

# 2,5-Hexanedione-Induced Adduction of Bovine Neurofilament Protein: Mass Spectrometric de Novo Sequencing, Identification of Endogenous and Exogenous Covalently Modified Sites

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## Overview

## Introduction

## Methods

## Conclusion

### Purpose:

• To understand and elucidate mechanisms of protein alkylation by neurotoxic *n*-hexane metabolite

### Methods:

- Peptide mapping (ESI MS)
- HPLC-MALDI-TOF-MS off line
- FAB MS
- NMR spectroscopy

### Results:

- The identification of previously unknown hexanedione-induced products under physiologically-relevant conditions.
- Investigations of the mechanistic implications of alkylation: double alkylation to form the isoindolium ion.
- *De Novo* sequencing to identify the partial sequence of NFH and sequence homologies/heterogeneities with other species.
- Identification of phosphorylation sites in NFH.

Over the past decades, there has been a debate about the mechanism for the peripheral neuropathy arising from repeated exposure to *n*-hexane. *n*-Hexane is metabolized mainly to 2,5-hexanedione (2,5-HD) by the P-450 system. 2,5-HD reacts with neurofilament proteins (NFs) in nerve fiber axons, which undergo retrograde degeneration resulting in extremity weakness and sensory loss in humans and other mammals [1]. It has been widely assumed that 2,5-HD reacts with  $\epsilon$ -amino groups of the lysines (K) of NFs to form 2,5-dimethylpyrroles (2,5-DMP) that can further oxidize and cross-link proteins via a free-radical mechanism. Both processes are believed important in 2,5-HD-neurotoxicity [1]. However, there is a surprising lack of agreement as to the underlying mechanisms and products of amino acid adduction by 2,5-HD. Elucidation of the amino acid alkylation products yields insight into the nature of protein alkylation and suggests alternative pathways to protein cross-linking.

2,5-HD reacts with neurofilament proteins (NFs) in nerve fiber axons, which undergo retrograde degeneration resulting in extremity weakness and sensory loss in humans and other mammals [2]. Neurofilament proteins (NFs) are major cytoskeletal components of neurons and are composed of three different polypeptide subunits: NFL (low molecular weight), NFM (intermediate molecular weight), and NFH (high molecular weight). NFs are reported to be highly phosphorylated *in vivo* [2, 3]. Since it is likely that modulation of the level of phosphorylation at these specific sites will affect the structural and functional interaction of filament assembly and interfilament spacing, it is important to unambiguously identify endogenous phosphorylation sites. Peptide mapping investigations on NFs, including MS and MS/MS experiments, were obtained utilizing Q-TOF and MALDI-TOF/TOF mass spectrometers.

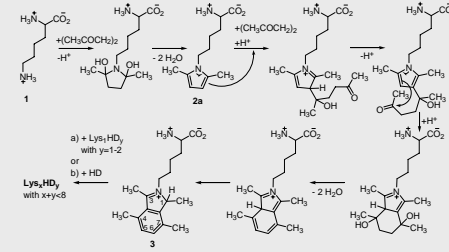
### Alkylation:

**Reagent:** *n*-hexanedione  
**Environment:** *in vitro*  
**Incubation conditions:** time-, concentration-, solvent-, molar ratio-, temperature-, pH-dependent  
**MS:** FAB-MS  
**NMR:** 600 MHz Bruker

### Peptide mapping:

**Proteases:** trypsin, Lys C, pepsin  
**Chemical cleavage:** BrCN  
**Environment:** *in-solution*  
**Incubation conditions:** time-dependent  
**MS:** LC-MS/MS (Quadrupole Time-of-Flight mass spectrometer, Q-TOF, Global MS from Micromass); MALDI-MS/MS (Applied Biosystems AB4700)  
**TOF/TOF mass spectrometer, Applied Biosystems)**  
**Data interpretation:** Mascot

## Mechanism to isoindolium Lys salt



Appropriately modified methodology was applied to show that K-2,5-HD adduction relevant to 2,5-HD-neuropathy is dynamic, and that chemically sensitive secondary alkylation products are formed and can be investigated in detail while maintaining physiologically-relevant conditions. Work up procedures, that are used to affect the oxidative products, are discussed and described here. The results of the highly sensitive, secondary alkylation product are consistent with 2,5-HD-adduction of the  $\epsilon$ -amino group of K forming a labile isoindolium-adducted K salt, 3, chemically similar to that of aromatic  $\gamma$ -diketones, e.g. 1,2-diacetylbenzene, thereby providing a link in their relative reactivities. The 2,5-DMP intermediate must first be transformed to the more reactive isoindole. It is thus postulated that charge carrying adducts such as intermediate 3 can be stabilized via ionic interactions with phosphate groups in NF. The extensive phosphorylation of NFs may therefore play a significant role in stabilizing the 2,5-HD-adducted NFP, or any other 2,5-HD-targeted proteins, yet, altering the net charge of the NFP complex via phosphate charge neutralization may cause changes in conformation and assembly that leads to aggregation. These results also indicate that complementary to mechanisms involving pre-formed pyroles, 2,5-HD-induced protein cross-linking may proceed via mechanisms with additional diketone molecules in a time-dependent manner. The observed time-dependence of the oxidative reactions is consistent with the necessity for an extended 2,5-HD-exposure period for development of 2,5-HD-related diseases *in vivo* [4]. With phosphorylation sites on NFM now established [3], a detailed understanding of 2,5-HD-adduction products of K opens an opportunity for determining the adducted sites and relevant epistasis on NFs and improved understanding of mechanisms underlying  $\beta$ -diketone neuropathy.

A limiting factor to the success of this study is the lack of information on the sequence and phosphorylation sites of bovine NFH. Mass spectrometric *de novo* sequencing of NFH, utilizing peptide maps with various enzymes and the application of simple homology comparisons with other mammalian proteins and error tolerant search for data interpretation, done on the NFP mixture and on purified NFH, yielded sufficient primary sequence information (308 amino acids) to subsequently investigate covalently modified sites. Compared to known NFH sequences, the highest homologies of bovine NFH are found with human NFH in the N-terminal region and with dog NFH in the KSP region of the protein; the KSP repeats in bovine NFH and NFH are dissimilar. MALDI analysis of CNBr cleavage of bovine NFH identified a methionine (M) located in the C-terminal region homologous only to rat NFH (M<sup>107</sup>). This result emphasizes the hypervariability of the KSP and C-terminal regions of NFs. Several endogenous phosphorylation sites within the KSP region of NFH were tentatively established and suggest the involvement of possible kinases in the phosphorylation of NFH similar to bovine NFM [3]. In regard to the alkylation of NFP we gained evidence for the cross-link formation of the NFP subunits and the involvement of other amino acids such as histidine.

## Results

### Part I. Mechanistic studies of hexanedione-induced alkylation

The regular pattern of molecular signals in the mass spectrum is indicative of sequential alkylation and intramolecular cross-linking. The distribution of molecular species observed is highly dependent on the reaction conditions (solvent, pH, temperature and time).

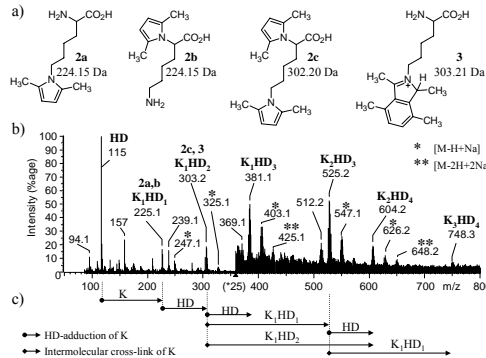


Figure 1. 2,5-HD-alkylated lysine (K) products after 4 weeks (25 fold scale increase 3360 Da) under physiologically-relevant conditions.

### Part II. *De Novo* Sequencing of NFH

308 amino acids (aa) are determined; extrapolation to human NFH approximates the sequence coverage: 373/1020 aa 37%; 271/450 aa 60% N-terminal region)

#### N-terminal region

Sequence 301-400

##### NFP sample

LEAAKYVTD AMSAGAEIT KYRQLGQAT TELEALKSTK DELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELEALKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET rat  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELEALKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET mouse  
 LEAAKYVTD AMSAGAEIS KYRQLGQAT TELELTKSTK DELSASGSLT sheep<sub>pan</sub>

##### NFH sample

LEAAKYVTD AMSAGAEIS KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET rat  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET mouse  
 LEAAKYVTD AMSAGAEIS KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET dog

##### NFP and NFH (higher priority) samples

LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET "human to bovine"  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET "rat to bovine"  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET "mouse to bovine"  
 LEAAKYVTD AMSAGAEIS KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET "dog->bovine"

• Peptide Mapping identified 271 amino acids and the highest homology is found with the human NFH sequence

#### KSP region

##### dog KSP

LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human KSP (determined from cDNA):  
 LEAAKYVTD AMSAGAEIT KYRQLGQAT TELELTKSTK RELSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human KSP:  
 AIVRSLRKYRQLGQATTELELTKSTKRELSSASGSLT ESRKHQDVAS YQAIQGLQR ELRNTYEMIA AQLREYVQLI NYVMALDIET human KSP:  
 KEEKREK K

• Peptide Mapping identified 102 amino acids and the highest homology is found with the dog NFH sequence

### Part III. Identification of the Modified Amino Acid Residues

Several endogenous phosphorylation sites within the KSP region of NFH (e.g. homologous to S<sup>11</sup> human NFH) were tentatively established. The data also allowed the opportunity to determine other covalent modifications, e.g. carbamylation, deamidation, oxidation, pyroglutamic acid formation; all of which were heterogeneous.

The determination of the exogenous 2,5-HD-induced-adduction sites on native NFs was a complicated endeavor. In addition to the 2,5-DMP-K-adducts, the formation of the 2,5-HD-induced isoindole-K-adducts, as well as information regarding the recently established N-terminal partial sequence of NFH and the partial sequence of NFH determined here, covalently modified sites in NFP, e.g. phosphorylation, oxidation, were considered in completing the data interpretation of the complex NFP mixture. MS peptide mapping of NFP treated with 2,5-HD under physiological conditions yielded little sequence coverage of the proteins. It was found that (i) there were many fewer peptides from enzymatic proteolysis than expected from the molecular weight of a simple 2,5-DMP-adducted structure. Both of the latter observations can be rationalized by cross-linking of the NFP subunits. The involvement of other amino acids in the alkylation process of NFs is a possible explanation for the lack of a successful interpretation of the MS detected but unidentified peptides. There is evidence for 2,5-HD-induced alkylation of histidine.

## Literature

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