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Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine

Hasmik Grigoryan[a], **Bin Li**[a], **Erica K. Anderson**[a], **Weihua Xue**[a], **Florian Nachon**[b], **Oksana Lockridge**[a], and **Lawrence M. Schopfer**[a,*]

a *Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198, hgrigoryan@berkeley.edu, binli@unmc.edu, ericaanderson@mail.unomaha.edu, weihuaxue@gmail.com, olockrid@unmc.edu*

b *Unité d'Enzymologie, Département de Toxicologie, Centre de Recherches du Service de Santé des Armées (CRSSA), 24 avenue des Maquis du Grésivaudan, 38702 La Tronche, France, fnachon@crssa.net*

Abstract

Organophosphorus esters (OP) are known to bind covalently to the active site serine of enzymes in the serine hydrolase family. It was a surprise to find that proteins with no active site serine are also covalently modified by OP. The binding site in albumin, transferrin, and tubulin was identified as tyrosine. The goal of the present work was to determine whether binding to tyrosine is a general phenomenon. Fourteen proteins were treated with a biotin-tagged organophosphorus agent called FP-biotin. The proteins were digested with trypsin and the labeled peptides enriched by binding to monomeric avidin. Peptides were purified by HPLC and fragmented by collision induced dissociation in a tandem ion trap mass spectrometer. Eight proteins were labeled and six were not. Tyrosine was labeled in human alpha-2-glycoprotein 1 zinc-binding protein (Tyr 138, Tyr 174, Tyr 181), human kinesin 3C motor domain (Tyr 145), human keratin 1 (Tyr 230), bovine actin (Tyr 55, Tyr 200), murine ATP synthase beta (Tyr 431), murine adenine nucleotide translocase 1 (Tyr 81), bovine chymotrypsinogen (Tyr 201) and porcine pepsin (Tyr 310). Only 1–3 tyrosines per protein were modified, suggesting that the reactive tyrosine was activated by nearby residues that facilitated ionization of the hydroxyl group of tyrosine. These results suggest that OP binding to tyrosine is a general phenomenon. It is concluded that organophosphorus-reactive proteins include not only enzymes in the serine hydrolase family, but also proteins that have no active site serine. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure. Another application is in the search for biomarkers of organophosphorus agent exposure. Previous searches have been limited to serine hydrolases. Now proteins such as albumin and keratin can be considered.

Keywords

FP-biotin; Organophosphorus Agent; Tyrosine; Non-cholinesterase; Mass Spectrometry

*[a] To whom correspondence should be addressed. Lawrence M. Schopfer, University of Nebraska Medical Center, Eppley Institute, 986805 Nebraska Medical Center, Omaha, Nebraska 68198-6805, Phone 402-559-6014, Fax 402-559-4651, Email E-mail: lmschopf@unmc.edu.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

1. Introduction

Organophosphorus agents (OP) are used as insecticides, fuel additives, plasticizers, lubricants, flame retardants, and chemical warfare agents [1,2]. These compounds are toxic to insects, fish, birds and mammals. Exposure can lead to a variety of symptoms culminating in seizures, respiratory arrest and death in acute cases [3]. The traditional targets for organophosphorus agents have long been considered to be the active site serine residues in acetylcholinesterase and butyrylcholinesterase. It is generally accepted that covalent inhibition of acetylcholinesterase is responsible for most of the clinically relevant symptoms observed upon high dose exposure to OP [3]. However, evidence has been accumulating over the past several years that suggests cholinesterases are not the only clinically relevant targets for OP, especially during low-dose exposure [1,2,4,5].

Investigations in several laboratories have been directed at identifying other proteins with which OP can react covalently. In addition to a variety of serine hydrolases [2,6], reactions of OP with other classes of enzymes and with receptors have been reported [2,7]. Results from our laboratory have demonstrated that non-enzymatic proteins such as transferrin [8], serum albumin [9–11] and tubulin [12] can be covalently modified by OP. For these latter proteins, the reactive amino acid is tyrosine.

Reaction of the organophosphorus agent diisopropylfluorophosphate (DFP) with a tyrosine residue in human serum albumin (and in bovine serum albumin) was reported by Sanger in 1963 [13]. Between 1965 and 1971, DFP was shown to react with tyrosine residues on bromelain [14], papain [15], and lysozyme [16]. These findings were consistent with the known reactivity of tyrosine with organophosphorus agents [17]. Interest in the reaction of OP with protein-bound tyrosyl residues appears to have waned after 1970. However, starting in 1999, a resurgence of interest was re-kindled with the recognition that serum albumin provided an alternative to butyrylcholinesterase as a biological marker for exposure to OP [18]. Researchers responsible for this work took advantage of improvements in mass spectrometry that simplified the identification of post-translational modifications on proteins. Subsequent studies on serum albumin 1) identified Tyr411 as the most reactive tyrosine residue on human serum albumin, confirming Sanger's assignment [9]; 2) demonstrated that multiple tyrosine residues from albumin could react with OP [19]; and 3) showed that OP-labeled tyrosine could be detected in rodents that had been treated with sub-lethal doses of OP [11] including nerve agents [20].

To our knowledge, reaction of OP with tyrosyl residues has not been confirmed for any proteins other than those mentioned. If this reaction is wide spread, as we suspect, then it opens a new arena for investigation when considering intoxication due to OP exposure. Reactions with tyrosine could be responsible for intoxication that is not consistent with inhibition of acetylcholinesterase. Demonstrating that tyrosine residues from a wide variety of proteins will react with OP is an essential step for the development of this concept.

In this work, we have expanded the list of proteins that react with OP at tyrosine to include: human alpha-2-glycoprotein 1 zinc, human kinesin 3C, human keratin 1, bovine actin, murine ATP synthase beta, murine adenine nucleotide translocase 1, bovine chymotrypsinogen and porcine pepsin. We suggest that covalent reaction of OP with tyrosine is a general phenomenon that can be expected to occur for a large number of proteins.

Our findings may have application to diagnosis and treatment of OP exposure. Proteins that have no active site serine may serve as biomarkers of exposure. In the future it may be possible to develop antibodies to the new OP-labeled biomarkers to use for screening OP exposure. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms of long-term effects of OP exposure.

2. Materials and Methods

2.1. Materials

Human alpha-2-glycoprotein 1 zinc was isolated from plasma. Human kinesin KIF3C motor domain was from Cytoskeleton Inc. (Denver, CO #KF01). Human epidermal keratin (#K0253), bovine actin (#A3653), bovine DNase (#D4527), porcine pepsin (#P6887), chicken lysozyme (#L6876), bovine RNase A (#R5125), bovine insulin (#I5500), diisopropylfluorophosphate (#D0879) and iodoacetamide (#I6125) were from Sigma (St. Louis, MO). Human IgG was from Fluka/Sigma (St. Louis, MO, #56834). ATP synthase beta and adenine nucleotide translocase 1 were isolated from a mouse-heart membrane preparation. Porcine gelatin was from USB (Cleveland, OH, #16045). Chymotrypsinogen was found as a component of the bovine DNase preparation. Sequencing grade modified trypsin (#V5113, porcine, reductively methylated, TPCK treated) was from Promega (Madison, WI). Dithiothreitol was from Fisher Biotech (Fair Lawn, NJ, #BP172-25, electrophoresis grade). Alpha-cyano-4-hydroxy cinnamic acid (CHCA) from Fluka (#70990) was recrystallized before use then dissolved to 10mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid. NeutrAvidin agarose beads were from Thermo Scientific (Rockford, IL, #29202). Monomeric avidin agarose beads were from Pierce (Rockford, IL, #20228)

FP-biotin was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana (Missoula, MT) [22]. Stock solutions of FP-biotin were made in dimethylsulfoxide and stored at −80°C.

2.2. Sample Preparation for mass spectrometry

Proteins were either purchased and were therefore relatively pure, or they were isolated from crude extracts. All pure proteins except kinesin were treated with FP-biotin by the following protocol. One mg/ml protein (approximately 10–25 μM) was dissolved in 10 mM ammonium bicarbonate, pH 8.3, containing 0.01% sodium azide. FP-biotin was added to a final concentration of 120 μM and the solution was incubated at 37°C for 24 hours. Negative controls were processed in the same manner as labeled samples except that FP-biotin was omitted. Samples were boiled for 10 minutes, reduced with 10 mM dithiothreitol for 2 hours at 60°C, alkylated with 50 mM iodoacetamide for 1 hour at room temperature in the dark, and dialyzed against 4 liters of 10 mM ammonium bicarbonate pH 8.3 for 24 hours at 8°C (with one change), and then digested with sequencing grade trypsin at a ratio of 1:100 (μg trypsin:μg sample) for 24 hours at 37°C. Digests were used directly for MALDI TOF TOF analysis or dried and resuspended in 5% acetonitrile, 0.1% formic acid for QTrap analysis.

Kinesin motor domain (1 mg or 13.3 nanomoles) was dissolved in 1 ml of 80 mM PIPES M ATP. FP-biotin buffer pH 7.0 containing 0.5 mM EGTA, 2 mM $MgCl_2$, 0.2 M NaCl and 20 μ (260 nanomoles) was added. The protein did not fully dissolve. The mixture was incubated at 37°C for 48 hours, with occasional mixing. The protein was denatured in 8 molar urea in the presence of 10 mM dithiothreitol and boiled for 3 minutes. The resultant solution was clear. Sulfhydryl groups were alkylated with 90 mM iodoacetamide at 37°C for 3 hours. The protein solution was freed of unbound FP-biotin and salts by dialysis against 4 liters of 25 mM ammonium bicarbonate, pH 8.5 for 36 hours at 4°C (with three changes). There were some particles in the preparation after dialysis. The preparation was then digested with trypsin (at a ratio of 50 to 1, μg protein to μg trypsin) for 11 hours at 37°C before a second aliquot of trypsin was added for another 14 hours of digestion. Trypsin was inactivated by reaction with 1 μl of 5.73 M diisopropylfluorophosphate overnight. Labeled peptides were enriched by extraction with monomeric avidin beads (1 ml, settled volume) equilibrated in 10 mM ammonium bicarbonate, pH 8.5. Loaded beads were washed with 10 ml of 1 M potassium phosphate, pH 7.5; 10 ml of 0.1 M Tris acetate, pH 8.6; and 30 ml of water before elution with 10% acetic

acid. The digest was examined with the MALDI TOF TOF 4800 mass spectrometer. Peptides that showed characteristic masses for FP-biotin under MSMS conditions were further purified by reverse phase HPLC (Phenomenex Prodigy 5 micron ODS column) using an acetonitrile/trifluoroacetic acid gradient. Fractions containing masses consistent with the putative FP-biotinylated peptides were dried, resuspended in 50% acetonitrile/0.1% formic acid, and infused into the QTrap 2000 mass spectrometer for improved MSMS fragmentation.

The FP-biotinylated tryptic peptide from human alpha-2-glycoprotein 1 zinc-binding protein was isolated from human plasma by extraction with NeutrAvidin Agarose beads as described [19].

Mouse heart membrane was prepared in 5 mM HEPES buffer, pH 7.4, from 5 mouse hearts as described [7]. The membrane preparation (at 2 mg/ml protein) was incubated with 100 μM FP-biotin at 37°C overnight, then it was denatured with 8 M urea, reduced with 10 mM dithiothreitol for 1 hour at 37°C, alkylated with 40 mM iodoacetamide for 1 hour at 37°C in the dark, and dialyzed against 10 mM ammonium bicarbonate buffer. Tryptic digestion was accomplished by incubation at 37°C overnight with Promega sequencing grade trypsin, at a trypsin to protein ratio of 1 to 37 (weight to weight). FP-biotinylated peptides were extracted from the digest with NeutrAvidin Agarose beads (1 ml settled volume) in 8 mM sodium phosphate buffer, pH 7.0, containing 155 mM sodium chloride (PBS). Beads were washed with 5–10 ml of PBS and then with 10 ml of water. Peptides were eluted with 50% acetonitrile/water containing 0.15% trifluoroacetic acid. It had been anticipated that this procedure would yield FP-biotinylated muscarinic receptor. However, the FP-biotinylated proteins identified in the preparation were ATP synthase beta and adenine nucleotide translocase I.

2.3. MALDI TOF TOF Analysis

Generally, 0.5–1 microliter of sample (approximately 20 pmole/μl for pure proteins, assuming no losses during processing) was air dried onto a 384 well Opti-TOF sample plate (Applied Biosystems, Foster City, CA, #1016491) and then overlaid with 1 μl of CHCA (10 mg/ml). Mass spectra and collision induced MSMS spectra were collected in positive ion reflector mode on a MALDI TOF TOF 4800 mass spectrometer (Applied Biosystems). The final spectrum was the average of 500 laser shots. The mass spectrometer was calibrated before each use with CalMix 5 (Applied Biosystems).

2.4. QTrap Analysis

For analysis using on-line HPLC separation of sample before introduction into the mass spectrometer, a 1 milliliter sample from the tryptic digest was dried by SpeedVac and redissolved in 0.4 ml of 5% acetonitrile containing 0.1% formic acid to yield approximately 3–5 pmole of peptide/μl, assuming no losses during processing. Ten microliters of this solution (30–50 pmole, assuming no losses during handling) were injected onto an HPLC nanocolumn (218MS3.07515 Vydac C18 polymeric reverse phase, 75 micron I.D. − 150 mm long; P.J. Cobert Assoc, St. Louis, MO). Peptides were separated with a 90 minute linear gradient from 5 to 60% acetonitrile at a flow rate of 0.3 μl/min and electrosprayed through a fused silica emitter (360 micron O.D., 75 micron I.D., 15 micron taper, New Objective, Woburn, MA) directly into the QTRAP 2000, a hybrid quadrupole linear ion trap mass spectrometer (Applied Biosystems). An ion-spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. Information dependent acquisition was used to collect MS, high resolution MS, and MSMS spectra. All spectra were collected in the enhanced mode, using the trap function. The three most intense MS peaks in each cycle with mass between 400 and 1700 *m*/*z*, charge of +1 to +4, and intensities greater than 10,000 cps were selected for high resolution MS and MSMS analysis. Precursor ions were excluded for 30 s after one MSMS spectrum had been collected. The collision cell was pressurized to 40 μTorr with pure nitrogen, and collision

energies between 20 and 40 eV were determined automatically by the software based on the mass and charge of the precursor ion. The mass spectrometer was calibrated on selected fragments from the MSMS spectrum of Glu-fibrinopeptide B.

The MSMS data were submitted to Mascot (Matrix Science, London, UK, http://www.matrixscience.com) for identification of labeled peptide [23]. The added mass of the FP-biotin (572 amu) has been incorporated into the UNIMOD database (http://www.unimod.org) for use as a variable modification in the Mascot algorithm. In addition, the MSMS chromatograms were searched with an extracted ion chromatographic protocol (Analyst 1.4.1 software, Applied Biosystems) for fragment ions at 329, 312 and 227 amu which are characteristic of the FP-biotin label [24]. Sequences of peptides identified by either procedure were confirmed manually.

3. Results

The structure of FP-biotin is shown in Figure 1. FP-biotin was used for these studies for two reasons. First, MSMS fragmentation creates fragments that are characteristic of FP-biotin. Characteristic fragments are found at 329, 312 and 227 amu (for fragmentation of FP-biotin at its internal amide linkages) [24]. In addition, tyrosine-FP-biotin immonium ion fragments are found at 708 amu (for the singly-charged immonium ion), 691 amu (for the immonium ion less amine), 355 amu (for the doubly charged immonium ion) and 346 amu (for the doubly charged immonium ion less amine). These ions provide a convenient, positive identification for the labeled peptides. They also can be used as target masses when the mass spectra from crude mixtures are searched for the labeled peptides using extracted ion techniques. Second, avidin agarose can be used to concentrate and enrich the biotinylated-peptides from crude mixtures.

In the current study, eleven peptides from eight proteins were found to be labeled (see Table 1). The parent ion mass of each peptide was consistent with a covalent reaction of FP-biotin with some residue on that peptide (added mass of 572 amu). As it turned out, each peptide was labeled on tyrosine. The labeled residue for each peptide was confirmed by manual analysis of the MSMS spectra, with the assistance of the MS-Product algorithm for assignment of internal fragments (University of California, San Francisco, http://prospector2.ucsf.edu). Four representative MSMS spectra are presented.

Figure 2 shows the MSMS spectrum of QYY*TVFDR from porcine pepsin where FP-biotin is on Tyr 310. A 5-residue y-ion sequence could be extracted which included the C-terminal arginine. The labeled tyrosine was part of an unresolved three amino acid y-ion fragment at the N-terminus. This three amino acid fragment contained two tyrosines. Fortunately, masses consistent with the unlabeled a2-ion (QY = 264.0 amu) and its deaminated consort at 247.0 amu appeared in the spectrum. Thus, by a process of elimination the label could be assigned to the tyrosine in position three from the N-terminus. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal fragments. Characteristic fragments from FP-biotin appeared at 226.8, 312.1, 329.2, 708.2, 691.1, 354.7 and 346.0 amu.

Figure 3 shows the MSMS spectrum of GY*SFVTTAER from bovine actin where FP-biotin is on Tyr 200. The peptide was identified by an eight amino acid y-ion sequence. The labeled tyrosine appeared in all b-ions, for example in the singly-charged b2-ion at 793.2 amu and in the doubly-charged b2-ion at 397.3 amu. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal fragments. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 226.8, 312.2, 329.4, 708.4, 691.2, 355.0 and 346.3 amu.

Figure 4 shows the MSMS spectrum of THNLEPY*FESFINNLR from human keratin 1 where FP-biotin is on Tyr 230. The peptide was identified by an eight amino acid y-ion sequence and a six amino acid b-ion sequence. The label was found as part of the Pro Tyr pair (833.0 amu) at the N-terminus of the proline internal fragment. The majority of the remaining masses in the spectrum could be assigned to dehydration/deamination products or internal fragments. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 312.1, 329.0, 708.1 and 691.2 amu.

Figure 5 shows the MSMS spectrum of YY*YDGKDYIEFNK from human alpha-2-glycoprotein 1 zinc-binding protein where FP-biotin is on Tyr 138. Though the signal-to-noise ratio was low, the peptide could still be identified by a seven amino acid y-ion sequence and a four amino acid b-ion sequence. The minimum signal-to-noise ratio for the sequence peaks had an acceptable value of three. The label was found as part of a trimer at the N-terminus that consisted of three tyrosines. Analysis of the b-ion sequence demonstrated that the label was on the b2-ion (899.4 amu) and therefore had to be on one of the first two N-terminal tyrosines. Though the b2 peak had low intensity, its identity was confirmed by the a2-ion at 871.4 amu which was substantially more intense. Since it could not be determined which tyrosine actually carried the label, the second tyrosine was arbitrarily marked as labeled. Characteristic fragments from the internal amide bonds of FP-biotin appeared at 227.0, 312.0, 329.2, 708.5 and 691.3 amu.

Labeled amino acids in the seven remaining peptides were identified using similar analyses of their MSMS spectra. The label was found as one of the sequence fragments in peptide WEAEPVY*VQR. The labeled amino acid was found as part of the b2-ion in peptides AY*LEEECPATLR and Y*FPTQALNFAFK. For both sequences, the only reasonable candidate for labeling was the tyrosine. For peptide ILQDY*K, the labeled tyrosine was part of the y2-ion at the C-terminus. Again, the only reasonable candidate for labeling was the tyrosine. If the lysine had been labeled, it would not have been recognized for cleavage by trypsin. The labeled tyrosine was part of a y-ion dimer at the N-terminus of peptide Y*TNANTPDR and as part of an unresolved three amino acid y-ion fragment at the N-terminus of two sequences (ASY*LEIYQEEIR and DSY*VGDEAQSK). In principle, either serine or threonine might have been labeled rather than tyrosine in all three peptides. However, there are three lines of evidence to indicate that the label is on tyrosine rather than serine or threonine. First, there was no evidence for the 591 amu fragment that is characteristic of a serine label (and presumably of a threonine label) [24]. Second, OP-labeled serine (like phosphoserine) readily loses the OP-label in the mass spectrometer, under the CID conditions employed in these experiments. This fragmentation leaves a dehydroalanine residue in its stead. A similar loss for OP-threonine would be predicted to yield dehydro-threonine. The masses of the observed fragments were consistent with the presence of both the intact serine (or threonine) and the OP label. No masses consistent with the loss of OP and the presence of either dehydroalanine or dehydro-threonine were detected. Third, masses for both tyrosine-FP-biotin immonium ions and tyrosine-FP-biotin immonium ions minus amine were detected in all three MSMS spectra, indicating the presence of FP-biotinylated tyrosine. For these reasons, the label was assigned to tyrosine in each case.

Non-sequence fragments characteristic of FP-biotinylated tyrosine at 329, 312, 691, 708 and 355 amu masses appeared in all 7 MSMS spectra and the 346 amu mass in 5 spectra. The presence of these ions in the MSMS spectra confirmed the presence of FP-biotin on the peptides.

Not all of the proteins that we treated with FP-biotin yielded labeled peptides. Proteins for which we could find no FP-biotinylated peptides included porcine gelatin, bovine RNase, bovine DNase I, human IgG, chicken lysozyme, and bovine insulin.

4. Discussion

4.1. General phenomenon

We have previously demonstrated that tyrosine in transferrin, serum albumin and tubulin (both alpha and beta) react in vitro with a variety of organophosphorus agents (FP-biotin, chlorpyrifos-oxon, diisopropylfluorophosphate, dichlorvos, soman, and sarin) [8–10,12,19]. In addition, SDS PAGE experiments have demonstrated the presence of at least 50 FP-biotin reactive proteins in mouse brain supernatant, again in vitro [25]. Many of these latter proteins were identified as members of the serine hydrolase family. Such proteins are the expected targets for FP-biotin [26]. However, many were not members of that family [25].

The current experiments clearly demonstrate that the organophosphorus agent FP-biotin is capable of reacting with tyrosines on a variety of non-serine hydrolase proteins. By inference, other OP should react with these tyrosines as well.

With the addition of these proteins to those that have already been identified, it stands to reason that the reaction of protein-bound tyrosine with FP-biotin is a widespread phenomenon. On the other hand, not all tyrosines in a protein are reactive nor do all proteins contain reactive tyrosines.

4.2. Only certain tyrosines react with OP

The question of what determines the selectivity for the reaction of some tyrosines with FP-biotin arises. In order for a tyrosine to react with FP-biotin, it must satisfy two criteria. First, the phenolic oxygen of the tyrosine must be exposed to the medium. The necessity for this criterion is self-evident.

Second, the phenolic oxygen must be capable of nucleophilic attack. Reactions of OP typically involve attack by a nucleophile on the phosphorous to displace the most labile of the OP ligands [27]. For tyrosine to serve as a nucleophile, it must be deprotonated at the pH of the reaction. Ashbolt and Rydon [17] have demonstrated that tyrosine alone can react with diisopropylfluorophosphate (13 mM diisopropylfluorophosphate, 3 mM tyrosine, at pH 7.8 and 37°C for 24 hours). Despite the fact that the concentration of OP in their experiments was 100-fold higher than in ours they demonstrated the potential of tyrosine to react with OP.

The high concentrations of OP used by Ashbolt and Rydon were necessary because of the low reactivity of tyrosine under their conditions. This low reactivity can be attributed to the pKa of tyrosine. The pKa of tyrosine is 10.1 [28]. At pH 8.3, where our reactions were conducted, only 1% of the typical tyrosine would be deprotonated. To promote a nucleophilic reaction of protein-bound tyrosine with OP, some mechanism for increasing the amount of deprotonated tyrosine would be advantageous. A simple way to accomplish this objective would be to lower the pKa value of the tyrosine.

It has been demonstrated that interactions between histidine and cysteine provide a viable method for lowering the pKa of cysteine in proteins. Decreases of 4 to 5 pH units have been reported [29,30]. It has been proposed that the decrease in cysteine pKa is due to through-space, charge-charge interactions [31]. Lowered pKa values for tyrosine in human transferrin [32] and UDP-galactose 4-epimerase [33] also have been reported. The decrease in pKa was ascribed to interaction with nearby positively charged residues. In the case of UDP-galactose 4-epimerase, NAD^+ was implicated. For human transferrin, four tyrosines were found to have pKa values around 7. Neighboring lysines were implicated. We suggest that this sort of process may be involved in activating the FP-biotin reactive tyrosines in the peptides described in this paper.

4.3. Labeled peptides are hard to find; knowing what to look for helps

Our ultimate goal is to identify the proteins that are modified by low dose exposure in an animal, and eventually in humans. Organophosphorus pesticides have no tag to help identify the labeled peptide. The task is to find a labeled peptide based on its mass, starting from a mixture of 30,000 proteins which upon digestion with trypsin will yield at least a million peptides. This is a very difficult assignment. The work we are doing with pure proteins aims to make it possible. By knowing what we are looking for, we can reduce the complexity of the starting material. For example, the most OP-reactive protein in human plasma is butyrylcholinesterase. By purifying butyrylcholinesterase from plasma before beginning a search for the labeled peptide, one can successfully find the labeled peptide and identify the modifying agent [34, 35].

Covalent modification of butyrylcholinesterase and acetylcholinesterase does not explain cognitive impairment and depression following pesticide exposure [36–38]. Therefore we are searching for unknown proteins modified by OP. When we first identified FP-biotinylated albumin in mice treated with a nontoxic dose of FP-biotin [11], and identified Tyr 411 of human albumin as the site modified by OP [9], we thought the OP-tyrosine adduct on albumin was an exception. Only with study of additional proteins has it become clear that covalent binding of OP to tyrosine is common. This principle allows us to expand our search. One way we have applied this principle is by adding OP-bound tyrosine as a variable modification in the UNIMOD database (http://www.unimod.org) of the Mascot search engine (http://www.matrix-science.com); all Mascot users can now search for OP-tyrosine adducts.

Knowing the exact mass of candidate OP-labeled peptides helps us find the labeled peptide. Even after a protein has been partially purified from plasma or from brain, there are still thousands of peptides in the tryptic digest. In a mixture of peptides some peptides dominate in a process called ion suppression, making it impossible for other ions to ionize in the mass spectrometer. Often, the labeled peptide cannot be found. An example of the ion suppression problem and the difficulty of finding a labeled peptide is the fact that we could not find the labeled peptide in pure chicken lysozyme even though it is known that tyrosine in chicken lysozyme is labeled by OP [21]. The solution to the problem of ion suppression is to fractionate the peptides. We separate the peptides offline by HPLC and check each fraction by MALDI-TOF to identify the fraction that includes the mass of interest. This step requires that we know the mass we are looking for. This is where our studies with pure proteins are very helpful. The partially purified peptide is then subjected to LC/MS/MS which adds another liquid chromatography purification step before the peptide is fragmented in the mass spectrometer. We search the data for labeled peptides using Mascot software, but we also search the data manually for peptides that Mascot might have missed. Mascot generally does not report peptides that contain fewer than 5 amino acids in its search results. For example, the OP-labeled tryptic peptide of bovine albumin YTR, was not reported by Mascot but it was found by manual examination of the data [24]. This step requires that we know what we are looking for. In conclusion, the information obtained from a study of pure proteins labeled with OP is the basis for in vivo studies that aim to identify proteins modified by OP.

4.4. In vivo studies

The issue that we currently have under investigation is whether reaction of OP with proteins occurs in vivo at OP concentrations low enough that signs of cholinergic toxicity do not appear. We have already demonstrated that albumin and carboxylesterase from mouse plasma together with nine other unidentified proteins can be labeled in vivo by FP-biotin, at concentrations that do not significantly inhibit acetylcholinesterase [11]. The reactive residue on albumin is tyrosine [9,20], while that on carboxylesterase is the active site serine [39]. In vivo studies in guinea pigs treated with soman and tabun identified OP-tyrosine adducts on albumin [20]. In

vivo studies to search for other proteins modified by OP are underway. The work with pure proteins in this report will aid in the identification of proteins modified in vivo.

4.5. Significance

Our findings may have application to diagnosis of OP exposure. Proteins that have no active site serine may serve as biomarkers of exposure. In the future it may be possible to develop antibodies to new OP-labeled biomarkers to use for screening OP exposure. The recognition of a new OP-binding motif to tyrosine suggests new directions to search for mechanisms to explain cognitive deficits and depression associated with exposure to OP.

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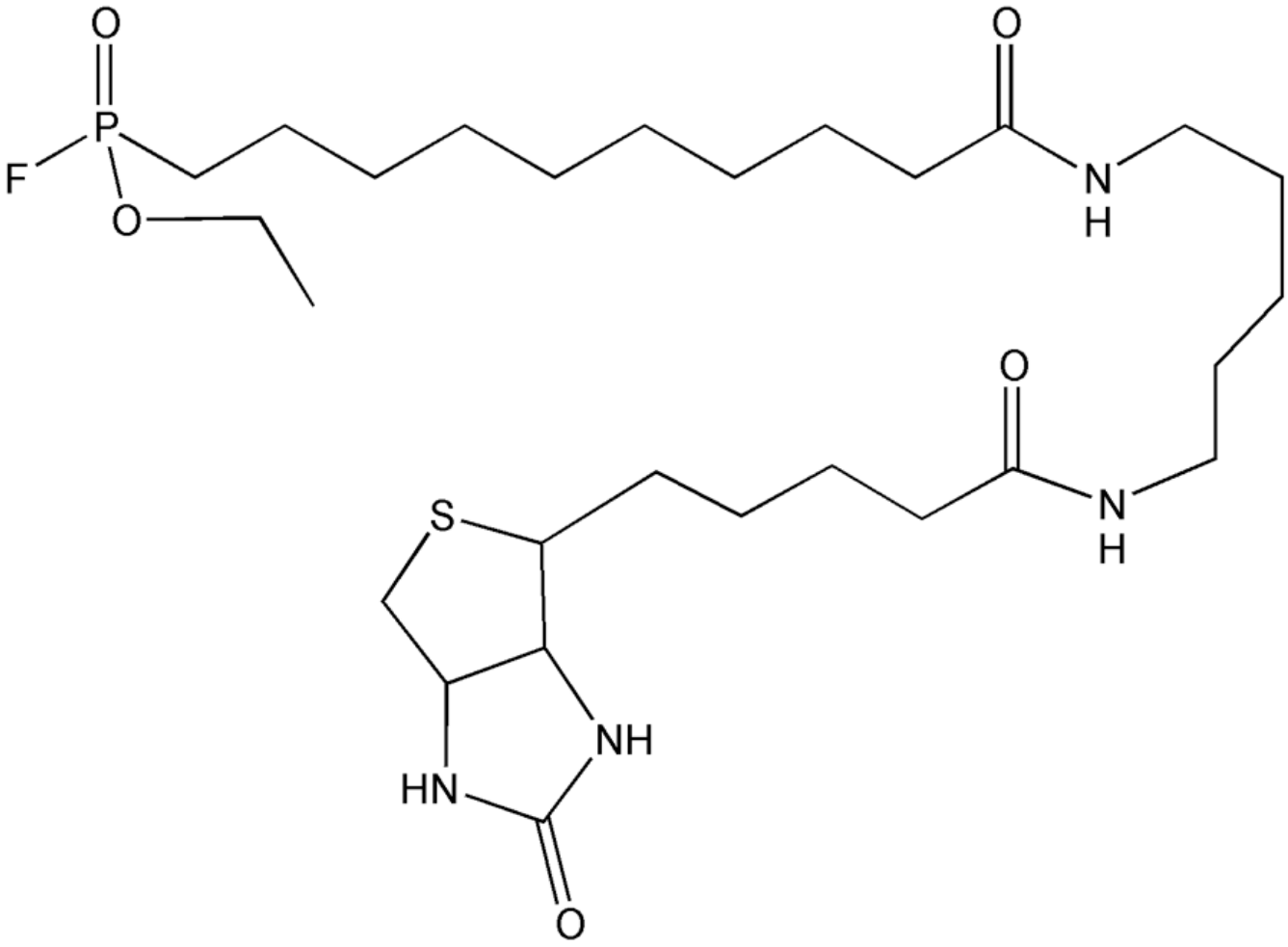

Figure 1.
The structure of FP-biotin, 10-(fluoroethoxyphosphinyl)-N-(biotinamidopentyl) decanamide.

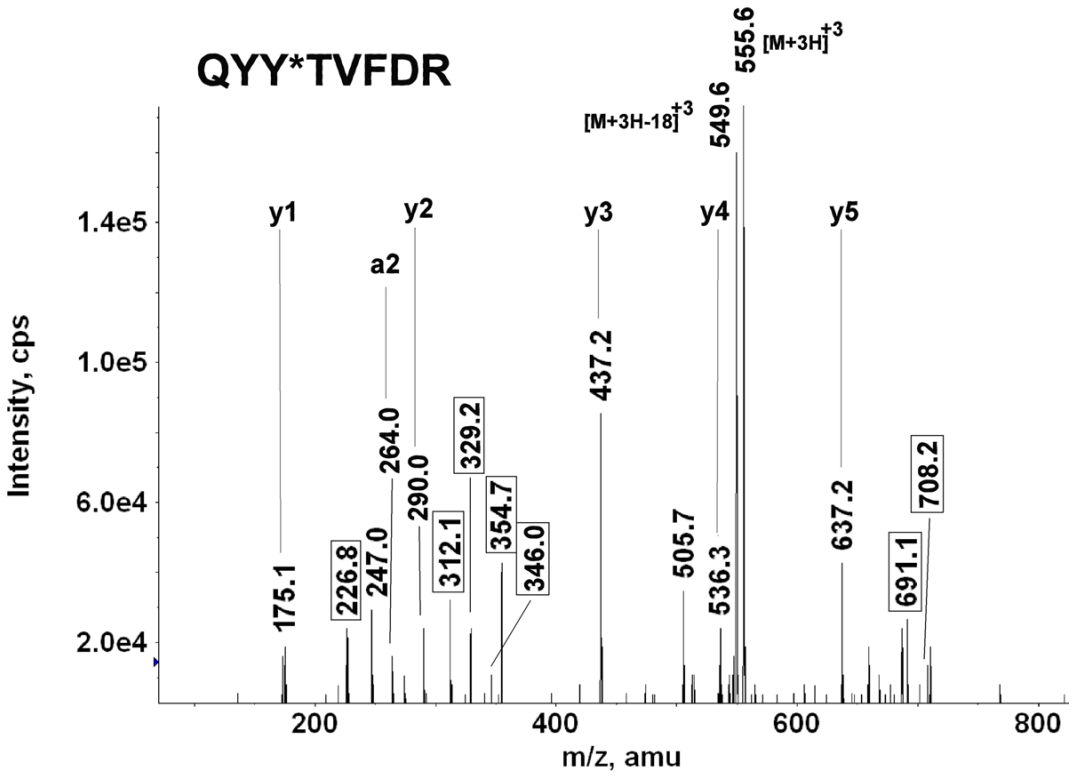

Figure 2.
The MSMS spectrum of peptide QYY*TVFDR from porcine pepsin (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply-charged parent ion has a mass of 555.6 amu.

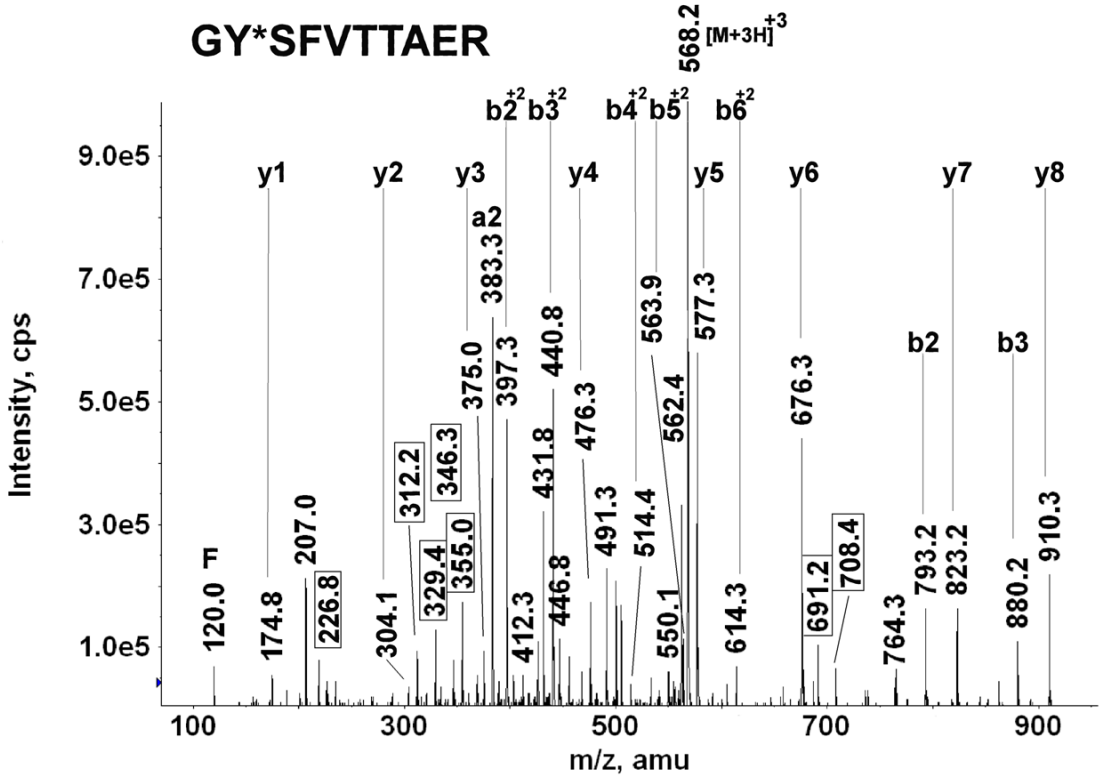

Figure 3.

The MSMS spectrum of peptide GY*SFVTTAER from bovine actin (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply-charged parent ion has a mass of 568.2 amu.

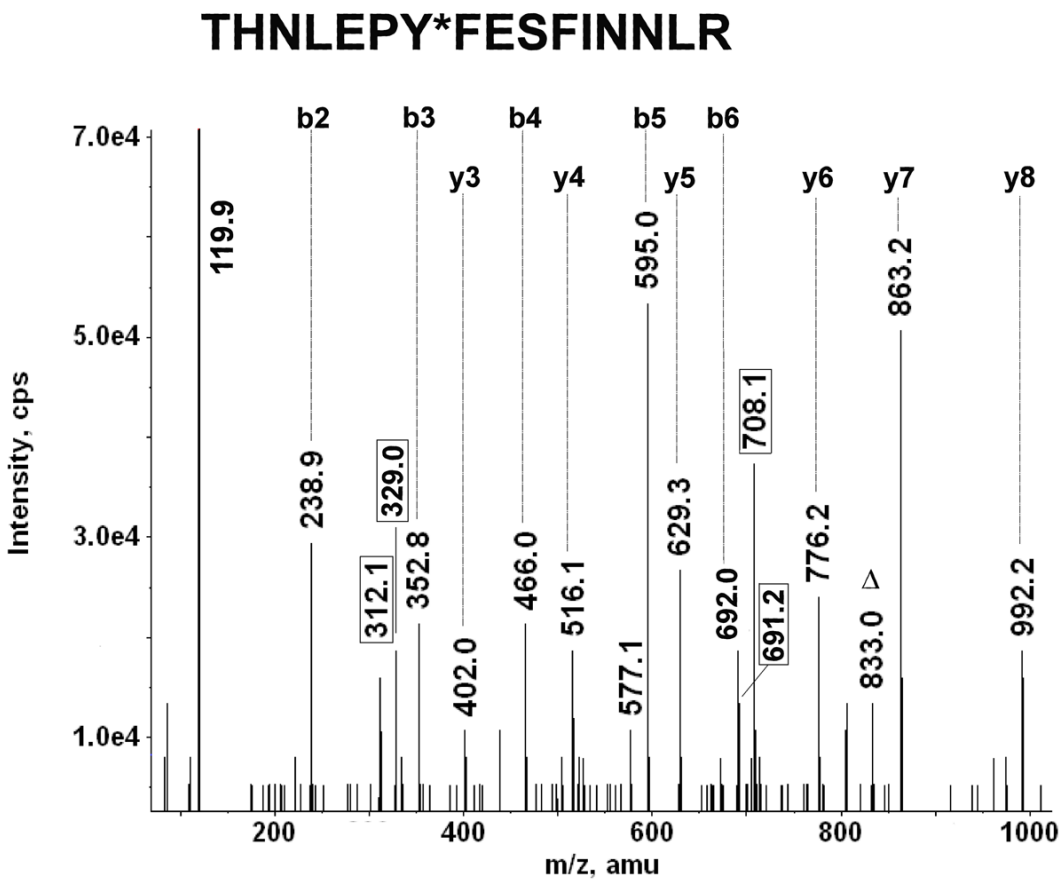

Figure 4.

The MSMS spectrum of peptide THNLEPY*FESFINNLR from human keratin 1 (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The mass marked with a triangle (833.0 amu) is the N-terminus of a proline internal fragment. The triply-charged parent ion has a mass of 642.6 amu which does not appear in the spectrum.

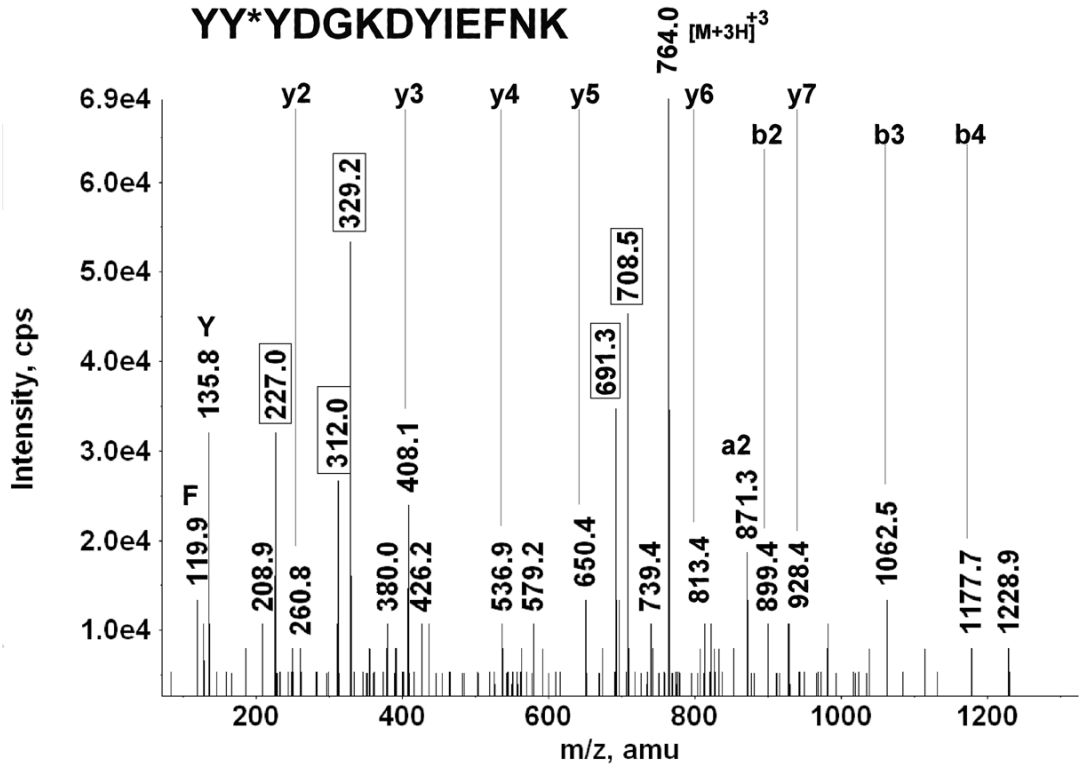

Figure 5.

The MSMS spectrum of peptide YY*YDGKDYIEFNK from alpha-2-glycoprotein 1 zinc binding protein (where Y* is the labeled tyrosine). The boxed masses identify the non-sequence, characteristic fragments from FP-biotin. The triply-charged parent ion has a mass of 764.0 amu.

Table 1

FP-biotin labeled peptides

Species	Protein	accession number gi	Peptide Sequence [a]	Tyr	FP-biotin characteristic ions				
					329	312	227	691	708
Human	alpha-2-glycoprotein 1 zinc	52790422	WEAEPVY*VQR	174	X	X	X	X	X
	alpha-2-glycoprotein 1 zinc	52790422	AY*LEEECPATLR	181	X	X	-	X	X
	alpha-2-glycoprotein 1 zinc	52790422	YY*YDGKDYIEFNK	138	X	X	X	X	X
	Kinesin 3C	41352705	ASY*LEIYQEEIR	145	X	X	X	X	X
	Keratin 1	119395750	THNLEPY*FESFINNLR	230	X	X	-	X	X
Bovine	Actin	62287933	DSY*VGDEAQSK	55	X	-	-	X	X
	Actin	62287933	GY*SFVTTAER	200	X	X	X	X	X
	Chymotrypsinogen	194674931	Y*TNANTPDR	201	X	X	X	X	X
Mouse	ATP Synthase beta	20455479	ILQDY*K	431	X	X	X	X	X
	Adenine Nucleotide Translocase 1	902008	Y*FPTQALNFAFK	81	X	X	X	X	X
Porcine	Pepsin	13096225	QYY*TVFDR	310	X	X	X	X	X

[a]The asterisk (*) indicates the labeled tyrosine. The residue number of the labeled tyrosine in the protein sequence is given. X indicates the presence of FP-biotin characteristic ions in MSMS spectra of the labeled peptide.