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Inactivation of rabbit muscle glycogen phosphorylase *b* by peroxynitrite revisited: Does the nitration of Tyr^{613} in the allosteric inhibition site control enzymatic function?

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ABSTRACT

There is increasing evidence that sequence-specific formation of 3-nitrotyrosine (3-NT) may cause functional changes in target proteins. Recently, the nitration of Tyr residues in glycogen phosphorylase *b* (Ph-b) was implicated in the age-associated decline of protein function [Sharov et al., Exp. Gerontol. 41 (2006) 407–416]; in another report, the nitration of one specific residue, Tyr^{613}, located in the allosteric inhibition site was hypothesized as a rationale for peroxynitrite inactivation [Dairou et al., J. Mol. Biol. 372 (2007) 1009–1021]. In this study, we have optimized the analysis of in-gel Ph-b digests by high performance liquid chromatography-electro spray ionization-tandem mass spectrometry, in order to achieve a quantitative analysis of nitration of individual Tyr residues at a high coverage of Tyr-containing sequences (92%). Our data do not confirm the role of Tyr^{613} nitration in the control of enzymatic function. Furthermore, we show here that the enzymatic activity of Ph-b does not directly correlate with the protein nitration levels, and that the modification of Cys and, potentially, other amino acid residues can better rationalize Ph-b inactivation by peroxynitrite.

There is increasing evidence that reactive nitrogen species nitrate specific tyrosine residues in proteins, and that the formation of 3-nitrotyrosine (3-NT)[1] causes functional changes in targeted proteins [1–4]. Biological nitration of protein tyrosine residues has been demonstrated in various diseases and biological aging [5–13]. Protein nitration appears to be a rather selective process since neither all tyrosine residues in proteins nor all proteins in a given proteome get nitrated both *in vivo* [6,14,15] and *in vitro* [15–18]. In addition, modifications of different Tyr residues may not be evenly important for protein function. Several sources of Tyr nitration *in vivo* have been established involving reactions of peroxynitrite and/or nitrogen dioxide, or nitrite catalyzed by peroxidases [19]. Regardless of the chemical mechanism, tyrosine nitration appears to be a complex process depending on the individual reactivity of Tyr residues in a protein, environment (pH, concentrations of reagents, solvent accessibility and diffusion coefficients), tissue specific protection, protein repair and turnover mechanisms. The knowledge of sequence location and the yields of respective Tyr nitration is therefore very important, both mechanistically and physiologically, to understand the role of specific protein damage and to design potential treatments of ensuing disorders. However, to date most protein nitration targets *in vivo* have been identified by anti-3-NT antibodies only, and, with a few exceptions [6,15,20–26], a sequence-specific analysis of 3-NT on individual proteins *in vivo* has not been done. Peptide mass mapping by liquid chromatography–mass spectrometry (LC–MS) has become a key approach in the qualitative and quantitative characterization of protein post-translational modifications. The development of quantitative approaches suitable for virtually complete sequence-specific characterization of "problematic" proteins is, therefore, a necessary step towards understanding a role of protein oxidative post-translational modifications, such as Tyr nitration, in the functional alteration of proteins. Earlier, we found that nanoHPLC-nanoESI-MS/MS analysis of in-gel digests obtained by 1-D SDS–PAGE can be successfully used for the characterization of 3-NT accumulation on even large membrane proteins, such as SERCA [27,28], and this technique is applied here for a comprehensive analysis of phosphorylase *b* (Ph-b).

Ph-b is a ubiquitous protein, which represents ca. 5% of total soluble protein in muscle tissue. Ph-b (gene name: *PYGM*) belongs to

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[1] *Abbreviations used:* 3-NT, 3-nitrotyrosine; Ph-b, phosphorylase *b*; LC–MS, liquid chromatography–mass spectrometry; GSD5, glycogen storage disease type 5; HPLC-ESI-MS/MS, high performance liquid chromatography-electro spray ionization-tandem mass spectrometry; BSA, bovine serum albumin; OBG, octyl-β-glucopyranoside; TFA, trifluoroacetic acid; CID, collision-induced dissociation; FT-ICR, Fourier transform ion cyclotron resonance; SASA, solvent accessible surface area; AMU, atomic mass units.

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the glycogen phosphorylase family, which catalyzes the formation of glucose-1-phosphate from the cellular carbohydrate storage form, glycogen:

$(1,4\text{-}\alpha\text{-D-glucosyl})_n$ + phosphate = $(1,4\text{-}\alpha\text{-D-glucosyl})_{n-1}$ + α-D-glucose-1-phosphate

The activity of Ph-b is controlled both allosterically (through the non-covalent binding of metabolites) and by covalent modification [29,30]. AMP allosterically activates, whereas ATP, ADP, and glucose-6-phosphate allosterically inhibit Ph-b. Phosphorylation of Ser^{14} (SwissProt and NCBI protein databases refer to this residue as Ser^{15}, which includes the original Met removed in the mature protein) converts Ph-b (a homodimer, T-form) to phosphorylase *a* (enzymatically more active, tetrameric R-form). High-resolution crystal structures of the protein in different conformational T and R states are now available [31,32] allowing for computational analysis of structure-activity relationships in the protein. Defects in the *PYGM* gene cause glycogen storage disease type 5 (GSD5), also known as McArdle disease, which is a metabolic disorder resulting in myopathy characterized by exercise intolerance, cramps, muscle weakness and recurrent myoglobinuria. The structure and enzymatic activity of Ph-b are extremely sensitive to the modification of even a single amino acid residue: at least 18 missense mutations of a single amino acid residue of human muscle Ph-b throughout the gene sequence have been identified, which cause functional deficiency of skeletal muscle [33,34]. The Ph-b sequence is highly conserved in mammals sharing 98% homology in the human, mouse and rabbit protein. Generally, Ph-b is an excellent model to study the selectivity of protein tyrosine nitration and represents a good challenge to test new methodology. The enzyme of 97 kDa contains 36 tyrosine residues (out of a total 842 amino acids), which can be differentially mapped to assess the selectivity of tyrosine nitration. The unusually high fraction of protein Tyr residues (4.3%) may be a reason for the susceptibility of the protein to nitration observed *in vivo* [15].

The aim of the current work was the sequence-specific characterization of Tyr nitration by peroxynitrite in rabbit muscle Ph-b, and a potential correlation to enzymatic activity. The motivation for the current study originated from two findings. First, Ph-b from rat skeletal muscle suffers an age-associated loss of function, which is accompanied (but not necessarily caused) by age-associated, sequence-specific accumulation of 3-NT on the protein [15,24]. Immunochemical evidence for Ph-b nitration *in vivo* was also reported by Kuo and others [26]. Second, in a recent study [17] the nitration of a critical residue, Tyr^{613}, located in the allosteric inhibitor site of the enzyme, has been suggested as a mechanism of Ph-b inhibition by peroxynitrite, based on ligand binding and MS analysis. However, this important conclusion was apparently made from qualitative MS analysis using MALDI-TOF peptide fingerprinting only, at relatively low sequence coverage (<50%). Furthermore, the experimental conditions in the cited work did not account for potential changes of peroxynitrite reactions in the presence of physiological concentrations of CO_2/HCO_3^- [19].

Therefore, we have optimized the analysis of in-gel Ph-b digests by high performance liquid chromatography-electro spray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS), focusing on a quantitative analysis of individual Tyr residue nitration at a high coverage of Tyr-containing sequences (92%). Our data do not confirm a role of Tyr^{613} nitration in the control of enzymatic function. Furthermore, we show here that the enzymatic activity of Ph-b does not directly correlate with the protein nitration levels, and that the modification of Cys and, potentially, other amino acid residues can better rationalize Ph-b inactivation by peroxynitrite.

Materials and methods

Chemicals

TPCK-treated sequence grade trypsin was from Promega (Madison, WI). Rabbit muscle phosphorylase *b*, bovine serum albumin (BSA), DTT, SDS, urea, NADP, glycogen from rabbit liver, adenosine-5′-monophosphate, glucose-6-phosphate dehydrogenase and sodium iodoacetate were purchased from Sigma (St. Louis, MO). Phosphoglucomutase was obtained from Boeringer Mannheim (Indianapolis, IN). Pre-cast Novex® tris-glycine-SDS gels, molecular weight standard Mark12, running and sample buffers were from Invitrogen (Carlsbad, CA). All other chemicals of highest commercially available grade were obtained from Fisher (Pittsburgh, PA, USA).

Reaction with peroxynitrite

Peroxynitrite was prepared by the reaction of ozone with cooled aqueous sodium azide as described previously [35], aliquoted and kept at −70 °C and pH 12 until use. The concentration of stock peroxynitrite (ca. 50 mM) was determined by absorbance (ε_{302} = 1,670 M^{-1} cm^{-1} [19]), and all dilutions were made in 0.1% NaOH (pH 12). Ph-b was reconstituted in a buffer containing 1 mM DTPA and either 25 mM Na_2HPO_4 and 25 mM $NaHCO_3$, or 50 mM Na_2HPO_4 alone (pH 7.4). A small volume of stock peroxynitrite was quickly added while vortexing to get desired final concentrations of peroxynitrite (bolus addition of peroxynitrite). For control experiments, peroxynitrite was added to the respective buffer 5 min prior to mixing with the protein solution (reverse-order-of-addition experiment). We did not observe any differences in nitration, electrophoretic mobility, and HPLC elution profile between control (not treated with peroxynitrite) and the reverse-order-of-addition samples.

Assay of phosphorylase b activity

Phosphorylase *b* activity was assayed at room temperature with an assay described earlier [15]. The test mixture contained 0.1 mM EDTA, 3 mM $MgCl_2$, 0.3 mg/ml NADP, 0.2 mg/ml glycogen, 30 μM adenosine-5′-monophosphate, 0.7 U/ml phosphoglucomutase, 3 U/ml glucose-6-phosphate dehydrogenase in 0.05 M potassium phosphate (pH 6.8). After recording the blank rate for 2 min, the reaction was initiated by the addition of phosphorylase *b* solution, and the absorbance at 340 nm was monitored for 8 min. The activity was determined as ΔA_{340}/min and calibrated through a standard commercial enzyme with known activity.

Gel electrophoresis

For reduction and alkylation of protein Cys residues, samples were incubated for 1 h with 6 M urea and 2 mM DTT at 37 °C followed by the addition of 5 mM sodium iodoacetate and incubation for an additional 1 h at room temperature under exclusion of light. Aliquots of Ph-b containing 20 μg protein were mixed with an equal volume of tris-glycine-SDS sample buffer, heated for 2 min in a boiling water bath, and loaded onto 1.5-mm thick 10-well Novex 4–12% Tris–glycine gradient gels (Invitrogen, Carlsbad, CA). After running gel electrophoresis at 200 V for 90 min, the gels were stained with 0.2% Coomassie R250 in 7.5% acetic acid/30% methanol/62.5% H_2O for 2 h, followed by destaining in 7.5% acetic acid/40% methanol/52.5% H_2O until the bands were visible and the background was clear.