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Nitration of a Critical Tyrosine Residue in the Allosteric Inhibitor Site of Muscle Glycogen Phosphorylase Impairs its Catalytic Activity

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Muscle glycogen phosphorylase (GP) is a key enzyme in glucose metabolism, and its impairment can lead to muscle dysfunction. Tyrosine nitration of glycogen phosphorylase occurs during aging and has been suggested to be involved in progressive loss of muscle performance. Here, we show that GP (in its T and R form) is irreversibly impaired by exposure to peroxynitrite, a biological nitrogen species known to nitrate reactive tyrosine residues, and to be involved in physiological and pathological processes. Kinetic and biochemical analysis indicated that irreversible inactivation of GP by peroxynitrite is due to the fast ($k_{inact} = 3 \times 10^4$ M^{-1} s^{-1}) nitration of a unique tyrosine residue of the enzyme. Endogenous GP was tyrosine nitrated and irreversibly inactivated in skeletal muscle cells upon exposure to peroxynitrite, with concomitant impairment of glycogen mobilization. Ligand protection assays and mass spectrometry analysis using purified GP suggested that the peroxynitrite-dependent inactivation of the enzyme could be due to the nitration of Tyr613, a key amino acid of the allosteric inhibitor site of the enzyme. Our findings suggest that GP functions may be regulated by tyrosine nitration.

Keywords: glycogen phosphorylase; tyrosine nitration; inhibition; peroxynitrite

Introduction

Glycogen phosphorylase (GP, EC 2.4.1.1) is an essential enzyme of glycogen metabolism in skeletal muscle. GP catalyzes the breakdown of the storage polysaccharide glycogen to produce glucose-1-phosphate (G1P), which can then be used for the production of ATP.[1] Impairment of muscle GP activity leads to McArdle's disease, a rare metabolic myopathy.[2] GP is a homodimer composed of two 97 kDa subunits that exist in two interconvertible forms: GPb is the dephosphorylated form and GPa is the Ser14-phosphorylated form.[3] GP is an allosteric enzyme whose activity can be regulated by reversible phosphorylation (GPa) or by binding of allosteric effectors to the GPa or GPb forms. Allosteric activators, e.g. AMP, or allosteric inhibitors, e.g. caffeine, alter the equilibrium between the less active/inactive T form (GPb without allosteric activators) and the active R form (Ser14-phosphorylated form GPa or GPb form with allosteric activators). The structures of the T and R form of GP have been characterized.[3] Mammalian GP enzymes are highly conserved (amino acid identity > 95%) and most studies of GP have been done with the rabbit enzyme, which shares 98% identity with the human and mouse enzymes.

Nitric oxide (NO) and reactive nitrogen species (RNS) are biological oxidants. They have been implicated in major physiological and pathophysiological processes, including vasorelaxation, apoptosis,

Abbreviations used: GP, glycogen phosphorylase; GSH, reduced glutathione; SIN1, 3-morpholinosydnonimine *N*-ethylcarbamide; 3NT, 3-nitro-tyrosine.

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inflammation and cancer, through the oxidative modification of DNA, proteins or lipids.[4–8] Peroxynitrite is one of the most reactive, and therefore most deleterious, NO derivatives involved in the oxidative modification of biological molecules.[4,5] Peroxynitrite affects protein functions by modifying essential reactive thiol groups[6] and/or tyrosine residues, thereby leading to the formation of oxidized thiol groups or of 3-nitrotyrosine (3NT).[7,8]

Peroxynitrite has numerous physiological functions, especially in skeletal muscle cells. These functions include contraction, glucose uptake, metabolism and signaling.[9–11] However, under pathophysiological conditions including inflammation, excess production of this nitric oxide-derived oxidant or failure of antioxidant systems contribute to muscle dysfunction and pathological situations through protein modification.[9–13] Peroxynitrite may be involved in muscle dysfunction through the inactivation of important muscle enzymes, including creatine kinase,[14] sarcoplasmic reticulum Ca^{2+}-ATPase,[11,15] and antioxidant enzymes.[9] Moreover, chronic production of peroxynitrite in skeletal muscle during aging processes modifies key enzymes, including GP, involved in muscle energy metabolism, which may lead to the decline of motor function.[16–18] Recently, enzymes involved in glucose metabolism, e.g. aldolase A and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), have been suggested to be regulated by biological oxidants and nitrative stress through the post-translational nitration of tyrosine and/or oxidation of cysteine residues.[19,20] GP nitration occurs in muscle.[18] However, no study has shown clearly whether peroxynitrite-dependent tyrosine nitration of GP leads to the irreversible inactivation of the enzyme *in vitro* or in skeletal muscle cells.

In this study, we show that exposure of GP (either in its T or R form) to the biological oxidant peroxynitrite leads to the irreversible impairment of its activity. Overall, we show that GP glycolytic functions were regulated by peroxynitrite through the nitration of a Tyr residue present in an important regulatory region of the enzyme. These findings indicate that the glycolysis pathway is possibly regulated by tyrosine nitration of key glycolytic enzymes, e.g. GAPDH, aldolase, enolase and GP, as suggested recently.[20]

Results

Effects of peroxynitrite and SIN1-generated peroxynitrite on GPb activity

We exposed purified GPb (1.25–20 μM), either in its T or R form, to various concentrations of peroxynitrite to analyze the sensitivity of GPb to this nitric oxide-derived oxidant. GPb (T form) inactivation by peroxynitrite depended on the concentrations of both reactants (second-order kinetics) (Figure 1). Such behavior has been shown for peroxynitrite-mediated inactivation of various enzymes.[19,21,22]

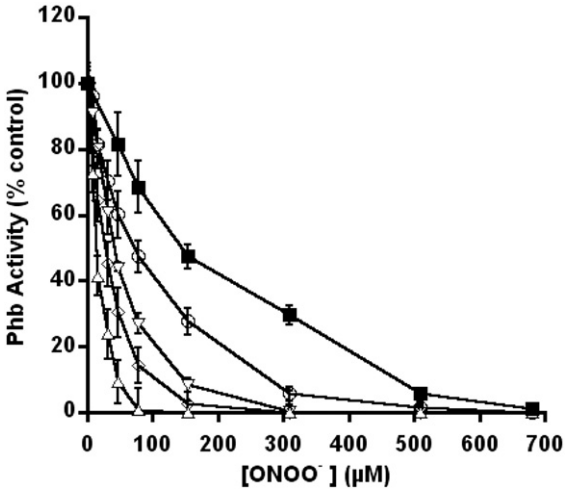

Figure 1. Dose-dependent inactivation of GPb activity by peroxynitrite. The purified T form of GPb (Δ, 1.25 μM; ◇, 2.5 μM; ▽, 5 μM; ○, 10 μM; ■, 20 μM final) was incubated with peroxynitrite ($ONOO^-$) at various concentrations for 10 min at 37 °C in 50 mM Gly-Gly buffer (pH 6.9), 2 mM EDTA (final volume of 50 μl). GPb activity was assayed in the presence of 2 mM AMP in a final volume of 1 ml. These results are representative of three independent experiments in which each treatment was done in quadruplicate.

Nearly 90% of GPb at a final concentration of 1.25 μM was inactivated with a final concentration of about 50 μM peroxynitrite, and nearly 90% of GPb at a final concentration of 20 μM was inactivated with a final concentration of peroxynitrite of about 500 μM. The same results were observed for the R form of GPb (in the presence of AMP) on exposure to peroxynitrite (data not shown). GPb (T or R form) was also exposed to SIN1 (final concentration 100–1000 μM), which mimics physiological peroxynitrite generation by releasing superoxide and nitric oxide at a constant rate, leading to the quantitative formation of peroxynitrite fluxes.[23–25] Both T and R forms of GPb were readily inactivated by SIN1 in a dose-dependent manner (data not shown). With 150 μM SIN1 (final concentration), nearly 90% inactivation was observed for GPb at a final concentration of 1.25 μM (data not shown). A concentrations of SIN1 higher than that of peroxynitrite was necessary because SIN1 generates at least four times less peroxynitrite than the parent peroxynitrite donor.[26,27] Decomposed peroxynitrite and SIN1 did not affect GPb activity (data not shown).

Peroxynitrite affects protein functions mainly through oxidation of thiol residues and nitration of tyrosine residues.[5,7,28] We tested whether peroxynitrite-dependent inactivation of GPb could be due to thiol residue modifications by exposing GPb (T or R form) at a final concentration of 1.25 μM to various concentrations of four reagents that modify cysteine residues. These reagents included *N*-ethylmaleimide, iodoacetamide, H_2O_2 and Hg^{2+}. We found that *N*-ethylmaleimide had no effect on GPb activity (Figure 2(a)). Similar results were observed with the