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Cresyl saligenin phosphate makes multiple adducts on free histidine, but does not form an adduct on histidine 438 of human butyrylcholinesterase

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Abstract

Cresyl saligenin phosphate (CBDP) is a suspected causative agent of “aerotoxic syndrome”, affecting pilots, crew members and passengers. CBDP is produced *in vivo* from *ortho*-containing isomers of tricresyl phosphate (TCP), a component of jet engine lubricants and hydraulic fluids. CBDP irreversibly inhibits butyrylcholinesterase (BChE) in human plasma by forming adducts on the active site serine (Ser-198). Inhibited BChE undergoes aging to release saligenin and *o*-cresol. The active site histidine (His-438) was hypothesized to abstract *o*-hydroxybenzyl moiety from the initial adduct on Ser-198. Our goal was to test this hypothesis. Mass spectral analysis of CBDP-inhibited BChE digested with Glu-C showed an *o*-hydroxybenzyl adduct (+106 amu) on lysine 499, a residue far from the active site, but not on His-438. Nevertheless, the nitrogen of the imidazole ring of free L-histidine formed a variety of adducts upon reaction with CBDP, including the *o*-hydroxybenzyl adduct, suggesting that histidine-CBDP adducts may form on other proteins.

Keywords

butyrylcholinesterase; histidine; TCP; CBDP; aging; *o*-hydroxybenzyl

1. Introduction

Exposure to a component of jet-engine oil, tricresyl phosphate (TCP), through leaky jet engine oil seals is hypothesized to cause “aerotoxic syndrome”, the common symptoms of which include dizziness, headache, nausea, disorientation, blurred vision, short-term memory loss, cognitive dysfunction and sleep disorders [1–3]. The toxicity of *ortho*-isomers of TCP is associated with its active metabolite cresyl saligenin phosphate (CBDP) [4].

Butyrylcholinesterase (BChE) is an excellent biomarker of exposure to tri-*o*-cresyl phosphate, and presumably to other *ortho*-containing TCP isomers. Recently we have found phosphoserine adducts formed by CBDP on plasma BChE of jet airplane passengers [5]. This finding has proven that exposure to *ortho*-isomers of TCP occurs on-board aircraft [5].

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In the present study we investigated the mechanism of reaction between human BChE and CBDP.

CBDP reacts rapidly with BChE inhibiting its enzymatic activity and forming organophosphorylated adducts on the active site serine (Ser-198). The initial adduct (a ring-opened adduct) undergoes two sequential hydrolysis reactions (“aging”) to release saligenin and *o*-cresol, leaving phosphate on the serine (Scheme 1) [6,7]. In 1979, R. Toia and J. Casida proposed a mechanism for aging of serine hydrolases inhibited with saligenin cyclic phosphorus esters [8]. According to their hypothesis histidine from the catalytic triad attacks the benzylic carbon of the initial ring-opened adduct, which results in transfer of the *o*-hydroxybenzyl moiety to the histidine to form *o*-hydroxybenzyl adduct. The plausibility of this hypothesis was supported by our finding of *o*-hydroxybenzyl adduct on free L-histidine upon reaction with CBDP. Our goal was to investigate the involvement of histidine in the aging of organophosphorylated BChE. This investigation is important for understanding the mechanism of aging of CBDP-BChE.

2. Materials and methods

2.1. Materials

2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide or cresyl saligenin phosphate (CBDP; CAS 1222-87-3) 99% pure was custom synthesized by Starks Associates (Buffalo, NY, USA). CBDP was dissolved in acetonitrile to 100 mM and stored at -80°C . The following were from Sigma-Aldrich, St. Louis, MO: S-butyrylthiocholine iodide (B-3253), 5,5'-dithiobis(2-nitrobenzoic acid) (D-8130), L-histidine (H-8125), L-alanine (A-7627), formic acid (Fluka 94318), and 2,5-dihydroxybenzoic acid (DHB) (Fluka 85707 and Acros 165200050). 20 mg/ml DHB matrix was prepared in 50% acetonitrile, 0.1% trifluoroacetic acid, 1% phosphoric acid and stored at -20°C . The following were from Fisher Scientific, Fair Lawn, NJ: trifluoroacetic acid (A11650), and acetonitrile (BP1170-4). Endoproteinase Glu-C (LS02128) was from Worthington Biochemical Corporation, Lakewood, NJ. Amicon stirred cell 10 ml capacity (model 8010) with YM30 membrane (13712) was from Millipore, Billerica, MA. Butyrylcholinesterase was purified from outdated human plasma as described [9].

2.2. Reaction of CBDP with free amino acids

Stock solutions of 100 mM L-histidine and L-alanine were prepared in 100 mM potassium phosphate buffer (pH 7.8). CBDP (1 mM) was incubated with 1 mM free amino acid in 10 mM potassium phosphate buffer (pH 7.8) at room temperature with constant rotation. After 24 hours, 1 μl aliquots were mixed with 1 μl DHB matrix and analyzed with a MALDI TOF/TOF mass spectrometer.

2.3. Reaction of butyrylcholinesterase with CBDP and digestion with Glu-C

Seven ml of 3 mg/ml BChE (245 nmol) in 10 mM potassium phosphate buffer pH 7.4 containing 0.02% sodium azide was mixed with 70 μl of 100 mM CBDP (7000 nmol) and incubated for 5 min at room temperature. After 5 min the BChE activity was completely inhibited. Butyrylcholinesterase activity was measured with 1 mM butyrylthiocholine as substrate and 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M potassium phosphate buffer pH 7.0 at 25°C , as described [10]. The incubation mixture was dialyzed in a 10 ml Amicon stirred cell against 10 mM ammonium bicarbonate pH 8.0 to remove excess CBDP, change the buffer and concentrate the sample to 1 ml. The 1 ml sample in 10 mM ammonium bicarbonate pH 8.0 was denatured in a boiling water bath for 10 minutes, mixed with 0.5 mg Glu-C (1:40 weight ratio of Glu-C to BChE) and digested overnight at 37°C . One μl of protein digest was diluted 1:10 in water, then 1 μl of the diluted digest was mixed

with 1 μ l DHB matrix and analyzed in a MALDI-TOF/TOF mass spectrometer. The protein digest was diluted with water to 2 ml to decrease viscosity and filtered through an Ultrafree-MC Millipore centrifugal filtration system (10 kDa cut off). A 5 μ l aliquot of the filtered sample was analyzed in the LTQ-Orbitrap mass spectrometer.

2.4. HPLC purification of the active site histidine peptide WMGVMHGYE

Peptides in 1.5 ml of the filtered Glu-C digest of CBDP-treated BChE were purified on a Waters 625 LC system as described [11]. The active site histidine containing peptide, WMGVMHGYE m/z 1109.3, eluted between 23–24 min. This fraction was dried in the SpeedVac, dissolved in 50 μ l of 0.1% formic acid and analyzed in the LTQ-Orbitrap mass spectrometer.

2.5. Analysis in a MALDI-TOF/TOF 4800 mass spectrometer

Essentially salt-free 1- μ l samples were spotted onto a 384-well Opti-TOF sample plate (cat. no. 1016491, Applied Biosystems, Foster City, CA, USA), dried in air, and overlaid with 1 μ l of DHB matrix. MALDI mass spectra were acquired on a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA) as described [11].

2.6. LC/MS/MS with the LTQ-Orbitrap mass spectrometer

Glu-C protein digests were dried in a vacuum centrifuge and dissolved in 0.1% formic acid to make a 1 pmol/ μ l solution. A 5- μ l aliquot was injected into the LC system of the LTQ-Orbitrap mass spectrometer and analyzed as described [12].

3. Results and discussion

3.1. Reaction of CBDP with free histidine produces multiple species

To determine the nature of the adducts formed by CBDP on histidine, 1 mM CBDP was reacted with 1 mM L-histidine and analyzed by mass spectrometry. The MALDI mass spectrum in Figure 1 indicates the presence of protonated histidine (m/z 156.0), and the protonated (m/z 277.0) and K^+ forms of CBDP (m/z 314.9 with potassium ion). In addition to free reactants, several products of reactions between CBDP and histidine are present (at m/z 262.1, 368.1, 432.1 and 474.1). Chemical structures for the new products are in Figure 1. Each structure was supported by MS/MS fragmentation of the corresponding parent ion. We have arbitrarily chosen N-3 of the imidazole ring as the linkage site in our illustrations. However, either the N-1 or N-3 nitrogen could be adducted. The pK_a value of the histidine side chain is 6.04. Thus, at pH 7.8 the imidazole ring is essentially neutral and both N-1 and N-3 possess a free electron pair to be used in nucleophilic attack on CBDP.

It is conceivable that the α -amino group of free histidine could react with CBDP, rather than the imidazole. To test this hypothesis, free alanine was treated with CBDP using the same conditions that were used for the histidine-CBDP reaction. With alanine, the α -amine is the only potential nucleophile. After 24 hours no peaks consistent with CBDP-alanine adducts were detectable in the mass spectrum strongly arguing that the α -amino group is not reactive under these conditions.

Scheme 2 summarizes the reaction of histidine with CBDP. Note that the masses are for the neutral charge state. Nucleophilic attack of histidine on the benzyl position of CBDP results in the ring-opened CBDP-histidine adduct (added mass of 276 amu, reaction A). Hydrolysis of the latter liberates *o*-cresyl phosphate and leaves *o*-hydroxybenzyl attached to histidine (added mass of 106 amu). Further attack of the free hydroxyl of *o*-hydroxybenzyl-histidine adduct on the benzylic carbon of another *o*-hydroxybenzyl-histidine gives rise to concatenated-[2]-*o*-hydroxybenzyl-histidine (reaction B). The reaction proceeds to form

concatenated-[3]-*o*-hydroxybenzyl-histidine, where each consecutive *o*-hydroxybenzyl moiety is attached through a C-O bond (reaction C). This process is somewhat similar to the polymerization reaction of cyclic ϵ -caprolactone and L,L-lactide initiated by free amino acids [13].

Interestingly, the *o*-cresyl phosphate (+170 amu) adduct was not formed in the reaction of CBDP with free histidine. However, formation of the *o*-cresyl phosphate adduct was observed in the reaction with free tyrosine [6], where the initial adduct formation involved nucleophilic attack on the phosphorus of CBDP. This further confirms that the initial attachment of CBDP to histidine occurs through the benzylic carbon, but not through phosphorus.

3.2. Mechanism of aging of the human butyrylcholinesterase-CBDP adduct does not involve the formation of *o*-hydroxybenzyl adduct on the active site histidine

We have previously shown that human BChE reacts rapidly with CBDP to form covalent adducts on the active site serine (Ser-198) [6,7]. Scheme 1 shows major steps of aging for the initial CBDP ring-opened adduct on Ser-198. The initial adduct (added mass of 276 amu) loses saligenin to yield the *o*-cresyl phosphoserine adduct (added mass of 170 amu). Further hydrolysis liberates *o*-cresol leading to the phosphoserine adduct (+80 amu). The *o*-cresyl phosphoserine and phosphoserine adducts were proven by mass spectral analysis [6]. The formation of the *o*-cresyl phosphoserine adduct (F. Nachon, unpublished data) and a final phosphoserine adduct were also observed in crystallography studies [7].

In 1979, R. Toia and J. Casida studied the mechanism of aging for chymotrypsin and trypsin inhibited by saligenin cyclic phosphorus esters [8]. They found that after the reaction was complete, saligenin remained covalently bound to the protein (approximately 20% of the amount of phosphoenzyme). Thus, they proposed that histidine in the catalytic triad attacks the benzylic carbon of the ring-opened adduct on the active site serine and traps saligenin by forming *o*-hydroxybenzyl-adduct. The catalytic triad of human BChE is similar to that of chymotrypsin and trypsin and consists of Glu-325, Ser-198 and His-438. It was therefore possible that His-438 of BChE would be modified by saligenin. To test this hypothesis 21 mg of human BChE were treated with CBDP to achieve a 1:28 molar ratio of protein to inhibitor. The high excess of CBDP was used to ensure that the active site serine as well as other reactive sites (if any) would be labeled. CBDP-treated BChE was digested with endoproteinase Glu-C. *In silico* digestion of human BChE (accession number P06276 in the SwissProt database) with Glu-C predicted that the active site histidine peptide would have the sequence WMGVMH₄₃₈GYE and a monoisotopic mass of 1109.45 m/z.

Analysis of the Glu-C digest of CBDP-treated BChE with MALDI mass spectrometry revealed the presence of a peak at m/z 1109.5. MS/MS fragmentation suggested that this signal was from a mixture of two different BChE peptides: WMGVMHGYE and QKYLTNLNTE. We were able to separate the two peptides by offline HPLC: QKYLTNLNTE eluted between 19–20 min, while the active site histidine peptide WMGVMHGYE eluted between 23–24 min (Figure 2, panel A and B). The identity of the peptides in each fraction was confirmed by MALDI MS/MS fragmentation.

Figure 2.B. shows a peak at m/z 1215.5 consistent with the mass of *o*-hydroxybenzyl adduct (added mass of 106 amu) on either WMGVMHGYE or QKYLTNLNTE. LC/MS/MS analysis of the 23–24 min fraction, as well as of the whole digest revealed that the *o*-hydroxybenzyl adduct was on QKYLTNLNTE. The MS/MS fragmentation spectrum of the doubly-charged parent ion at m/z 608.3 is shown in Figure 2, panel C. The spectrum confirms the identity of this peptide as QK*YLTNLNTE and proves that the adducted residue is Lys-499. Supporting ions are y₂, y₃–18 (loss of water), y₅, b₂*/y₃, b₃*, b₄*, b₅*, b₆*/y₇, b₇*, as well as b ions

that have lost ammonia (b^*-17). The asterisk indicates ions retaining *o*-hydroxybenzyl. The presence of the added 106 amu mass on the b_2 ion (QK) clearly establishes the location of the adduct. Thus, it was concluded that the *o*-hydroxybenzyl adduct was formed on Lys-499. It is not feasible that the *o*-hydroxybenzyl moiety was transferred from the CBDP-adduct on the active site serine to Lys-499 because the distance between the active site and Lys-499 is 29 Å.[7] Therefore, a direct reaction of CBDP with lysine is indicated.

No evidence was found for an adduct on the active site histidine peptide. It is possible that the geometry of the CBDP-adduct on Ser-198 of BChE impedes the reaction between CBDP and His-438 of BChE rather than promoting it. Since we did find *o*-hydroxybenzyl adducts on free L-histidine, it is still possible that the catalytic histidine in chymotrypsin and trypsin does react with the CBDP adduct on the active site serine to create an *o*-hydroxybenzyl-histidine adduct. Histidines in albumin react with CBDP to form *o*-hydroxybenzyl adducts [12].

Significance

In summary, we demonstrated the reactivity of CBDP with free histidine. If the same adducts form on proteins *in vivo*, they could induce antibodies. Antibodies could be used to track the modified proteins and therefore identify a mechanism of CBDP toxicity in aerotoxic syndrome.

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Abbreviations

BChE	butyrylcholinesterase
CBDP	2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide
TCP	tricresyl phosphate
DHB	2,5-dihydroxybenzoic acid
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
LTQ	linear trap quadrupole

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Highlights

- Free L-histidine makes multiple adducts with CBDP.
- His-438 of human BChE is not alkylated by CBDP.
- Lys-499 of human BChE forms *o*-hydroxybenzyl adduct upon reaction with CBDP.

Figure 1. MALDI mass spectrum of a mixture of histidine and CBDP after 24-h reaction in potassium phosphate buffer, pH 7.8. Peaks marked by masses represent reactants and reaction products. The structures are consistent with the indicated masses. Peak at m/z 107.2 corresponds to protonated *o*-cresol. Other major peaks (non-labeled) come from either the DHB matrix or represent K^+ forms of CBDP-histidine adducts.

Figure 2.

Panels A and B. MALDI mass spectra of Glu-C digest of CBDP-treated human BChE after HPLC purification. MS spectra were acquired in positive mode at 6000 V with DHB matrix. The values shown indicate monoisotopic masses. The amino acid sequences of the peptides were determined by manual inspection of the MS/MS fragmentation spectra, with the aid of the MS-Product algorithm from the Proteomics Toolkit from DB Systems Biology (<http://db.systemsbio.net:8080/proteomicsToolkit/FragIonServlet.html>). A mass at m/z 1215.5 (panel B) is consistent with the o-hydroxybenzyl adduct on lysine-499 in peptide QKYLTLNTE. **Panel C.** LTQ-Orbitrap MS/MS fragmentation of the parent ion [M+2H]²⁺ at m/z 608.3 yielded y- and b-ion series consistent with peptide QK*YLTLNTE. The asterisk * indicates +106 amu shift in ion mass as compared to native peptide ions.

Scheme 1.

Reaction of CBDP with butyrylcholinesterase. The masses are for the neutral charge state.

Scheme 2.

Reaction of CBDP with histidine. The masses are for the neutral charge state.