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Inactivation of NEIL2 DNA glycosylase by pyridoxal phosphate reveals a loop important for substrate binding

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ABSTRACT

Pyridoxal-5′-phosphate (PLP), in addition to its known metabolic functions, inactivates many DNA-dependent enzymes through conjugation to their critical amino groups. We have investigated the ability of PLP to inhibit bifunctional DNA repair glycosylases, which possess a catalytic amine. Of six enzymes tested, only endonuclease VIII-like protein 2 (NEIL2) was significantly inhibited by PLP. The inhibition was due to Schiff base formation between PLP and the enzyme. PLP-conjugated NEIL2 completely lost its ability to bind damaged DNA. Liquid chromatography/nanoelectrospray ionization tandem mass spectrometry of the products of proteolysis of pyridoxylated NEIL2 identified Lys50 as the site of modification. Thus, the β2/β3 loop where Lys50 is located in NEIL2 is important for DNA binding, presumably lies next to a phosphate-binding site, and may represent a target for regulation of the enzyme activity.

1. Introduction

Pyridoxal-5′-phosphate (PLP) is a necessary cofactor of enzymes involved in many reactions of amino acid metabolism, such as transamination, elimination, etc. [1]. By virtue of its aldehyde moiety, PLP is normally conjugated to a Lys residue in the active site of PLP-dependent enzymes as a Schiff base, which is then transferred onto the incoming amino group of the substrate molecule.

While the importance of PLP in metabolism is well established, the possibility and consequences of its conjugation to proteins not involved in general metabolic processes has received much less attention. Nevertheless, inhibition or inactivation by PLP was shown for a diverse set of DNA- and RNA-dependent enzymes, including RNA and DNA polymerases of various origins [2–5], reverse transcriptases [6], integrases [7], and topoisomerases [8]. In all these cases, the inhibition was attributed to PLP conjugation to an amine nucleophile in or near the active site of the enzyme. It is possible that PLP, which carries a phosphate and an aromatic pyridine moiety, resembles nucleoside monophosphates to certain extent and thus has an increased affinity for active sites of DNA- and RNA-dependent enzymes.

DNA repair glycosylases are enzymes that initiate base excision repair, a pathway for removal of damaged bases from DNA, thereby maintaining genome integrity [9]. DNA glycosylases catalyze nucleophilic substitution at C1′ of the damaged deoxynucleoside. These enzymes can be either monofunctional, which use a water molecule as a nucleophile and release an apurinic/apyrimidinic (AP) site product, or bifunctional, which use an enzyme's amino group as a nucleophile, form a Schiff base with C1′, catalyze further elimination of the 3′-phosphate (β-elimination) or both 3′- and 5′-phosphates (β,δ-elimination), and release a nicked DNA product [9]. According to their sequence and three-dimensional structure, DNA glycosylases are classified into three large superfamilies: uracil-DNA glycosylase (Ung), endonuclease III (Nth), or formamidopyrimidine-DNA glycosylase/endonuclease VIII (Fpg/Nei) superfamily, and three smaller families outside the large superfamilies: methylpurine-DNA glycosylase, bacteriophage T4 endonuclease V (DenV), and HEAT-like repeat family [10]. Bifunctional DNA glycosylases are restricted to the Nth superfamily, Fpg/Nei superfamily, and DenV family.

All DNA glycosylases highly distort DNA upon binding, with the target dNMP flipped out of the duplex and inserted into the enzyme's active site. Possibly due to this structural feature, DNA glycosylases often possess increased affinity for dNMPs [11].

As the amine nucleophile is critical for the activity of bifunctional DNA glycosylases, we have investigated whether these important enzymes are sensitive to PLP. Unexpectedly, we have found that only one of the studied DNA glycosylases, NEIL2, is substantially inactivated by PLP. Moreover, the PLP conjugation site is

Abbreviations: AP, apurinic/apyrimidinic; LC, liquid chromatography; MS, mass spectrometry; PLP, pyridoxal phosphate; THF, (3-hydroxytetrahydrofuran-2-yl)methyl phosphate.

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not at the catalytic nucleophile but is located in a loop likely involved in DNA binding.

2. Materials and methods

2.1. Enzymes and oligonucleotides

Escherichia coli Fpg, Nei, and Nth, and murine NEIL1 and NEIL2 proteins were purified as described [12–15]. DenV was a kind gift of Dr. R.S. Lloyd (Oregon Health and Science University). Endoproteinase Asp-N was from Roche, lysozyme, from Sigma–Aldrich, and T4 polynucleotide kinase and *E. coli* Ung, from New England Biolabs. Oligonucleotides were synthesized from commercially available phosphoramidites (Glen Research). Their sequences were 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ where X = uracil, (3-hydroxytetrahydrofuran-2-yl)methyl phosphate (THF), 8-oxoguanine, or 5,6-dihydrouracil for the modified strand, and 5′-(AGAGGAAAGGAGCGAAGGGAGAG)-3′ for the complementary strand. To prepare an AP substrate, the uracil-containing duplex (100 nM) was treated with Ung (0.1 U/μl) in 25 mM potassium phosphate (pH 6.8)/1 mM EDTA for 30 min at 37 °C, and used immediately.

2.2. Enzyme modification by pyridoxal phosphate

The reaction mixture included 1 μM enzyme (NEIL2 or other bifunctional glycosylase), 10 mM PLP, 25 mM potassium phosphate (pH 6.8), 1 mM EDTA, and 1 mM DTT. The enzymes were allowed to bind PLP at 25 °C for 30 min, and the complexes were reduced by adding freshly dissolved $NaBH_4$ to 50 mM. After 15 min, the unreacted $NaBH_4$ was quenched by 400 mM glucose followed by 30 min on ice. Controls were subject to the same treatment except PLP was omitted. If the non-reduced modification was required, NaCl substituted for $NaBH_4$. The reaction mixture was used directly in the activity assay.

2.3. Enzyme activity assay

The reaction mixture included ^{32}P-labeled substrate (20–100 nM), 25 mM potassium phosphate (pH 7.5), 30 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, and various amounts of NEIL2 (or other glycosylase), either treated or not with PLP. The reactions were incubated for 10 min at 37 °C and quenched by adding an equal volume of the formamide dye and heating for 1 min at 95 °C. The products were resolved by electrophoresis in 20% polyacrylamide gel containing 7.2 M urea and quantified by phosphorimaging (Molecular Imager FX, Bio-Rad).

2.4. NEIL2 inactivation kinetics

NEIL2 was treated with 0.5–10 mM PLP for 1–30 min as described above omitting the glucose quenching step. After the incubation with $NaBH_4$, the AP:C substrate was added to 50 nM, and the reaction was allowed to proceed for 10 min at 30 °C. The reactions were quenched by adding an equal volume of the SDS–dye and heating for 5 min at 95 °C. The products were resolved by electrophoresis in 12% polyacrylamide gel (Laemmly system), and quantified by phosphorimaging. The apparent inactivation constants (k_{app}) were extracted from the slopes of the residual activity plots in the coordinates $\ln(([E{\cdot}S]_t - [E{\cdot}S]_\infty)/([E{\cdot}S]_0 - [E{\cdot}S]_\infty))$ vs. time. Forward (k_{on}) and reverse (k_{off}) modification rate constants were determined from the slope and the intercept, respectively, of the plot of k_{app} vs. PLP concentration.

2.5. NEIL2 binding assay

The reaction mixture included ^{32}P-labeled THF:C ligand (1 nM), 25 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mg/ml BSA, and NEIL2 (2, 10, 50, 250, or 1000 nM), the latter either modified or not with PLP and dialyzed against the binding buffer thereafter. The enzyme was allowed to bind the ligand for 6 min at 8 °C, and the complexes were analyzed by electrophoresis in 8% non-denaturing polyacrylamide gel.

2.6. Mass spectrometry analysis of modified NEIL2

NEIL2 (10 μg) was modified or not with PLP and precipitated with cold acetone. For MALDI analysis, the precipitate was dissolved in 1 mM Tris–HCl (pH 8.0), desalted using a ZipTip (Millipore), and analyzed using a MALDI-TOF Reflex-III system (Bruker Daltonics). Alternatively, the precipitate was dissolved in the SDS–dye and subjected to electrophoresis in the Laemmly system. The bands were revealed by Coomassie Blue staining, excised, and the proteins were digested in-gel overnight at 37 °C with 20 μg/ml endoproteinase Asp-N in 50 mM NH_4HCO_3 (pH 8.5). The eluates were analyzed by nanoelectrospray ionization tandem mass spectrometry (LC/MS/MS) on an ABI Qstar Pulsar i Q-TOF system (Applied Biosystems). Samples were desalted and preconcentrated at 30 μl/min on a 300 μm × 5 mm μ-Precolumn Trapping Cartridge (LC Packings). Peptides were then separated on a Waters NanoEase Symmetry column (3.5 μm C_{18}, 75 μm × 15 cm) with a linear gradient from 2% to 30% solvent B in 60 min at a flow rate of 300 nl/min. Solvent A was 2% acetonitrile with 0.1% formic acid, solvent B was 98% acetonitrile with 0.1% formic acid. Mass analysis employed a MS survey scan for 1 s with the mass range m/*z* 350–1600. The information-dependent acquisition MS/MS scan range was *m/z* 100–1400 with a 2-s accumulation time. MS/MS spectra were submitted to the Mascot v1.7 search engine (Matrix Science) for protein identification using the Swissprot database.

3. Results

3.1. Reaction of bifunctional DNA glycosylases with pyridoxal phosphate

Since PLP is capable of forming Schiff bases with amino groups, we first addressed the question whether it can interfere with the activity of bifunctional DNA glycosylases, which possess an amine catalytic nucleophile. We have reacted DNA glycosylases Fpg, Nei, NEIL1, NEIL2, Nth, and DenV with PLP in the presence of $NaBH_4$, included to prevent the hydrolysis of the newly formed Schiff base by reducing it to the corresponding amine. Of these enzymes, the first four belong to the Fpg/Nei superfamily and possess an N-terminal Pro as the catalytic nucleophile, Nth is the prototypic member of the Nth superfamily, using a Lys side chain as the nucleophile, and DenV is a unique member of its own family, with an N-terminal Thr as the nucleophile. The residual activity in the reaction of cleavage of their common substrate, DNA containing an AP site, was estimated in comparison with the enzymes treated with $NaBH_4$ alone (Fig. 1A). The conditions of excess enzyme (500 nM) over substrate (20 nM) were used on the assumption that if PLP modifies the catalytic amine, the enzymes must be fully inactivated. However, only a slight loss of substrate cleavage was seen for Fpg, Nei, NEIL1, Nth and DenV under these conditions. The PLP treatment did not change the nature of the reaction products (β- or β,δ-elimination) as can be judged from their mobility. In a striking contrast, NEIL2 was nearly fully inhibited by conjugation to PLP (Fig. 1A). Moreover, reduction with $NaBH_4$ was not necessary for this inhibition (Fig. 1B). PLP-conjugated NEIL2 showed much lower activity than the intact enzyme over a wide range of enzyme/substrate ratios (Fig. S1A). At low enzyme/substrate ratios the activities of other tested enzymes were also partially impaired,

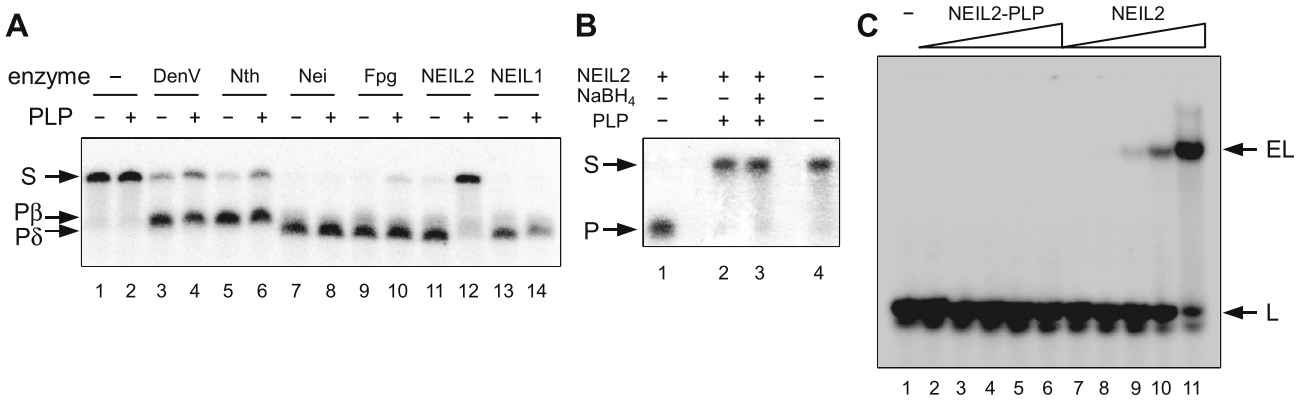

Fig. 1. (A) Activity of unmodified bifunctional DNA glycosylases and DNA glycosylases conjugated to pyridoxal phosphate. 1 and 2, control (AP substrate, no enzyme); 3 and 4, DenV; 5 and 6, Nth; 7 and 8, Nei; 9 and 10, Fpg; 11 and 12, NEIL2; 13 and 14, NEIL1. AP substrate (S, 20 nM) is cleaved by the enzymes (500 nM) treated (even lanes) or not treated (odd lanes) with PLP for 30 min in the presence of $NaBH_4$ to produce cleavage products of β-elimination (Pβ) or β,δ-elimination (Pδ). (B) Inhibition of NEIL2 by PLP in the absence of $NaBH_4$ reduction. 1, fully active enzyme; 2, enzyme pre-treated with PLP but not reduced with $NaBH_4$; 3, enzyme pre-treated with PLP and reduced with $NaBH_4$; 4, no enzyme. The reaction conditions were the same as in Panel A except the absence of $NaBH_4$ where indicated. (C) Binding of unmodified and PLP-conjugated NEIL2 to the uncleavable THF:C ligand. Arrows indicate the mobility of the free THF:C ligand (L) and the enzyme–ligand complex (EL). 1, no enzyme, 2–6, NEIL2-PLP (2–1000 nM), 7–11, unmodified NEIL2 (2–1000 nM). See Section 2 for the reaction conditions.

both with AP substrates and damaged base-containing substrates, but not to the same extent as for NEIL2 (Fig. S1B and C). Overall, of all tested glycosylases, NEIL2 was the most profoundly inactivated by PLP. Hence, we have addressed the mechanism of NEIL2 inactivation in more detail.

3.2. Kinetics of NEIL2 inactivation by pyridoxal phosphate

The most obvious possibility for the inactivation of NEIL2 by PLP is modification of some critical amino functionality in this enzyme. Since this type of inactivation involves formation of a covalent bond, a process slow in comparison with simple inhibitor binding, one could expect that the degree of NEIL2 inactivation will depend on the duration of pre-incubation with PLP. We have directly followed the relative amount of the active enzyme using the cross-linking assay. NEIL2 was incubated in the presence of PLP for various times, and then the formed Schiff base complexes were reduced with $NaBH_4$. Since $NaBH_4$ also reduces aldehydes very quickly, formation of the NEIL2·PLP covalent conjugate stops approximately at the moment of borohydride quenching, and the fraction of the remaining active NEIL2 can be used to assess the extent of the enzyme modification. The relative amounts of the active enzyme were determined using cross-linking to the AP substrate (Fig. 2A). With the increasing time of pre-incubation with PLP, the fraction of the remaining active NEIL2 decreased. The data were linearized in semi-logarithmic coordinates, $\ln(([E{\cdot}S]_t - [E{\cdot}S]_\infty)/([E{\cdot}S]_0 - [E{\cdot}S]_\infty))$ vs. time (Fig. 2B), allowing determination of the apparent inactivation constant from the slope of the linear plot ($k_{app} = (7.32 \pm 0.76) \times 10^{-2}$ min^{-1} for the data in Fig. 2B, corresponding to 1 mM PLP). After plotting the k_{app} values against PLP concentrations, they could be fitted with a straight line intercepting the ordinate axis. When the inhibitor concentration is much higher than the enzyme concentration, such behavior is indicative of slowly developing tight binding of the inhibitor [16]. The slope

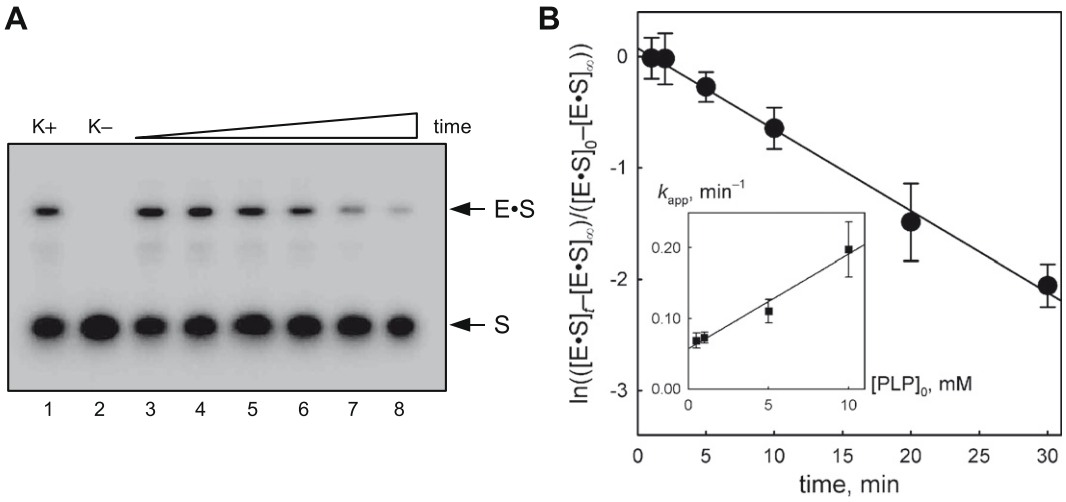

Fig. 2. Time course of inactivation of NEIL2 by PLP. (A) Cross-linking of NEIL2 (50 nM) to the AP:C substrate (50 nM) after pre-incubation with 1 mM PLP for 1–30 min (3–8). 1, pre-incubation in the absence of PLP; 2, no enzyme. Arrows indicate the mobility of the free AP:C substrate (S) and the enzyme–oligonucleotide covalent complex (E·S). (B) Decrease in the remaining fraction of active NEIL2 plotted against the time of modification with PLP (1 mM). Mean ± S.E. are shown (three independent experiments). The inset shows the dependence of the apparent inactivation rate constant on the concentration of PLP; mean ± S.E. are shown (three independent experiments for each PLP concentration).

of the fitted line then provides the association rate constant ($k_{on} = (1.34 \pm 0.15) \times 10^{-2}$ mM^{-1} min^{-1}), while the intercept gives the dissociation rate constant ($k_{off} = (5.72 \pm 0.84) \times 10^{-2}$ min^{-1}). Although an inhibition constant may be formally derived from these values as k_{off}/k_{on} (4.3 mM), it would have little meaning in the context of formation of a labile covalent bond between PLP and NEIL2. Nevertheless, this constant is near the lower bracket of the range of stability constants for conjugates between PLP and various amines (~1–200 mM [17]).

3.3. Pyridoxylation of NEIL2 interferes with DNA binding

In order to understand possible reasons for NEIL2 inactivation by PLP conjugation, we have investigated the ability of unmodified and pyridoxylated NEIL2 to bind a duplex containing THF, the AP site analog that cannot be cleaved by bifunctional DNA glycosylases but binds them with high affinity [18]. Unmodified NEIL2 formed a complex with this ligand in a concentration-dependent manner (Fig. 1C, lanes 7–11). After PLP conjugation, the ability of NEIL2 to bind the THF-ligand was completely lost (Fig. 1C, lanes 2–6).

3.4. Identification of the modification site in NEIL2

A MALDI analysis showed that the mass difference between unmodified and PLP-conjugated NEIL2 was 230.90. The calculated mass difference for the monoadduct is 231.03 Da, indicating a 1:1 stoichiometry of PLP conjugation to the protein, and suggesting that PLP was specifically conjugated to one residue instead of modifying all available amino groups of NEIL2.

To positively identify the site of PLP modification, the PLP-conjugated NEIL2 and unmodified NEIL2 were digested with endoproteinase Asp-N, which specifically cleaves proteins N-terminal to Asp and to some of Glu residues. The reaction products were analyzed by LC/MS/MS (Fig. 3A). In the control, NEIL2 was the first hit with the score 256, seven peptides and 12% sequence coverage (both Asp and Glu included). Notably, the unmodified ^{43}DAQVHGKKLFLRF^{55} peptide was detected as the +4 charge state of a 1557.8-Da species at 38.9 min in the LC/MS/MS run. In the PLP-conjugated NEIL2, we inferred that the modified peptide was present in the sample by the appearance of the +3 charge state of the singly modified peptide at m/z 597.33 (calculated m/z 597.31) in the MS mode (Fig. 3A). Product ion analysis of this species showed that the apparent mass shift of the expected peptide ^{43}DAQVHGKKLFLRF^{55} was 231.09 Da, giving a good indication of pyridoxylation of one Lys residue in this peptide.

Sequence information was obtained for this peptide. The initial loss of the phosphate produced a +3 ion at m/z 564.67 (Fig. 3B). Singly charged y_1 to y_5 sequence ions were readily observed along with b_1 to b_7 ions. The modification seems to be at K50 because a strong singly charged b_7 ion at m/z 736.38 and its complementary fragment y_6 containing the pyridoxyl unit (y_6 + Pyr) at m/z 956.58 appeared in the spectrum. This was supported by the observation of the doubly charged $(b_8 + Pyr)^{+2}$ at m/z 499.29. The y sequence ions continued with y_7 + Pyr to y_{12} + Pyr for basically complete coverage in the +1 and +2 y ion series.

The modification at K49, the target residue for NEIL2 acetylation [19], would have resulted in an y_7 ion at m/z 951 or a doubly charged y_7 ion at m/z 476 and a $(b_7 + Pyr)^{+2}$ ion at m/z 435. The spectrum does contain a peak at m/z 435.29, but it is attributed to the singly charged y_3 ion, and does not show a significant signal at the other two m/z values predicted for K49 modification. There was some evidence for a doubly substituted peptide at m/z 674.31 (+3 charge state of the 2019.92-Da species); however, fragmentation did not provide sequence information, rather the major ion in the product ion scan was m/z 213.13 (loss of 461 Da, possibly two PLP residues). Overall, it seems that Lys50 is the major site for PLP conjugation in NEIL2.

4. Discussion

NEIL2 joins the list of DNA-dependent enzymes that can be inactivated by PLP [2–8]. Since the role of PLP in metabolic reactions is well established, it is generally thought that its interaction with non-metabolic enzymes is fortuitous and due to the amino-reactive aldehyde group. However, there is circumstantial evidence that PLP may be involved in regulation of non-metabolic proteins *in vivo*. For example, it has been shown that pyridoxylation of nuclear corepressor RIP140 enhances its activity and modulates its functions in the induction of adipocyte differentiation [20]. Inhibition of cell proliferation by vitamin B_6 has been attributed to inactivation of DNA polymerases by PLP [5].

NEIL2 is a DNA glycosylase specific for oxidatively damaged pyrimidines [21]. It belongs to the Fpg/Nei superfamily characterized by two-domain structure and several conserved structural motifs [21,22] (Fig. S2). In their C-terminal domain, all Fpg/Nei proteins carry two DNA-binding motifs, a helix–two turns–helix motif and a zinc finger or a zincless β-hairpin. The N-terminal domain bears the catalytic nucleophile and an intercalation loop, which is inserted into the DNA helix to flip out the damaged deoxynucleoside [22]. In addition, an absolutely conserved Lys residue lies in a short loop between two β-strands (β2/β3) of the N-terminal domain. This Lys, together with two absolutely conserved Asp and Arg residues from the C-terminal domain, binds DNA phosphates near the lesion [22]. Although the structure of NEIL2 is unknown, the overall sequence similarity of NEIL2 to other members of the Fpg/Nei superfamily suggests that these major structural motifs are preserved in NEIL2.

Several DNA glycosylases, including NEIL2, are regulated by post-translational modification. Acetylation of human NEIL2 at Lys49 (which corresponds to the absolutely conservative Lys in the β2/β3 loop) by p300 histone acetyltransferase, significantly reducing the enzyme's activity, has been reported [19]. Mutations of Lys49 in NEIL2 or the equivalent Lys52 in Nei also inactivate these enzymes [19,22]. Our results regarding Lys50 underscore the importance of the β2/β3 loop for the activity of Fpg/Nei proteins and suggest that the loss of NEIL2 activity after acetylation may also be due to impaired DNA binding.

The observed narrow specificity of PLP conjugation to NEIL2 suggests that the modification process is at least partly mechanism-specific. Although there are 21 lysines and the N-terminal amino group in murine NEIL2, we have observed PLP addition only to Lys50 (and possibly Lys49). The most plausible interpretation is that PLP binds in a positively charged DNA-binding groove of the enzyme, perhaps in a partly hydrophobic base-binding pocket, and forms a Schiff base with a Lys residue nearby, which happens to lie in the β2/β3 loop. Thermodynamic studies suggest that the DNA-binding groove of Fpg/Nei proteins possesses a site with an increased affinity for dNMPs [11], where PLP may bind. Lack of modification at other lysines most likely reflects the absence of appropriate binding sites adjacent to them.

It is important to underscore that our biochemical data do not prove that pyridoxylation is relevant to *in vivo* regulation of NEIL2 activity, although the kinetics of PLP addition to NEIL2 is comparable with kinetics of some physiologically relevant processes, e.g., pyridoxylation of the active site of D-serine dehydratase, a PLP-dependent enzyme [23]. Yet NEIL2 clearly stood out of other bifunctional DNA glycosylases in its sensitivity to inacti-

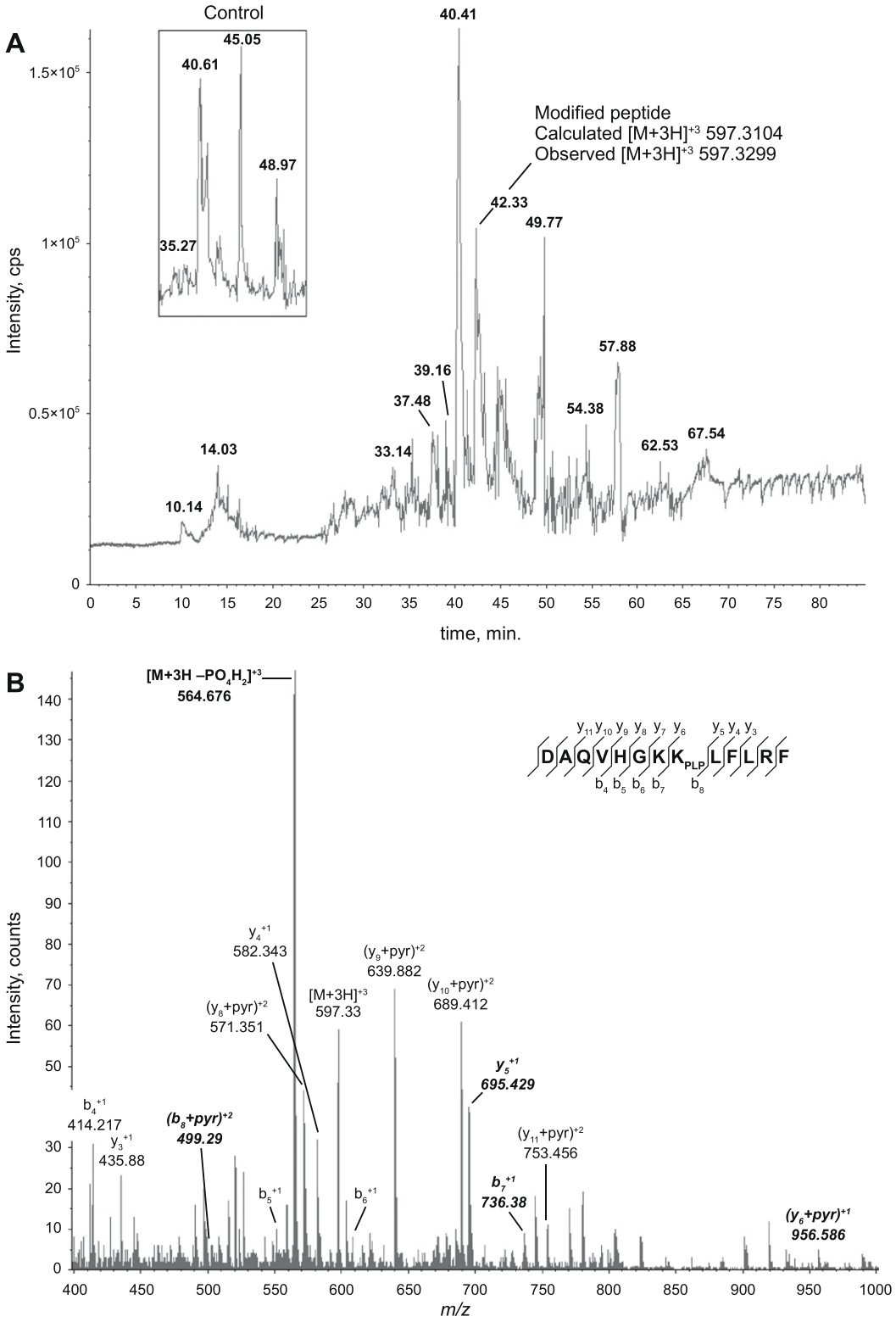

Fig. 3. Mass spectrometry sequencing of the PLP-modified peptide in NEIL2. (A) LC/MS total ion current chromatogram of the peptides derived from endoproteinase Asp-N digestion of control (inset) and modified NEIL2. A new component is observed in the modified sample at 42.33 min. The mass spectrum of this peak displayed a triply charged molecular ion at m/z 597.33. The calculated triply charged m/z of a single PLP-conjugated ^{43}DAQVHGKKLFLRF^{55} peptide is 597.31 suggesting that this peptide has been modified. (B) Part of the MS/MS spectrum of the triply charged species at m/z 597.33 showing the m/z range 400–1000 highlighting the initial loss of phosphate and important sequence ions. Singly charged b_7 ion at m/z 736.38 and the complementary (y_6 + Pyr) ion at m/z 956.58, as well as the $(b_8 + Pyr)^{+2}/y_5^{+1}$ complementary pair are highlighted. Peptide sequence and the observed sequence ions in the spectrum are indicated.

vation by PLP. Thus, although our data do not imply that pyridoxylation may regulate NEIL2 activity *in vivo*, they confirm the importance of the β2/β3 loop as a potential regulatory site in this protein.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.121.

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