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Organophosphorus Pesticides Promote Protein Cross-Linking

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ABSTRACT: Exposure to organophosphorus pesticides (OP) can have chronic adverse effects that are independent of inhibition of acetylcholinesterase, the classic target for acute OP toxicity. In pure proteins, the organophosphorus pesticide chlorpyrifos oxon induces a cross-link between lysine and glutamate (or aspartate) with loss of water. Tubulin is particularly sensitive to OP-induced cross-linking. Our goal was to explore OP-induced cross-linking in a complex protein sample, MAP-rich tubulin from *Sus scrofa* and to test 8 OP for their capacity to promote isopeptide cross-linking. We treated 100 μg of MAP-rich tubulin with 100 μM chlorpyrifos, chlorpyrifos oxon, methamidophos, paraoxon, diazinon, diazoxon, monocrotophos, or dichlorvos. Each sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Five gel slices (at about 30, 50, 150, and 300 kDa, and the top of the separating gel) were removed from the lanes for each of the eight OP samples and from untreated control lanes. These gel slices were subjected to in-gel trypsin digestion. MSMS fragmentation spectra of the tryptic peptides were examined for isopeptide cross-links. Sixteen spectra yielded convincing evidence for isopeptide cross-linked peptides. Ten were from the chlorpyrifos oxon reaction, 1 from dichlorvos, 1 from paraoxon, 1 from diazinon, and 3 from diazoxon. It was concluded that catalysis of protein cross-linking is a general property of organophosphorus pesticides and pesticide metabolites. Data are available via ProteomeXchange with identifier PXD034529.
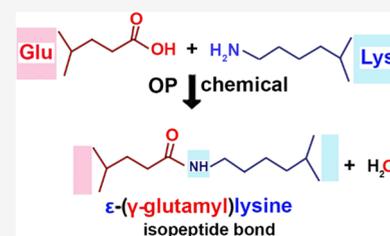
1. INTRODUCTION

The classical route by which exposure to organophosphates (OP) causes health problems is via inhibition of acetylcholinesterase.[1] However, repeated exposure to levels of OP that are too low to inhibit acetylcholinesterase can lead to chronic neurological problems. In adults, such exposure is manifested as deficits in memory, attention, reaction time, and fine motor skills[2−4] as well as increased risk for Alzheimer's disease,[3] Parkinson's disease,[4,5] and neuropsychological abnormalities.[6] In children, such exposure can lead to brain abnormalities,[7] neurodevelopmental defects,[8] and tremors.[9]

Although OPs can react with the active site serine from a variety of esterases and proteases, we suggest that OP modification of nonesterase proteins may be responsible for these chronic conditions.[10]

Treatment of pure proteins with chlorpyrifos oxon[11,12] or the nerve agent VX[13] induced the formation of isopeptide cross-links between the side chains of glutamic acid (or aspartic acid) and lysine. This is envisioned as a two-step process. First, a reaction between a lysine and the organophosphorus compound yields a phosphoryl-lysine adduct (see Figure 1, panel A). Second, the phosphoryl-lysine adduct reacts with γ-carboxyl of glutamate (or aspartate) to form an isopeptide cross-link, with the release of the phosphoryl moiety (see Figure 1, panel B). Both reactions are promoted by a vicinal acidic group. The overall reaction of isopeptide bond formation results in loss of water from the peptides.[11] Isopeptide bond formation does not occur in the absence of an

organophosphorus compound, clearly implicating the organophosphorus in the mechanism. These mechanisms are patterned after the mechanism proposed by Kang and Baker for the spontaneous formation of isopeptide bonds in Gram-positive bacterial pilus structures.[12]

It is important to point out that two of the OP used in this study are organophosphorothioates (chlorpyrifos and diazinon) that contain a phosphoryl-sulfur double bond P=S. Organophosphorothioates are intrinsically less reactive than organophosphates. However, adduct formation should proceed via the same pathway, albeit at a slower rate. A more thorough discussion of organophosphorothioates can be found in Discussion section 4.2 "Organophosphorus compounds."

Formation of isopeptide bonds can be mediated by transglutaminase enzyme activity or induced by OP. Transglutaminase creates a γ-glutamyl-ε-lysine linkage between Gln and Lys, with loss of NH$_3$. OP induction also creates a γ-glutamyl-ε-lysine linkage, but the amino acid in the cross-link is glutamic acid rather than glutamine. Another difference is that the OP-induced link between lysine and glutamic acid is accompanied by loss of H$_2$O rather than ammonia.
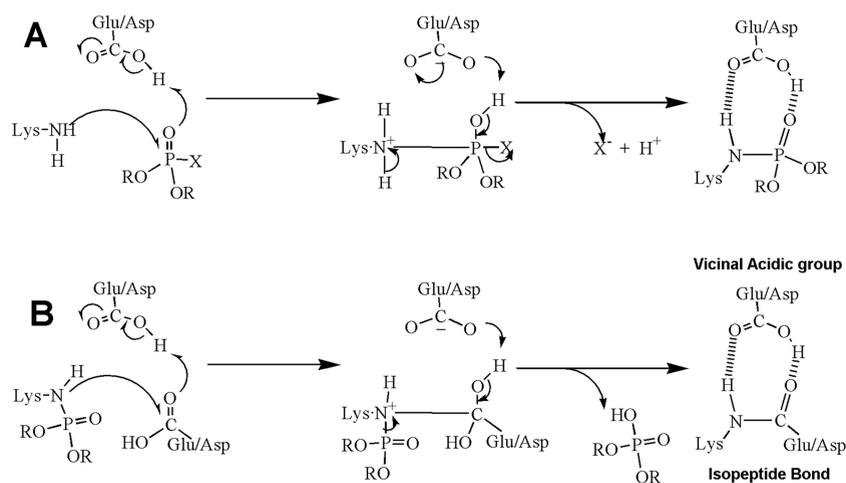
Figure 1. Proteins are cross-linked by a zero-length isopeptide bond between glutamic acid (or aspartic acid) and lysine following exposure to organophosphorus chemicals. Panel A illustrates the covalent modification of lysine by OP with release of a leaving group (X) and a proton. Adduct formation is promoted by a vicinal acidic group. Panel B illustrates the reaction of the OP-lysine adduct with a glutamic (aspartic) acid with release of the organophosphorus residue. This reaction would be promoted by the same vicinal acidic residue. The isopeptide bond is stabilized by hydrogen bonds with the nearby acidic amino acid. Panel B is adopted from Schopfer and Lockridge.[11]

Early methods for detecting isopeptide bonds were focused on identifying the γ-glutamyl-ε-lysine residue. Preparations were extensively digested and then examined by amino acid composition analysis[13] or amino acid sequencing.[14] γ-Glutamyl-ε-lysine cross-links were also detected by anti-isopeptide antibodies. These methods are unable to differentiate between lysine–glutamate and lysine–glutamine isopeptide bonds. Mass spectral methods that are capable of such discrimination have received limited use.[14,15] We have adopted mass spectrometry for identifying isopeptide cross-links and have successfully identified both lysine-glutamine[16] and lysine-glutamate cross-links.[11,17] We found that tubulin is particularly sensitive to OP-induced isopeptide cross-linking.[17]

In the present report, we use MAP-rich tubulin, a more complex protein mixture than we have used before. In addition, we reacted the protein with eight OP (chlorpyrifos, chlorpyrifos oxon, methamidophos, paraoxon, diazinon, diazoxon, monocrotophos, or dichlorvos) to determine whether the OP-induced isopeptide cross-link is a general property of OP or is restricted to a select few OP. Support for the observed cross-links was obtained by demonstrating that the lysine residues involved in the cross-links were also labeled by the OP.

2. EXPERIMENTAL PROCEDURES

2.1. Materials.

- MAP-rich tubulin *Sus scrofa* from porcine brain (Cytoskeleton Inc. ML116, Denver, CO) stored at −80 °C
- Trypsin (Promega, Sequencing grade, V5113, Madison, WI) stored at −80 °C
- Chlorpyrifos oxon 0.01 M stock solution in acetonitrile stored at −80 °C (CAS:5598-15-2 Chem Service Inc. MET-11459B, Westchester, PA)
- Chlorpyrifos 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 2921-88-2 Chem Service Inc. N-11459, Westchester, PA)
- Methamidophos 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 10265-92-6 Chem Service Inc. N12393 Westchester, PA)
- Paraoxon, ethyl 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 311-45-5 Chem Service Inc. N12816 Westchester, PA)

- Diazinon 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 333-41-5 Chem Service Inc. N11621, Westchester, PA)
- Diazoxon 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 962-58-3 Chem Service Inc. ME-11621, Westchester, PA)
- Monocrotophos 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 6923-22-4 Chem Service Inc. N12493, Westchester, PA)
- Dichlorvos 0.01 M stock solution in acetonitrile stored at −80 °C (CAS 62-73-7 Chem Service Inc. N11675, Westchester, PA)

2.2. Reaction of MAP-Rich Tubulin with Organophosphates.

Two milligrams of MAP-rich tubulin was dissolved in 4 mL of 20 mM Tris/Cl buffer, pH 8.5, containing 0.01% sodium azide to make 0.5 mg of MAP-rich tubulin per mL. Two microliters of 10 mM organophosphate (chlorpyrifos, chlorpyrifos oxon, methamidophos, paraoxon, diazinon, diazoxon, monocrotophos, or dichlorvos, in acetonitrile) were mixed with 0.2 mL of 0.5 mg/mL MAP-rich tubulin to give 100 μM OP and 100 μg MAP-rich tubulin. Reactions were incubated at 37 °C for 48 h, in a humidified chamber, in the dark.

Organophosphates are relatively stable to hydrolysis at pH 8.5. For example, dichlorvos has a half-life of 13.6 days at pH 8.5 and 25 °C, chlorpyrifos oxon has a half-life of 20.9 days at pH 8 and 23 °C, and diazoxon has a half-life of 18 days at pH 9.0 and 20 °C.[18] These values suggest that most of the organophosphates used in our experiments would still be present after 48 h at pH 8.5 and 37 °C. A duration of 48 h was chosen for reaction to allow the expected phospho-lysine adduct time to react with neighboring glutamate (aspartate) residues.

Eight hundred and fifty microliters of 20 millimolar ammonium bicarbonate, pH 8 was added to 160 μL of each reaction mixture to give 0.08 mg/mL MAP-rich tubulin. The mixtures were injected into 3 mL Slide-A-Lyzer dialysis cassettes, 7000 molecular weight cut-off (Thermo Scientific/Pierce Protein Research Products #66370, Waltham, MA), and dialyzed against 450 mL of 20 mM ammonium bicarbonate at 4 °C, overnight, to remove the unreacted OP. Dialysis was repeated with fresh buffer. Each sample was dialyzed in its own container to be certain the cross-link could be attributed to a particular OP. The dialyzed MAP-rich tubulin samples were tested for residual OP by incubating 10 μL of reaction mixture with 10 μL of butyrylcholinesterase (20 unit/mL) and checking for inhibition of butyrylcholinesterase activity. Six of the 8 OP are BChE inhibitors. No inhibition was detected, indicating that reactive OP was not present in the dialyzed samples.

Note that organophosphates are toxic. Gloves and face protection should be used when working with OP.

2.3. SDS-PAGE. Dialyzed samples were concentrated to about 2 μg/μL MAP-rich tubulin by vacuum centrifugation in a Savant SpeedVac (model SC100, Thermo Fisher, Waltham, MA). Seventy micrograms of MAP-rich tubulin from each sample (35−54 μL) were mixed with 1/3 volume of sample loading buffer (0.1 M Tris/Cl pH 6.8 containing 15% glycerol, 3% SDS, 0.01% Coomassie blue G250, and 0.3 M dithiothreitol) and heated in a boiling water bath for 3 min. Each mixture was loaded into a lane of a 4−22% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15 well, 0.75 mm thick, 16×18 cm slab) with a 4% stacking gel (Hoefer vertical slab gel, series SE600, Hoefer Inc., Holliston MA). Control samples were prepared by dissolving 1 mg of MAP-rich tubulin in 0.7 mL of 20 mM Tris/Cl, pH 8.5. Fifty and 25 μg of MAP-rich tubulin were mixed with sample loading buffer, denatured in a boiling water bath for 3 min, and loaded into lanes on the SDS-PAGE. Electrophoresis was run for 3000 volt-hours (300 volts for 10 h) at room temperature. Gels were stained with Coomassie Blue R-250 and destained with water.

2.4. In-Gel Digestion. In-gel tryptic digestion (with dithiothreitol reduction and iodoacetamide alkylation) was performed on gel slices as previously described.[19] Extracted peptides were dried by vacuum centrifugation in a Savant SpeedVac centrifuge and redissolved in 10 μL of water.

2.5. Data Acquisition with Orbitrap Fusion Lumos Tribrid Mass Spectrometer. Peptide separation was performed with a Thermo RSLC Ultimate 3000 ultra-high pressure liquid chromatography system (Thermo Scientific, Waltham, MA), at 36 °C. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in 80% acetonitrile. Peptides were loaded onto an Acclaim PepMap 100 C18 trap column (75 μm × 2 cm; Thermo Scientific, 165535) at a flow rate of 4 μL/min and washed with 100% solvent A for 10 min. Then, the peptides were transferred to a Thermo Easy-Spray PepMap RSLC C18 column (75 μm × 50 cm with 2 μm particles, Thermo Scientific, ES803) and separated at a flow rate of 300 nL/min using a gradient of 9−25% solvent B in 27 min, 25−35% solvent B in 5 min, 35−99% solvent B in 4 min, hold at 99% solvent B for 4 min, from 99 to 9% solvent B in 4 min, hold at 9% solvent B for 16 min.

Eluted peptides were sprayed directly into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). Data were collected using data-dependent acquisition. A survey full scan MS (from 350 to 1800 m/z) was acquired in the Orbitrap in positive ion mode, with a resolution of 120,000. The AGC target (Automatic Gain Control for setting the ion population in the Orbitrap before collecting the MS) was set at 4×10^5, and the ion filling time was set at 100 ms. The 25 most intense ions with charge state of 2−6 were isolated in quadrupole mode, in a 3 s cycle, and fragmented using high-energy collision-induced dissociation with 35% normalized collision energy. Fragment ions were detected in the Orbitrap with a mass resolution of 30,000 at 200 m/z. The AGC target for MSMS was set at 5×10^4, ion filling time was set at 60 ms, and dynamic exclusion set for 30 s after 1 time with a 10 ppm mass window. Data were reported in *.raw format.

2.6. Search for Cross-Linked Peptides with Batch-Tag Web Software in Protein Prospector Version 6.2.1. The *.raw data files from the Orbitrap were converted to *.mgf files using MSConvert (ProteoWizard Tools from SourceForge). The *.mgf files were analyzed using Batch-Tag Web on the Protein Prospector website https://prospector.ucsf.edu [prospector.ucsf.edu] last accessed June 2022. Data were searched for isopeptide cross-links Lys-to-Glu and Lys-to-Asp.

The search parameters for cross-links were as follows. (1) Database: User protein. (2) User Protein Sequence: Neurofilament heavy polypeptide (XP-005670835); Microtubule-associated protein 2 isoform X8 (XP-013839898); Microtubule-associated protein 1B isoform X1 (XP-003134080); Microtubule-associated protein tau isoform X16 (XP-020922473); Tubulin α1A (NP-001302639); and Tubulin β4B (XP-003122400) pasted into the user protein window in the FASTA format. (3) Precursor Charge Range: 2, 3, 4, 5. (4)

Masses: monoisotopic. (5) Parent Tol: 20 ppm. Frag Tol: 30 ppm. (6) Instrument: ESI-Q-high-res. (7) Digest: Trypsin. (8) Max missed cleavages: 2. (9) Constant Mods: carbamidomethylation(C). (10) Variable Mods: Oxidation (M). (11) Expectation Calc Method: None. (12) Mass Modifications: range −18 to 4000 Da. Formation of isopeptide bonds between amino acids is accompanied by loss of 18 Da due to loss of water, which sets the lower mass limit. Protein Prospector cross-link searches treat one peptide as a variable modification, which accounts for the upper mass limit. (13) Check mark in amino acid boxes D, E, and K. (14) No check mark in boxes N terminus and C terminus; and a check mark in the Uncleaved box. Checking the uncleaved box avoids false candidates in which a C-terminal lysine is reported as the cross-linked lysine. (15) Cross-linking: Link Search Type: User-Defined Link. (16) User-Defined Link Parameters: Link AAs: E, D, Protein C term>K, Protein N-term. (17) Bridge Elem Comp: H-2 O-1.

Batch Tag created a list of isopeptide cross-linked candidates that was viewed with the Search Compare algorithm.

2.7. Screening Search Compare Results. To reduce the number of cross-link peptide candidates and aid in the identification of cross-linked peptides, the Search Compare list was screened manually. Candidate cross-linked peptides were selected for further evaluation if they met the following criteria: charge state 2, 3, 4, 5; Score > 20; score difference > 1; % matched intensity > 40%; and at least 4 amino acids in each peptide. Choice of these parameters is empirical and was based on experience.

2.8. Manual Evaluation of Cross-Linked Peptide Candidates. Ultimately, cross-linked peptides were confirmed by manual evaluation of their MSMS spectra. For a cross-link candidate to be accepted as a cross-linked peptide, there must be amino acid sequence support for both peptides and there must be at least one cross-link specific amino acid, defined by two cross-link specific ions. Sequence support consists of the following features.

(1) A series of noncross-link specific masses in the MSMS spectrum must correspond to an amino acid sequence from each peptide in a cross-link candidate. Suitable sequences include an N-terminal sequence, a C-terminal sequence, or an internal fragment. Sequences must be at least 2 amino acids long (3 amino acids or more is better). For example, in Figure 3, the green peptide is supported by the b-ion sequence DVNAAI and the blue peptide is supported by the y-ion sequence RAYNNAA.

(2) At least one cross-link specific amino acid is essential. A cross-link specific amino acid is an interval in the MSMS spectrum from a cross-link candidate that is defined by two cross-link specific masses and corresponds to an amino acid that is part of the cross-link candidate sequence. A cross-link specific mass is a mass that includes residues from both peptides. A series of cross-link specific amino acids is better. For example, in Figure 5, the blue amino acid L is defined by the cross-link specific $y9^{+3}$ and $y10^{+3}$ ions.

(3) Neutral loss of amino acids can occur from the parent ion. Neutral losses commonly come from the N-terminal. This is referred to as a ladder sequence by Protein Prospector. With cross-linked peptides, ladder sequences can consist of N-terminal residues from both peptides. Neutral losses can sometimes be C-terminal amino acids. This is otherwise referred to as a [bn 1 + 18] fragment.[20] Any C-terminal residue can be lost, provided that a basic residue such as arginine, lysine, or histidine is present in the sequence.[21] Parent ions that have lost an N- or C-terminal amino acid are cross-link specific ions because they contain residues from both peptides. For example, in Figure 6, the green DDL^{+2} peptide at 516.27− 687.84 m/z is a ladder sequence.

2.9. MS-Tag from File. Dr. R.J. Chalkley (University of California San Francisco) pointed out that cross-linked candidates can sometimes be better fit to a linear sequence from a different protein (personal communication). This possibility can be tested with the Protein Prospector "MS-Tag from File" algorithm. This algorithm can

search a single spectrum to find other matches based solely on the Batch-Tag scoring system. Each MSMS spectrum that appeared to define a cross-linked peptide was examined with MS-Tag from File. Two databases were used: the 6-protein database employed in the original Batch Tag search and SwissProt.2017.11.01, *Sus scrofa*. In both cases, the Link Search Type parameter was set to No Link.

2.10. Adduct Formation by Organophosphates on Lysine. MSMS data were searched for peptide adducts formed by the OP using the Proteome Discoverer v2.2 with the Sequest HT database search engine (last used Oct 2020). Searches employed Processing Method "PWF fusion basic Sequest HR" and Consensus method "CWF Basic."

2.11. Replicate Analysis. Fifty-five gel bands were digested with trypsin, and the tryptic peptides were analyzed by LC/MSMS. Cross-link analysis yielded 160 candidates. Manual evaluation of these mass spectra was extremely time consuming. We did not perform the standard technical and biological replicates because of the highly time-consuming nature of this analysis. Consequently, our results must be considered qualitative.

3. RESULTS

3.1. SDS-PAGE. Proteins from OP-treated MAP-rich tubulin were separated by SDS-PAGE and stained with Coomassie Blue R-250 (Figure 2 panels A and B). The intense band at 50 kDa was tubulin. Band intensity in the 150 kDa range was greater than control for the samples treated with chlorpyrifos oxon, diazoxon, and dichlorvos. In addition,
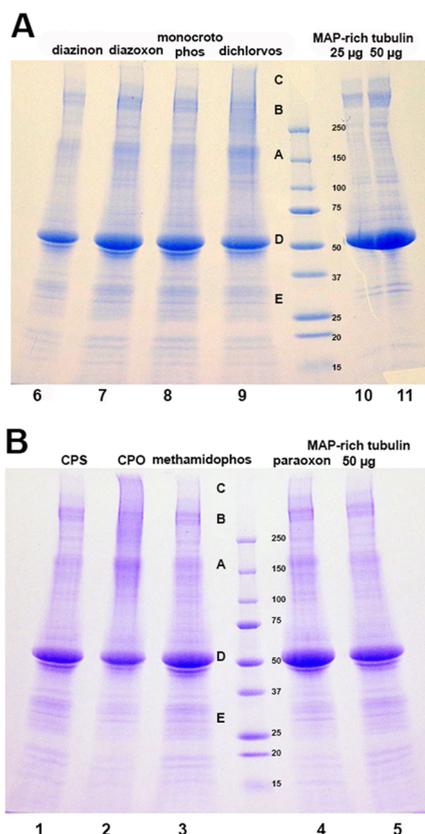
Figure 2. SDS-PAGE for organophosphate-treated MAP-rich tubulin. Gels were stained with Coomassie blue. Panel A shows the results for diazinon, diazoxon, monocrotophos, and dichlorvos, plus untreated MAP-rich tubulin. Panel B shows the results for chlorpyrifos (CPS), chlorpyrifos oxon (CPO), methamidophos, and paraoxon, plus untreated MAP-rich tubulin. Regions marked A, B, C, D, and E were excised and subjected to in-gel trypsinolysis.

band intensity in the 250 kDa region was more intense for the chlorpyrifos oxon-treated sample. Gel bands in areas marked A, B, C, D, and E were removed and digested with trypsin.

3.2. Mass Spectral Analysis. Fifty-five gel bands were analyzed (40 from OP-treated samples and 15 from controls). Protein Prospector/Batch Tag Web identified about 2500 potentially cross-linked peptides in the treated samples. Filtering the Search Compare files with the criteria described in the Experimental Section "Screening Search Compare results" reduced the number to 160. The MSMS spectra from these samples were examined manually. Sixteen cross-linked peptides were identified (see Table 1). Ten cross-links were induced by chlorpyrifos oxon, 3 by diazoxon, and 1 each by dichlorvos, paraoxon, and diazinon. The lysine involved in the cross-link was found to be labeled by the OP in eleven cases. For each of these sixteen data sets, "MS Tag from File" analysis found no single peptide that fit the data as well as the cross-link. No convincing cross-linked peptides were found in the untreated control samples.

Treatment of MAP-rich tubulin with chlorpyrifos oxon yielded 10 cross-linked peptides. A cross-link between K163 in peptide LSVDYGK$_{163}$K from tubulin α1A and E158 in peptide IREE$_{158}$YPDR from tubulin β4B appeared four times (in charge states 3 and 4, from gel slices A and B). The analogous cross-link between K163 in peptide LSVDYGK$_{163}$K and E158 in peptide EE$_{158}$YPDR appeared two times (in charge states 3 and 4, from gel slice B). A diethylphospho-adduct was found on lysine 163, supporting its involvement in cross-linking. The MSMS spectrum for this latter cross-linked peptide pair was previously published.[22] A cross-link between K336 in peptide DVNAAIATIK$_{336}$TK from tubulin α1A and E158 in peptide EE$_{158}$YPDR from tubulin β4B appeared two times (in charge state 3, from gel slices A and B). K336 in peptide DVNAAIATIK$_{336}$TK from tubulin α1A was also cross-linked to D98 in peptide ED$_{98}$AANNYAR from tubulin α1A two times (in charge state 3 from gel slices A and B). The MSMS spectrum for this latter cross-linked pair is presented in Figure 3. A diethylphospho-adduct was found on lysine 336, supporting its involvement in cross-linking. Cross-links between peptides from the same protein, for example, tubulin α1A above, might involve peptides from the same monomer or peptides from two separate monomers.

Lysine 163 is also acetylated, but the function of this acetylation is unknown.[23]

Treatment of MAP-rich tubulin with diazoxon yielded three cross-linked peptides. Each peptide pair appeared only once. There was a cross-link between K562 in peptide KE-SK$_{562}$EETPEVTK from MAP 1B isoform X1 and D1001 in peptide EKRESIASGDD$_{1001}$R from MAP 1B isoform X1. There was a cross-link between K2289 in peptide ESSDK$_{2289}$VSR from MAP 1B isoform X1 and D34 in peptide FLD$_{34}$SK from MAP 1B isoform X1. Also, there was a cross-link between K163 in peptide LSVDYGK$_{163}$K from tubulin α1A and E852 in peptide SPAE$_{852}$VK from neurofilament heavy polypeptide. The MSMS spectrum for this latter cross-linked pair is presented in Figure 4. A diethylphospho adduct was found on lysine 163, but on neither of the other lysines.

Treatment with dichlorvos, paraoxon, and diazinon yielded 1 cross-linked pair each. Each of these cross-linked pairs was unique. Dichlorvos induced a cross-link between D1028 in peptide GDAEQSEEEGEEEED$_{1028}$K from MAP 1B isoform X1 and K444 in peptide VVEK$_{444}$SEK from neurofilament heavy polypeptide. Paraoxon induced a cross-link between

Table 1. Summary of K-DE Cross-Linked Peptides

XLink peptide with charge [a]	proteins	NCBI #	OP [b]	gel slice	score total [c]	OP [a] Lys
LSVDYGK$_{163}$K $^{+4}$ / │–18 / IREE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	A	25.4	Y
LSVDYGK$_{163}$K $^{+3}$ / │–18 / IREE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	A	60.3	Y
LSVDYGK$_{163}$K $^{+4}$ / │–18 / IREE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	B	36.6	Y
LSVDYGK$_{163}$K $^{+3}$ / │–18 / IREE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	B	27.0	Y
LSVDYGK$_{163}$K $^{+3}$ / │–18 / EE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	B	27.0	Y
LSVDYGK$_{163}$K $^{+4}$ / │–18 / EE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	B	24.6	Y
DVNAAIATIK$_{336}$TK $^{+3}$ / │–18 / EE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	B	60.6	Y
DVNAAIATIK$_{336}$TK $^{+3}$ / │–18 / EE$_{158}$YPDR	tubulin α-1A / tubulin β-4B	NP_001302639.1 / XP_003122400	CPO	A	60.4	Y
DVNAAIATIK$_{336}$TK $^{+3}$ / │–18 / ED$_{98}$AANNYAR	tubulin α-1A / tubulin α-1A	NP_001302639.1 / NP_001302639.1	CPO	A	78.6	Y
DVNAAIATIK$_{336}$TK $^{+3}$ / │–18 / ED$_{98}$AANNYAR	tubulin α-1A / tubulin α-1A	NP_001302639.1 / NP_001302639.1	CPO	B	63.1	Y
GDAEQSEEEGEEEED$_{1028}$K $^{+3}$ / │–18 / VVEK$_{444}$SEK	MAP 1B X1 / NFH	XP_003134080.1 / XP_005670835	DCV	A	27.1	N
LDDE$_{183}$AR $^{+2}$ / │–18 / AEK$_{1046}$AEK	NFH / NFH	XP_005670835 / XP_005670835	PON	D	25.3	N
TAVCamD$_{355}$IPPR $^{+4}$ / │–18 / TLK$_{115}$NRPCamLSPK	MAP Tau X16 / tubulin β-4B	XP_020922473 / XP_003122400	DIN	E	35.9	Y
LSVDYGK$_{163}$K $^{+3}$ / │–18 / SPAE$_{852}$VK	tubulin α-1A / NFH	NP_001302639.1 / XP_005670835	DIA	D	25.3	Y
EKRESIASGDD$_{1001}$R $^{+3}$ / │–18 / KESK$_{562}$EETPEVTK	MAP 1B X1 / MAP 1B X1	XP_003134080.1 / XP_003134080.1	DIA	E	22.1	N
FLD$_{34}$SK $^{+2}$ / │–18 / ESSDK$_{2289}$VSR	MAP 1B X1 / MAP 1B X1	XP_003134080.1 / XP_003134080.1	DIA	E	26.9	N

[a]Numbering indicates the site of the cross-link and includes the leader sequence. Cam stands for carbamidomethylated cysteine. [b]CPO = chlorpyrifos oxon; DCV = dichlorvos; PON = paraoxon; DIN = diazinon; and DIA = diazoxon. [c]Score total equals the sum of the score and the score diff. [d]MSMS spectra for putative OP-labeled peptides were analyzed by manual sequencing. Y indicates that an OP-lysine adduct was identified, and N indicates that an OP-lysine adduct was not identified.

E183 in peptide LDDE$_{183}$AR from neurofilament heavy polypeptide and K1046 in peptide AEK$_{1046}$AEK from neurofilament heavy polypeptide. Diazinon induced a cross-link between D355 in peptide TAVCamD$_{355}$IPPR from MAP Tau X16 and K115 in peptide TLK$_{115}$NRPCamLSPK from tubulin β-4B. MSMS spectra for these cross-linked pairs are presented in Figures 5, 6, and 7. No organophosphorylation of the cross-link associated lysines was detected.

4. DISCUSSION

4.1. Organophosphate-Induced Peptide Cross-Linking: A New Mechanism for Toxicity. The foregoing results demonstrate that several organophosphates (chlorpyrifos oxon, paraoxon, dichlorvos, diazinon, and diazoxon) can induce cross-links between lysine-and-glutamate or lysine-and-aspartate. Chlorpyrifos oxon appears to be the most effective.

All of the organophosphorus compounds used in this study are pesticides (diazinon, dichlorvos, chlorpyrifos, methamidophos, or monocrotophos) or are activated forms of pesticides (chlorpyrifos oxon from chlorpyrifos, diazoxon from diazinon, and paraoxon from parathion). Use of most of these pesticides

has been restricted because of their reported toxicity in epidemiological studies.[1−9] Methamidophos was discontinued in 2009. Diazinon (diazoxon) is still allowed in agriculture but not for residential use. Parathion (paraoxon) and dichlorvos are still in general use. Monocrotophos is largely banned world-wide, but it is still extensively used in India. Chlorpyrifos (chlorpyrifos oxon) has been banned from use on food crops and in residential settings in the United States and Europe, although it is still used in other countries. Thus, although exposure to organophosphate pesticides has been reduced, there is still ample opportunity for toxic exposure under some conditions.

In general, mechanisms of chemical toxicity involve binding of an agent to an enzyme, which inhibits enzymatic activity. Organophosphates react in this classical way with the active-site serine in serine esterases and proteases.[24] In addition, organophosphates can bind to lysine and tyrosine residues on a variety of proteins.[25] Such adduct formation is another potential pathway for toxicity. We have demonstrated that organophosphorus toxicants can promote protein cross-linking. This reaction provides a third potential mechanism for toxicity.
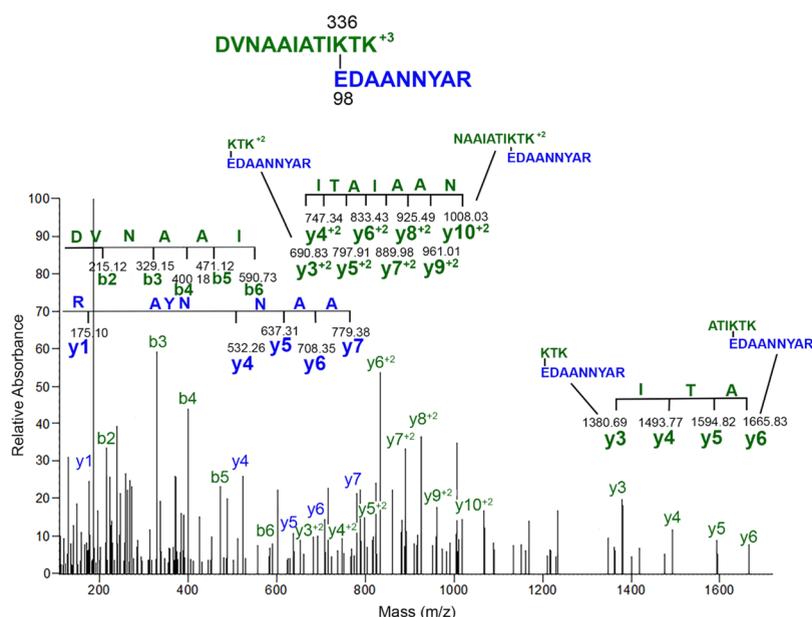
Figure 3. MSMS spectrum for the chlorpyrifos oxon-induced, cross-linked peptide DVNAAIATIK$_{336}$TK-E$_{98}$DAANNYAR that is cross-linked between K336 and E98. The mass of the triply-charged parent ion was 750.39 m/z. A y-ion sequence, AANNYAR, from the blue peptide and a b-ion sequence, DVNAAI, from the green peptide were present. A singly charged series of cross-link specific masses, ITA (1380.69−1665.83 m/z) from the green peptide was present. A series of doubly charged cross-link specific masses, ITAIAAN (690.83−1008.03 m/z) from the green peptide was also present. Most of the unlabeled masses in the spectrum were due to loss of H$_2$O, NH$_3$, or CO from the labeled masses.
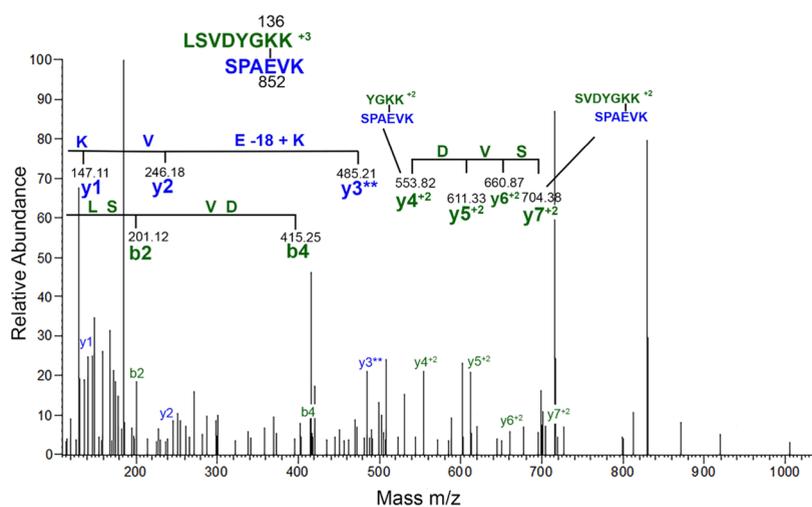
Figure 4. MSMS spectrum for the diazoxon-induced, cross-linked peptide pair LAVDYGK$_{136}$K-SPAE$_{852}$VK that is cross-linked between K136 and E852. The mass of the triply charged parent ion was 507.61 m/z. A pair of y-ions, VK, linked to an E − 18 + K cross-link interval (marked as y3**) from the blue peptide was present. A b-ion sequence, LSVSD, from the green peptide was present. A doubly charged y-ion series of cross-link specific masses, SVD (553.82−704.38 m/z), from the green peptide was present. Some of the unlabeled peaks correspond to loss of H$_2$O, NH$_3$, or CO from the labeled masses, but the major unlabeled peaks fit a sequence, TNDGGAL/I, that is consistent with the immunoglobulin heavy chain junction region.

The cross-linked proteins form abnormal interactions that may disrupt function. High molecular weight protein aggregates can be produced by protein cross-linking. Organophosphate-promoted protein cross-linking may account for neurodegenerative diseases associated with chronic organophosphate exposure.

4.2. Organophosphorus Compounds. Among the eight organophosphorus compounds used in this study, 6 were organophosphates (containing a phosphoryl-oxygen double bond P═O; chlorpyrifos oxon, methamidophos, paraoxon, diazoxon, monocrotophos, and dichlorvos) and 2 were organophosphorothioates (containing a phosphoryl-sulfur double bond P═S; chlorpyrifos and diazinon). The organophosphorothioates are much less reactive than the organophosphates because of the poor electron-withdrawing ability of the sulfur atom bound to the phosphorus. This would be expected to slow the rate of initial adduct formation (see Figure 1). Consistent with this prediction, a slower rate for inhibition of cholinesterases by organophosphorothiones is generally observed, that is, adduct formation is much slower than the equivalent reaction with organophosphates. However, adduct formation by diazinon on the active site serine of
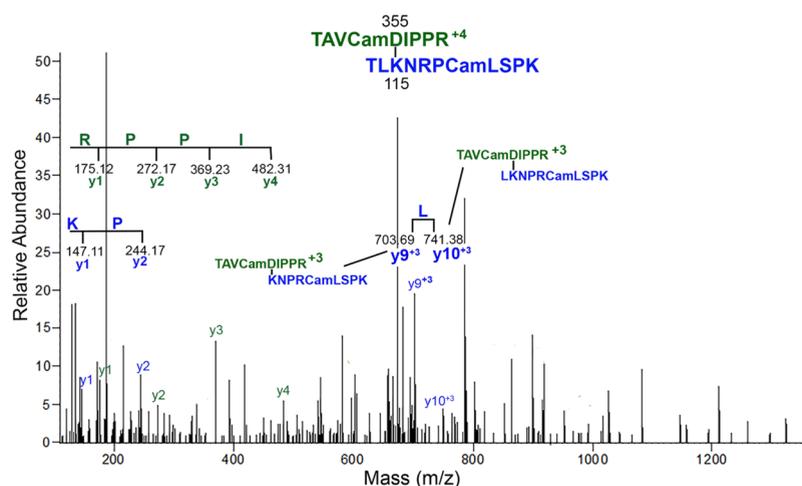
Figure 5. MSMS spectrum for the diazinon-induced, cross-linked peptide TAVCamD$_{355}$IPPR-TLK$_{115}$NRPCamLSPK that is cross-linked between D355 and K115. The mass of the quadruply charged parent ion was 581.55 m/z. A y-ion sequence, IPPR, from the green peptide and a y-ion amino acid pair, PK, from the blue peptide were present. A triply charged cross-link specific amino acid, L (703.69−741.38 m/z), was present. Some of the unlabeled peaks correspond to loss of H_2O, NH_3, or CO from the labeled masses, but the major unlabeled peaks fit a sequence, EQ/KL/IL/IAGQ/K, that is consistent with the myosin light chain.
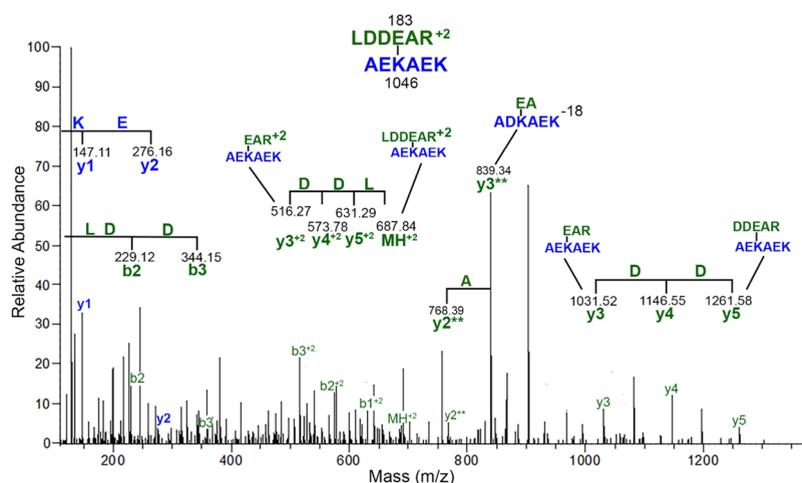
Figure 6. MSMS spectrum for the paraoxon-induced, cross-linked peptide LDDE$_{183}$AR-AEK$_{1046}$AEK that is cross-linked between E183 and K1046. The mass of the doubly-charged parent ion was 687.84 m/z. A b-ion sequence, LDD, from the green peptide and a y-ion amino acid pair, EK, from the blue peptide were present. A doubly charged ladder sequence, LDD (516.27 m/z to parent ion 687.84 m/z), from the green peptide was present. A singly charged cross-link specific sequence, DD (1031/52−1261.58 m/z), from the green peptide was present. A singly charged cross-link specific amino acid, A (768.39−839.34 m/z), that involved loss of residues from both ends of the green peptide and loss of water (indicated by y2**-y3**) was present. Some of the unlabeled peaks correspond to loss of H_2O, NH_3, or CO from the labeled masses, but most of the major unlabeled peaks appear to be random and could not be assigned to the cross-linked peptide pair.

butyrylcholinesterase has been reported.[26] Adduct masses were consistent with a diethoxyphosphate and with a diethoxythiophosphate. A diethoxythiophosphate adduct could arise from a direct nucleophilic attack of the active site serine on diazinon. The diethoxyphosphate adduct could result from a thionothiolo rearrangement[27] followed by nucleophilic attack. Once formed, the diethoxyphosphate adduct would be expected to promote cross-linking, just like it does when chlorpyrifos oxon is the initial reactant (see Figure 1).

4.3. Cross-Linking of Tubulin. Most of the isopeptide cross-links we found from MAP-tubulin were in alpha and beta tubulin. In α-1A tubulin, Lys 163, Lys 336, and Asp 98 appeared in isopeptide cross-links. In β-4B tubulin, Glu 158, and Lys 115 appeared in isopeptide cross-links.

Chemical cross-linking also occurred at these residues. For example, reaction of tubulins alpha and beta with disuccini-

midyl suberate caused cross-linking at Lys 163 and Lys 336 in alpha tubulin. Reaction with 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide cross-linked Asp 218 and Lys 163 in alpha tubulin. Succinimidyl 6-(4,4′-azipentanamido) hexanoate cross-linked Lys 163 from alpha tubulin with Lys 163 from beta tubulin, and it cross-linked Lys 154 to Glu 158 in beta tubulin.[28] Incubation of Tau with microtubules in the presence of bis(sulfosuccinimidyl)suberate resulted in covalent binding of Tau to Lys 336 and Lys 338 of alpha tubulin.[29] Thus, Lys 163 and Lys 336 in alpha tubulin appear to be particularly prone to cross-linking.

A curious observation can be drawn from Table 1. Chlorpyrifos oxon, paraoxon, and diazoxon yield different cross-linked peptide pairs despite the fact that all three generate the same lysine-adduct. Rationalization of this observation is speculative. Tubulin is a dynamic molecule,
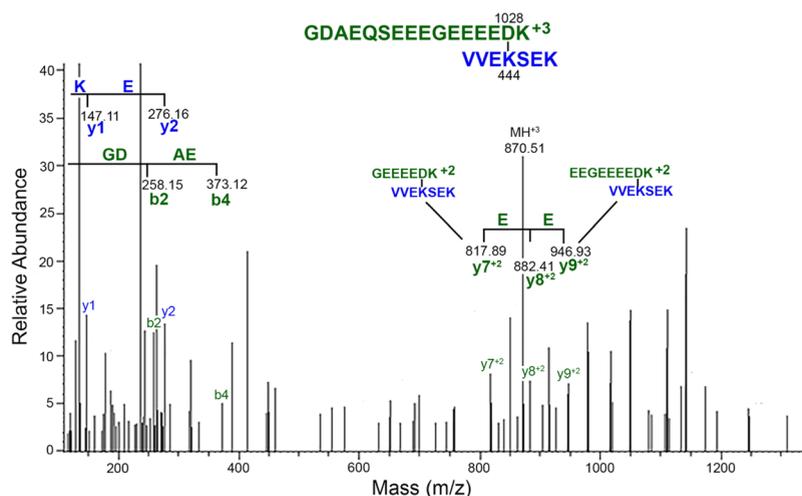
Figure 7. MSMS spectrum for the dichlorvos-induced, cross-linked peptide GDAEQSEEEGEEEED$_{1028}$K-VVEK$_{444}$SEK that is cross-linked between D1028 and K444. The mass of the triply charged parent ion was 870.37 *m/z*. A b-ion sequence, GDAE, from the green peptide, and a y-ion amino acid pair, EK, from the blue peptide are present. A doubly charged cross-link specific sequence, EE (817.89−946.93 *m/z*), from the green peptide was present. Some of the unlabeled peaks correspond to loss of H$_2$O, NH$_3$, or CO from the labeled masses, but most of the major unlabeled peaks fit to a contaminant sequence, WEEQ/KW, that is consistent with immunoglobulin heavy chain, junction region.

prone to assuming multiple isoforms. One might propose that the different structures of chlorpyrifos oxon, paraoxon, and diazoxon select different isoforms of tubulin for reaction. Thus, different cross-link partners become available for reaction with the lysine adduct.

The locations of the tubulin cross-link residues were determined from the crystal structures of alpha and beta tubulin. All of the cross-linked locations were on the surface, making them readily available for reaction with other proteins.

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Notes

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to ProteomeXchange Consortium[30] via the PRIDE[31] partner repository with the dataset identifier PXD034529.

ABBREVIATIONS

LC-MSMS, liquid chromatography tandem mass spectrometry; MSMS spectrum, fragmentation mass spectrum; OP, organophosphorus toxicants; CPO, chlorpyrifos oxon; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MAP, microtubule associated proteins; NCBI, National Center for Biotechnology Information; NFH, neurofilament heavy polypeptide

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