Acta Alimentaria 52 (2023) 4, 646–655
DOI: 10.1556/066.2023.00178

AKADÉMIAI KIADÓ

Phosphorylation promoted the glycolytic rate in postmortem meat by tightening the structure of glycogen phosphorylase

Y. Bai, X. Li*, C. Ren, X. Zheng, C. Hou, L. Chen and D. Zhang

Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Key Laboratory of Agro-Products Processing, Ministry of Agriculture and Rural Affairs, Beijing 100193, P. R. China

PRELIMINARY COMMUNICATION

Received: July 26, 2023 • Accepted: September 21, 2023
Published online: October 24, 2023

ABSTRACT

The activity and structural variation of glycogen phosphorylase (GP) at different phosphorylation levels during incubation at 4 °C were explored in this study. The GP was assigned into four treatments to obtain high/low phosphorylation levels, which were (1) treated with glycogen phosphorylase kinase (Phk) to obtain high phosphorylation level, (2) treated with protein kinase A to obtain high phosphorylation level, (3) treated with alkaline phosphatase to obtain low phosphorylation level, and (4) control. Compared with the control group, the content of α-helix and β-sheet increased and the secondary structure of GP changed from disorder to order after phosphorylation. The activity of GP was increased and its structure was more tightly in the Phk group than that in the control group. The phosphorylation at Ser277, Ser430, Ser809, Thr304, Tyr298, and Tyr525 resulted in tighter spatial structure. In conclusion, phosphorylation of GP enhanced its catalytic activity by making the secondary and spatial structure more orderly, which is of great significance for controlling meat quality by regulating glycolysis.

KEYWORDS

glycogen phosphorylase, protein phosphorylation, activity, protein structure simulation

* Corresponding author. Tel.: +86 10 62819392. E-mail: xinli.caas@gmail.com

1. INTRODUCTION

Glycolysis plays an important role in the formation of meat quality postmortem (Hammelman et al., 2003). Both excessive glycolysis and inadequate glycolysis result in inferior meat such as PSE and DFD meat, which leads to economic losses. Proper glycolytic rate is critical to maintain meat quality and avoid deterioration of meat quality (Chen et al., 2018). Protein phosphorylation is one of the most important protein posttranslational modifications that regulates biological processes, including glycolysis (Li et al., 2021).

As an enzyme that catalyses the degradation of glycogen, glycogen phosphorylase (GP) regulates glycolytic rate early postmortem. The active form of glycogen phosphorylase (GPa) catalyses the decomposition of glycogen. Correspondingly, there is an inactive form (GPb) of GP (Schmid et al., 2008). The two forms of GP, which can be converted to each other, are regulated by phosphorylation and allosteric during conversion (Barford and Johnson, 1989). It was found that covalent phosphorylation played a role in regulation of GP activity (Browner and Fletterick, 1992), which was catalysed by glycogen phosphorylase kinase (Agius, 2015). The binding of phosphate to serine of GP may cause the changes in its structure and activity. Phosphorylation of GPb can be activated through allosteric mechanism (Sprang et al., 1988).

Protein phosphorylation is closely related to meat quality (Li et al., 2021). The phosphorylation proteome of mutton with different tenderness showed that GP was the main protein involved in the regulation of energy metabolism. The phosphorylation level of GP in high tenderness group was higher (Li et al., 2017; Chen et al., 2018). Numerous studies have shown that GP activity regulates glycolysis in early postmortem muscle (Laville et al., 2009). The phosphorylation level of GP positively correlated with the rate of glycolysis (Li et al., 2017; Bai et al., 2020). The phosphorylation of GP positively regulated its activity (Bai et al., 2020). Therefore, protein phosphorylation may regulate glycolysis by changing the activity of GP and influence meat preservation consequently. However, the mechanism by which phosphorylation regulates GP activity is unclear. Therefore, to clarify the regulation mechanism of GP phosphorylation on glycolysis in meat, this study explored the effect of GP phosphorylation on its structure and activity variation.

2. MATERIALS AND METHODS

2.1. Experimental design

Equal amounts of GP (P6635, Sigma, USA) were incubated with kinase or phosphatase to regulate its phosphorylation levels as follows: (1) adding glycogen phosphorylase kinase (Phk, P2014, Sigma, USA) as high phosphorylation level group; (2) adding protein kinase A (PKA, P2645, Sigma, USA) as high phosphorylation level group; (3) adding alkaline phosphatase (AP, P0114, Sigma, USA) as low phosphorylation level group; (4) control group. The content of Phk, PKA, and AP were 5, 10, and 20 U per 100 μg protein, respectively. The content of added ATP was 2 μM per 100 μg protein. All groups were incubated at 4 °C in triplicates. Samples were collected at 0.5, 2, 6, 12, 24, 48, and 72 h of incubation.

2.2. Phosphorylation levels of glycogen phosphorylase

The phosphorylation level of GP was determined according to Chen et al. (2018).

2.3. Activity of glycogen phosphorylase

The activity of GP was determined according to Bai et al. (2020).

2.4. Secondary structure of glycogen phosphorylase

The determination of protein secondary structure by Fourier transform infrared (FTIR) spectroscopy was carried out by referring to Feng et al. (2020).

2.5. Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The phosphorylation sites of GP were analysed by LC-MS/MS, and the specific experimental parameters and methods were in accordance with Li et al. (2017). The results of the phosphorylation sites identified were shown in the Supplementary Table S1 (Supplementary files are available on the server of the Publisher).

2.6. Simulation of glycogen phosphorylase structure

The structure simulation of GP was carried out using Discovery Studio™ (DS) program with reference to Wang et al. (2023). The accession number of GP in Uniprot database is P00489. The data in structural simulation are shown in the Supplementary Table S2–S7 (Supplementary files are available on the server of the Publisher).

2.7. Statistical analysis

The data were analysed using SPSS Statistic 21.0 (IBM, Armonk, NY, USA). A general linear model and Fisher's Protected Least Significant Difference (LSD) test ($P < 0.05$) were used. All results were presented as means and standard deviation.

3. RESULTS AND DISCUSSION

3.1. Glycogen phosphorylase phosphorylation level

As shown in Fig. 1, the phosphorylation levels of GP in PKA and Phk groups increased during incubation. While the phosphorylation levels of GP in control and AP groups decreased. The phosphorylation level of GP in Phk group was higher than that in the other three groups within 12–72 h of incubation, and the phosphorylation level of GP in AP group was lowest within 2–72 h of incubation ($P < 0.05$).

The phosphorylation level of GP results indicated that Phk, PKA, and AP effectively catalysed the phosphorylation and dephosphorylation of GP.

3.2. Glycogen phosphorylase activity

The activity of GP in Phk and PKA groups increased during incubation, while decreased in AP and control groups (Fig. 2). The activity of GP in Phk group was nearly 8% higher than that in

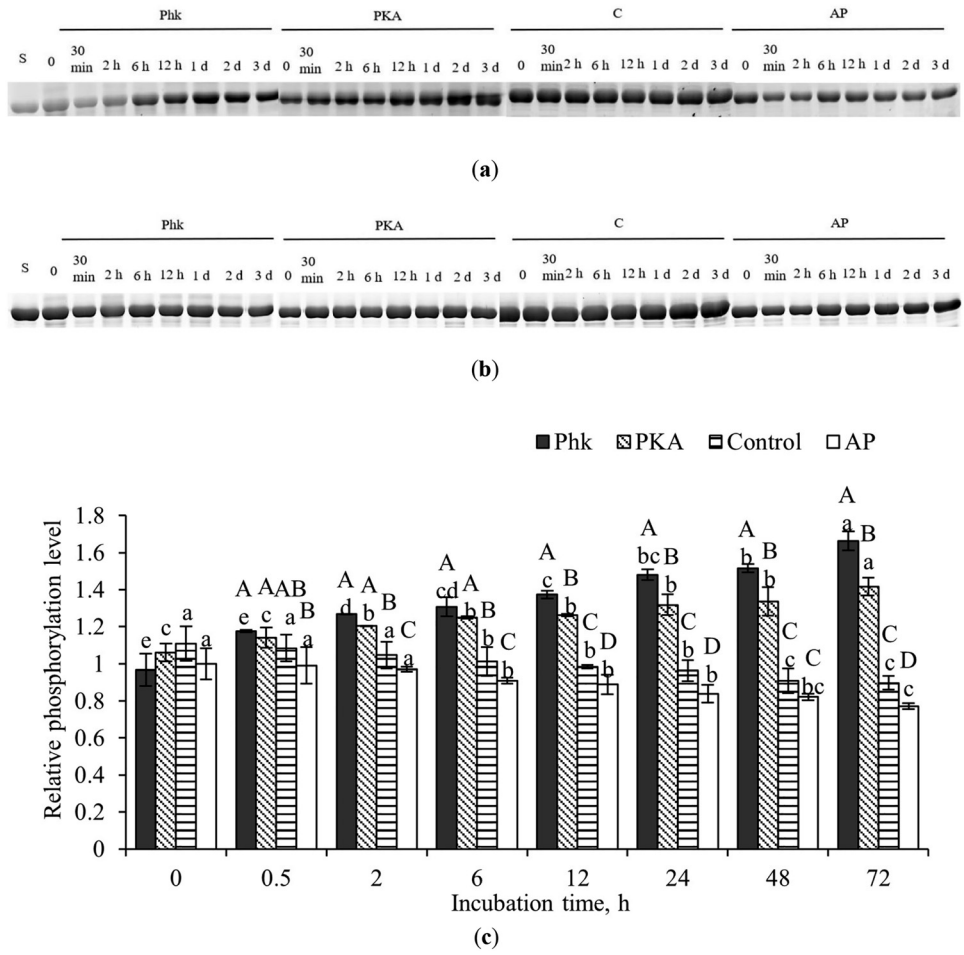

Fig. 1. Phosphorylation levels of glycogen phosphorylase during incubation at 4 °C *in vitro*. a–b: SDS-PAGE gels stained with Pro-Q Diamond (a) and SYPRO Ruby (b). c: Relative phosphorylation level. A–D: Different letters indicate significant differences between groups ($P < 0.05$). a–e: Different letters indicate significant differences at different incubation times ($P < 0.05$). S: standard glycogen phosphorylase, Phk: glycogen phosphorylase kinase; PKA: protein kinase; AP: alkaline phosphatase

PKA group. The activity of GP in the four groups were significantly different during 2–72 h of incubation ($P < 0.05$).

The changes of GP activity among the four groups were consistent with the changes of phosphorylation level during incubation. It also confirmed the positive correlation between phosphorylation level and activity of GP, which was consistent with the results in meat (Bai et al., 2020). The reason for the positive correlation was that the activity of GP was regulated by covalent modification of amino acids, and phosphorylation directly enhanced its activity (Huang et al., 2012). Protein phosphorylation changes the glycolytic rate by promoting the activity of GP, thus improving meat quality.

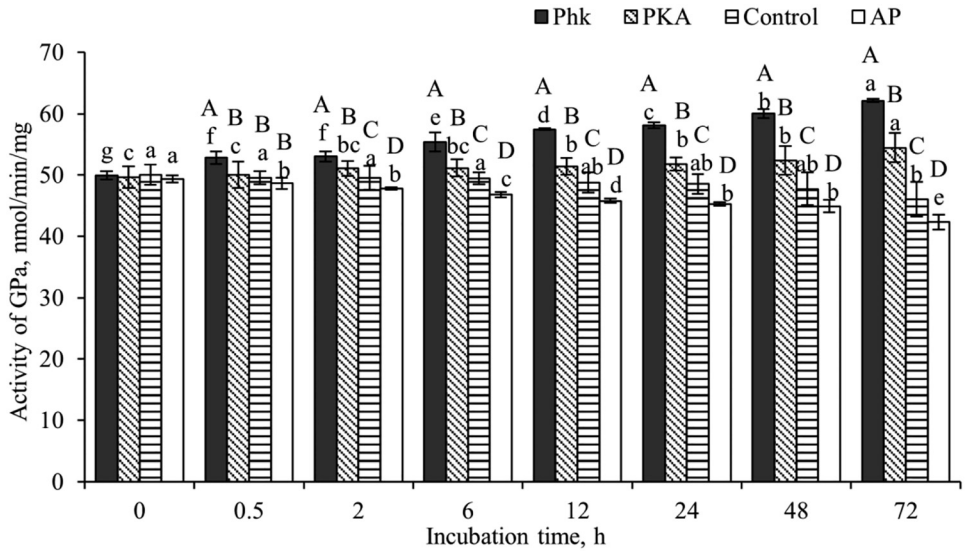

Fig. 2. Activity of glycogen phosphorylase during incubation at 4 °C *in vitro*. A–D: Different letters indicate significant differences between groups ($P < 0.05$). a–g: Different letters indicate significant differences at different incubation times ($P < 0.05$). Phk: glycogen phosphorylase kinase; PKA: protein kinase; AP: alkaline phosphatase

3.3. Secondary structure of glycogen phosphorylase

The secondary structure of the phosphorylated GP was dominated by the α-helix and β-sheet (Table 1). The content of α-helix in PKA group was higher than that in other groups ($P < 0.05$). The highest amount of β-sheet was found in Phk group. The content of nonregular coil in AP group was significantly higher than that in other groups.

The secondary structure of protein is divided into ordered and disordered structure. The ordered structure is represented by α-helix and β-sheet, and the disordered structure is represented by β-turn and nonregular coil (Makori et al., 2021). Among them, α-helix and β-sheet are the most important secondary structure of multiple proteins. The α-helix structure is maintained by intramolecular hydrogen bonds between carbonyl oxygen (C=O)

Table 1. The changes of glycogen phosphorylase secondary structure after phosphorylation/dephosphorylation (%)

Group	α-helix	β-sheet	β-turn	Nonregular coil	Total
Phk	29.82 ± 0.02^{b}	39.54 ± 0.02^{a}	11.36 ± 0.02^{c}	19.28 ± 0.01^{b}	1
PKA	41.84 ± 0.02^{a}	36.03 ± 0.03^{b}	8.66 ± 0.02^{c}	13.47 ± 0.01^{b}	1
Control	28.05 ± 0.01^{c}	25.67 ± 0.01^{c}	29.98 ± 0.01^{a}	16.30 ± 0.02^{b}	1
AP	13.13 ± 0.02^{d}	36.37 ± 0.01^{b}	25.77 ± 0.03^{b}	24.73 ± 0.04^{a}	1

[a–d]: Different letters mean significant difference at different groups ($P < 0.05$).
Phk: glycogen phosphorylase kinase; PKA: protein kinase; AP: alkaline phosphatase.

and amino hydrogen (NH) (Cao and Xiong, 2015). The β-sheet structure is usually stabilised by hydrogen bonds between peptide chains (Jheng et al., 2017). The higher content of ordered structure in Phk and PKA groups indicated that phosphorylation promoted the transformation of GP from disordered structure to ordered structure, leading to more stable structure and higher activity. The higher content of β-sheet in Phk group indicated that β-sheet plays a significant role in maintaining structural stability of GP and enhancing its activity.

3.4. Phosphorylation sites of glycogen phosphorylase

The number of phosphorylation sites identified in Phk, PKA, control, and AP groups were 14, 14, 10, and 7, respectively (Table 2 and Supplementary Table S1). The difference of phosphorylation sites between Phk and PKA groups was the Ser809 and Tyr525 in Phk group, and Thr304 and Tyr298 in PKA group. The three extra phosphorylation sites in control group were Ser2, Ser6, and Thr484 in comparison with AP group.

The higher phosphorylation level of GP in Phk group indicated that the phosphorylation level induced by serine was higher than that by threonine and tyrosine. The Ser2, Ser6, Ser277, Thr304, Tyr204, and Tyr298 were adjacent to the allosteric effect sites (Newgard et al., 1989), which was related to the binding of effectors. The proximity of Ser430, Thr341, Thr395, Thr484, and Tyr473 to glycogen storage sites was associated with glycogen decomposition (Sprang et al., 1988). Some amino acid residues in the N-terminal domain and the C-terminal domain (Ser514, Ser524, Ser809, Ser831, and Tyr525) combined to form deep slits, protecting and isolating the catalytic sites and creating an environment conducive to phosphorylation. In brief, the structure of GP changed after combination with phosphate groups, which may affect the binding between the enzyme and glycogen, leading to the changes of catalytic activity. Phosphorylation of GP regulates the glycolytic rate and meat quality by changing its structure.

Table 2. Number of phosphorylation sites of glycogen phosphorylase in four groups

Group	Total number of phosphorylation sites	Phosphorylation sites
Phk	14 (8 Ser, 3 Thr, 3 Tyr)	Ser 2, Ser 6, Ser 277, Ser 430, Ser 514, Ser 524, Ser 809, Ser 831 Thr 341, Thr 395, Thr 484 Tyr 204, Tyr 473, Tyr 525
PKA	14 (7 Ser, 4 Thr, 3 Tyr)	Ser 2, Ser 6, Ser 277, Ser 430, Ser 514, Ser 524, Ser 831 Thr 304, Thr 341, Thr 395, Thr 484 Tyr 204, Tyr 298, Tyr 473
Control	10 (5 Ser, 3 Thr, 2 Tyr)	Ser 2, Ser 6, Ser 514, Ser 524, Ser 831 Thr 341, Thr 395, Thr 484 Tyr 204, Tyr 473
AP	7 (3 Ser, 2 Thr, 2 Tyr)	Ser 514, Ser 524, Ser 831 Thr 341, Thr 395 Tyr 204, Tyr 473

3.5. Simulation of glycogen phosphorylase structure

Three models were obtained by analysing and comparing the amino acid sequence of GP (Supplementary Table S2). Model 2 was selected for structure simulation according to the probability density function (PDF), profile-3D, and Ramanchandran Plot (Supplementary Table S3 and S4) (Shen and Sali, 2006). The 3D structure simulation pictures of GP with different phosphorylation sites are shown in Fig. 3. The changes of dihedral angles were mainly reflected in serine (Supplementary Table S5).

The simulated spatial structure and stability were consistent with the secondary structure in Phk and PKA groups (Table 3). The results showed that phosphorylation regulated the activity of GP by changing its structure at different domains through phosphorylation sites. The structure of GP after phosphorylation in Phk group was the most stable, which may be the result of high phosphorylation level. The polypeptide fragments containing phosphorylation sites are disordered in the dephosphorylated GP, but they are folded into a twisted 3_{10} helix after phosphorylation. Phosphorylation changed the structure in N-terminal residues from an extended conformation to

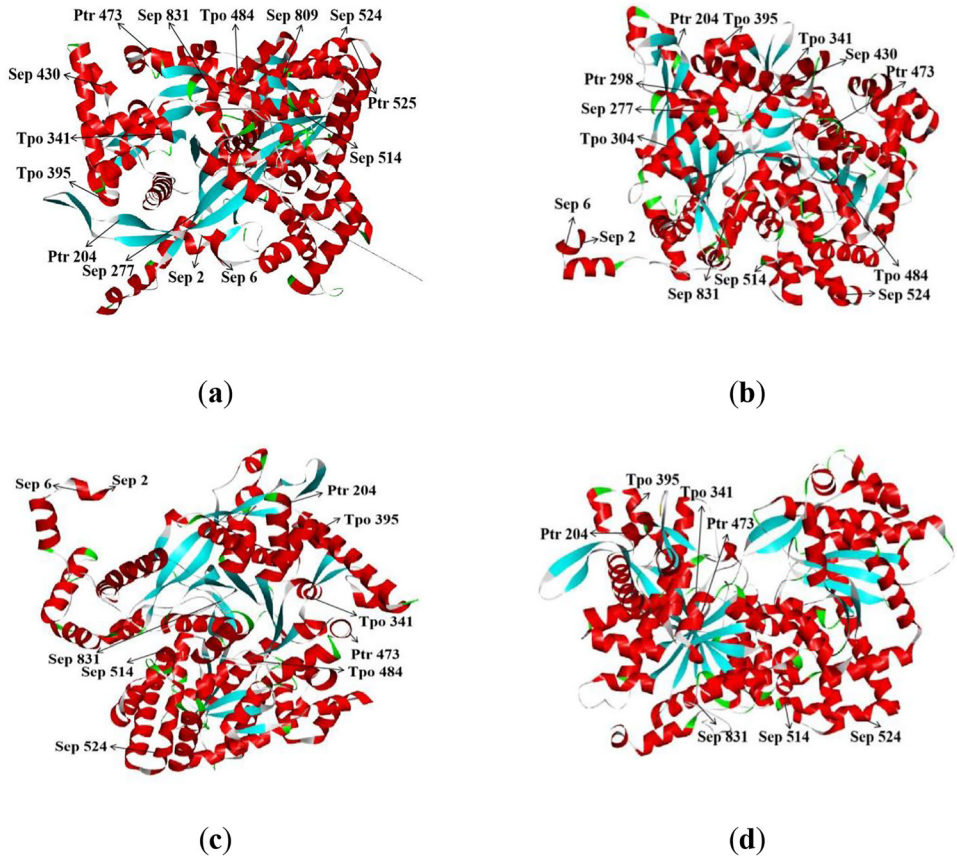

Fig. 3. 3D structure simulation pictures of glycogen phosphorylase with different phosphorylation level after mutations in serine, threonine, and tyrosine. a: Phk group; b: PKA group; c: control group; d: AP group

Table 3. Trajectory analysis of constructed glycogen phosphorylase 3D structures by amino acid mutations performed in DS program based on phosphorylation sites in four groups

Group	Bond energy (kcal/mol)	Electrostatic energy (kcal/mol)	Kinetic energy (kcal/mol)	Potential energy (kcal/mol)	Total energy (kcal/mol)
Phk	2,700.20	−399,744	74,993.80	−439,525	−364,532
PKA	2,698.66	−346,536	65,503.80	−382,055	−316,551
Control	2,653.34	−346,159	65,656.40	−382,184	−316,528
AP	2,682.91	−344,898	65,402.70	−380,838	−315,435

a twisted helix, and its contact changed from intra-subunit to inter-subunit (Johnson, 1992), which resulted in tighter structure. Phosphorylation promoted the activity of GP by making its spatial and secondary structure more tightly and protecting the catalytic or allosteric sites of GP. Protein phosphorylation makes the structure of GP more stable by making its spatial structure tighter, which is conducive to exerting catalytic activity and to regulate meat quality.

4. CONCLUSIONS

Phosphorylation of GP promoted its activity during incubation at 4 °C *in vitro*, and the phosphorylation effects of Phk was significantly higher than that of PKA. After phosphorylation, the secondary structure and spatial structure of GP changed from disorder to order. Phosphorylation promoted the activity of GP by making its secondary and spatial structure tighter, which provided a theoretical basis for regulation on glycolysis and meat quality by phosphorylation.

Conflict of interest: The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank the financial support of the Key Program from National Natural Science Foundation of China (32030086), Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2023-IFST) and the Central Public-interest Scientific Institution Basal Research Fund (S2020JBKY-16) in China.

SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2023.00178.

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