic patients is highly restricted in terms of the hapten structure; the presence of two methyl groups hinders the binding interaction between the hapten and the specific T-cell receptors.

In conclusion, we have identified cysteine as a selective target for PPD and 2,5-dimethyl-1,4-benzoquinonediimine modification in a model peptide and protein and verified that it can function as an antigenic determinant. The reaction with individual cysteine residues in protein is selective and will be determined by a number of physicochemical parameters including the pK_a of individual amino acids. In future experiments, it will be vital to explore the role of these PPD-modified cysteine residues on the T-cell response. Possible experimental approaches include the following: (1) the use of defined protein or peptide conjugates, (2) blocking protein cysteine residues prior to PPD exposure, and (3) preventing PPD oxidation in T-cell assays. Scheme 1 recapitulates our chemical investigations and underlines the exquisite specificity of lymphocytes toward different antigenic determinants.

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