Phosvitin phosphorus is involved in chicken embryo bone formation through dephosphorylation

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ABSTRACT The aim of this study was to investigate the role of phosvitin in bone formation in chicken embryos. The yolk P content, P/N ratio and secondary structure of phosvitin, alkaline phosphatase (ALP) activity of the tibia, and body length were determined during incubation. A high correlation was found between the phosphate group content of phosvitin and both secondary structure and bone metabolism (ALP activity in the tibia, body length). The ALP activity and body length growth slightly lagged behind changes in the P/N ratio and the secondary structure of phosvitin. The phosphate content of phosvitin decreased, the γ-random coil and β-turn gradually transformed into α-helices, and the secondary structure of protein tended to become more orderly; these changes mainly occurred on d 13 to 16. Bone formation of the chicken embryos occurred primarily on d 14 to 18, whereas ALP activity and body length growth increased substantially. The results indicate that phosvitin P is involved in chicken embryo bone formation through dephosphorylation.

Key words: phosvitin, chicken embryo development, bone formation, dephosphorylation

INTRODUCTION

The balance of physiological phosphate levels is crucially important for bone health. Bench and clinical studies have confirmed that phosphate is a major factor in maintaining bone health, and a deficiency of phosphate results in bone pathology and clinical illness (Amanzadeh and Reilly, 2006; Tiosano and Hochberg, 2009; Marks et al., 2010). Moreover, as a constituent of hydroxyapatite, P is indispensable to bone formation and growth, and a continuous translocation of P from the circulatory system to the mineral formation sites occurs during this process (Vieira, 2007).

Phosphoprotein is a type of compound protein that binds covalently with phosphate radicals. Phosphoprotein is closely related to bone and dentin mineralization. Phosphate groups bind covalently onto proteins such as osteopontin (Gericke et al., 2005), bone sialoprotein (Gordon et al., 2007), dentin phosphoprotein (MacDougall et al., 1992; Hao et al., 2009), and dentin matrix protein (He et al., 2003; He and George, 2004) play a direct role in the nucleation of Ca-P crystals during mineralization (Glimcher, 1989; Salih et al., 2002; Hao et al., 2009).

Phosvitin is an abundant highly phosphorylated protein. A detailed analysis of its amino acid sequence indicated that serine accounts for more than 55% of the total amino acids of phosvitin, of which almost 98% are phosphoserines (Byrne et al., 1984). Phosvitin has a very strong divalent metal binding capacity (Hegenauer et al., 1979; Grogan and Taborsky, 1986; Oscar et al., 2008) as well as a calcium phosphate crystal nucleation property (Onuma, 2005), giving it biological functions in common with bone phosphoproteins. However, the name phosvitin indicates that it contains a high amount of P, and it contains approximately 80% of an egg yolk’s P (Yamamoto et al., 1996). Chicken embryonic development is special. All nutrients needed by the embryo come from the egg (Moran, 2007); thus, phosvitin serves as the main source of P for chicken embryo bone formation (Richards, 1997). These results suggest that phosvitin plays an important role in bone formation; however, it is not clear how phosvitin is involved in this physiological process.

MATERIALS AND METHODS

Materials

Fertile eggs were obtained from the veterinary hospital of Huazhong Agriculture University. The eggs used were from White Leghorn hens. They were selected for uniformity of size, shape, and shell texture.
and were incubated soon after laying. An alkaline phosphatase (ALP) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). All other regents used were of analytical grade.

**Experimental Design**

All experiments were conducted on fertile eggs. In total, 300 newly laid eggs from White Leghorn hens were selected from a single flock; they weighed between 54 and 62 g. The fertile eggs were incubated in an EIF/CDM4200 automatic hatching machine under controlled conditions: temperature, 1 to 18 d 38°C, 19 to 21 d 37.5°C; RH, 1 to 21 d 60 to 65%; and automatic egg turning every 2 h. Incubated fertile eggs were opened carefully, and the yolk, albumen, and embryos were separated. The yolk P content was analyzed every 2 d. Phosvitin’s P/N ratio and secondary structure were analyzed daily from 9 to 14 d, and at 16, 18, and 20 d of incubation. Tibia ALP activity and the growth rate of the embryo’s body length were analyzed daily from d 12 to 21.

**Analytical Methods**

**Isolation and Purification of Phosvitin.** The phosvitin samples were prepared in our laboratory using the method described by Zhang et al. (2011). Briefly, yolk samples were isolated from fertile eggs after different lengths of incubation. Subsequently, the sediment (granules) was separated from the supernatant (plasma) at 12,000 × g for 10 min, 4°C, first with an equal mass of distilled water for 10 min and then with an equal mass of 0.17 M NaCl solution for 10 min. The granules were dissolved in a 1.74 M NaCl solution (1:10 wt/vol), the pH was adjusted to 4.0, and then the solution was homogenized with 3% PEG6000 wt/wt and centrifuged at 12,000 × g for 10 min, 4°C. The supernatant was exhaustively dialyzed against distilled water, centrifuged at 12,000 × g for 10 min, 4°C, to remove insoluble particles, and lyophilized. Further purification was performed using Toyopearl DEAE-650M column (DEAE) through a gradient elution to obtain a protein free of polyvalent metals.

**Determination of the Total P Content of Egg Yolk.** Yolk samples were digested using HNO3 and HClO4; then, the total P content was determined using the molybdenum blue method (Murphy and Riley, 1962). This analysis was performed on 3 replicates for each sample.

**Determination of the P and N Content of Phosvitin.** Phosvitin samples were digested using concentrated sulfuric acid; then, the P content was determined by the molybdenum blue method, and the N content was quantified using an AA3 Continuous Flow Analytical System (Zhang et al., 2006). Three replicates were performed.

**Secondary Structure Analysis.** The Fourier transform infrared spectroscopy (FT-IR) spectra were used to determine the secondary structures of phosvitin. The FT-IR spectra were analyzed using an Avatar 330 Fourier transform infrared spectrometer (US Nicolet, Shanghai, China) with a DTGS detector (wave number range of 650–4,000 cm⁻¹, resolution of 4.0 cm⁻¹). A total of 32 scans were collected for each spectrum. To minimize the effect of base line, the FT-IR spectra were autoscaled. Then, FT-IR data were also pretreated by Fourier self-deconvolution, second derivative, and band curve-fitting. And the relative contents of different secondary structures were determined according to area under the curve after band curve-fitting. Two replicates were performed.

**Measurement of ALP Activity.** We accurately weighed 0.1000 g of chick tibia isolated from a chicken embryo, removed tibial epiphysis and soft tissues, added 1 mL of saline, and used a glass homogenizer to obtain tissue homogenates; centrifugation at 3,000 × g for 10 min at 4°C was used to separate the supernatant, which was used to quantify the ALP activity. Three replicates were performed.

**Measurement of Embryo Body Length.** The separated chicken embryo was arranged flat on a dish in a fully extended state and then directly measured with a Vernier caliper. The distance from the top of the head to the tail was measured as the embryo body length. Three replicate measurements were performed.

**RESULTS AND DISCUSSION**

**Changes in Yolk P During Incubation**

The changes in yolk P content during incubation are illustrated in Figure 1. The total P content decreased gradually during incubation. A sharp drop in the total P content of the yolk was apparent after 9 d of incubation, and more than half disappeared by d 17. The fastest rate of decline occurred at d 13 to 17. Embryos consumed most of the P in the yolk during incubation.

To determine whether this P was part of phosvitin, we designed the following experiment.

**Changes in the P/N Ratio of Phosvitin During Incubation**

This study required accurate characterization of the phosphate group content of phosvitin. This study adopted the P/N ratio to identify changes in the phosphate radical content of phosvitin during incubation (from d 9–14, 16, 18, and 20). The P/N ratio is the ratio of the P content and N content of phosvitin, where the P content is indirectly expressed as the phosphate group content of phosvitin and the N content is directly expressed as the molar content of phosvitin. The P/N ratio is a relative value, thus avoiding the deviation caused by different extraction yields. The greater
the P/N ratio, the more phosphate groups that phosvitin has.

Changes in the total P content of egg yolk during incubation indicated that a drop in the total P content of the yolk was not apparent until after 9 d of incubation. Because phosvitin contains approximately 90% of the P of the yolk, changes in yolk P indirectly reflect the changes in phosvitin. To reduce the workload, we measured the phosvitin phosphate group content on d 9 to 14, 16, 18, and 20. Figure 2 shows the changes in the P/N ratio of phosvitin during incubation.

The observed changes in the P/N ratio of phosvitin during incubation indicate that the phosvitin P/N ratio was basically stable from d 9 to 13. Then, a rapid decline occurred until d 20, when the minimum value of 0.06 was reached. The decrease was rapid from d 13 to 16 and then continued at a slower pace.

During incubation, as phosvitin is degraded to provide P to enhance the absorption of Ca and accumulation of bones, the P content may decrease. Our result indicated that there was a drop during chick embryonic development. Vargas et al. (2009) also reported that the chick embryo exhibited a regular pattern and reabsorption of calcium during the latter half of incubation, which supported our conclusion.

Assuming that dephosphorylation led to the reduction in the P/N ratio, the secondary structure of phosvitin would have changed. Thus, the next experiment addressed the secondary structure of phosvitin during incubation.

**Phosvitin Secondary Structure Analysis During Incubation**

The phosvitin isolated and purified from yolks of eggs incubated for different lengths of time was analyzed by FT-IR conformational analysis. The spectrograms are shown in Figure 3. All phosvitin samples investigated displayed strong similarities in the spectrogram, but the infrared absorption decreased at some wavelengths. This change suggests that only specific amino acid residues changed, without significant changes in most amino acids. The $\text{PO}_4^{3-}$ group has a characteristic absorption peak at 1,100–920 cm$^{-1}$; the characteristic absorption peak of the $\text{PO}_4^{3-}$ group of phosphoserine in phosvitin is 980 cm$^{-1}$. This absorption peak intensity decreased significantly, and the $\text{PO}_4^{3-}$ group of phosvitin decreased during incubation.

To analyze the amide I band component, the Fourier self-deconvolution second derivative spectra must be curve-fitted. The amide I band component and each secondary structure element were assigned based on Dong et al. (1990), who obtained the secondary structure of phosvitin during incubation. The results are shown in Table 1. An obvious change trend can be observed in the secondary structure of phosvitin. The content of the $\gamma$-random coil is relatively stable at d 9 to 13 of incubation, at approximately 9%; however, it then disappeared after 13 d of incubation. The $\alpha$-helix exhibits a clear upward trend during incubation, with a sudden rise on d 14 followed by stability at approximately
31%. The β-turn exhibits a downward trend during incubation. The decline is obvious from d 12 to 16, with the largest drop appearing between d 13 and 14. The β-sheet exhibited no significant change and fluctuated near 18%. These data suggest that the γ-random coil and β-turn gradually transformed into α-helices during incubation and that the secondary structure of phosvitin tended to become more orderly. A circular dichroism spectrometer was also used to measure the change in the secondary structure of phosvitin; the results are consistent with those from the infrared spectrum and thus are not listed here.

When evaluating the changes in phosvitin’s secondary structure and P/N ratio, both changes occurred primarily between d 13 and 16. The P/N ratio decreased, whereas the α-helix increased, and the secondary structure tended to become more orderly. We considered this structural change to be caused by dephosphorylation. Other studies have shown that phosphorylation can impede the formation of the amphipathic α-helix; this conclusion is also supported by molecular dynamics simulations (Vassall et al., 2013). Missimer et al. (2012) postulated that phosphorylation destabilizes the peptide’s secondary structure by disrupting the salt bridges that support its helical conformation. Thus, the assumption that dephosphorylation leads to the reduction in the P/N ratio is supported.

During chick embryonic development, phosvitin serves as the main source of P for chicken embryo bone formation; it contains approximately 80% of the P in egg yolk, so the P generated through dephosphorylation must be involved in bone formation. To further study the correlation between phosphate groups from phosvitin and bone formation, the following experiment was designed.

**Changes in the ALP Activity of Embryo Tibia During Incubation**

Alkaline phosphatase is an osteoblast marker enzyme that can produce free phosphate through the decomposition of phosphate compounds (Li et al., 1997). Phosphate combines with the calcium present in the cellular matrix to form calcium phosphate, which is involved in regulating the calcification process. Alkaline phosphatase is a sign of mature osteoblasts and is an important indicator of bone metabolism (Alborzi et al., 1996; Zhang et al., 2012). Alkaline phosphatase activity can reflect the status of bone metabolism. The greater the ALP activity is, the faster the bone metabolism and formation are.

Changes in the ALP activity of the embryo tibia during incubation are shown in Figure 4. The ALP activity initially increased, reaching a maximum of 1.12 U/g of protein at d 15; then, this activity decreased sharply on d 16 to 18, stabilized, and finally decreased again until hatching, reaching values as low as 0.39 U/g of protein. These results show that the speed of bone formation...
first increased, then decreased, with the speed reaching its maximum on d 15.

Comparing the change in ALP activity with the result of the phosvitin P/N ratio, a high correlation was observed. The rate at which the P/N ratio decreased was positively correlated with ALP activity. The P/N ratio declined most rapidly when ALP activity was greatest; the lower the ALP activity was, the more slowly the P/N ratio decreased. The phosvitin secondary structure also changed correspondingly.

**Changes in the Growth Rate of Embryo Body Length**

The change in the growth rate of embryo body length, which can reflect the embryo bone metabolism at the macro level, is illustrated in Figure 5. The growth rate fluctuated at approximately 0.6 cm/d during d 12 to 16. After that, the growth rate increased significantly at d 17 to 18, at 1.1 cm/d, and then dropped significantly to as low as 0.01 cm/d until hatching. The re-
Results showed that bone metabolism is active on d 12 to 18 of incubation and is fastest on d 17. The growth rate of body length was positively correlated with ALP activity, and the 2 measures exhibited the same trend. However, growth rate lagged behind ALP activity.

Conclusion

By studying the change in the P/N ratio, this study found that the amount of P in phosvitin decreased during incubation. This study proved that dephosphorylation, combined with the change in secondary structure, led to the reduction in the P/N ratio, with α-helix increasing and the secondary structure of phosvitin tending to become more orderly. Additional experiments revealed that the rate at which the P/N ratio decreased was positively correlated with ALP activity and the growth rate of body length, reflecting the degree to which phosvitin phosphorylation is closely related to the bone metabolism of chicken embryos. Faster bone metabolism is linked to a more rapid decrease in the phosvitin phosphate group content.

In summary, there were correlated changes in the P/N ratio and secondary structure of phosvitin, ALP activity, and body length growth. The ALP activity and body length growth slightly lagged behind changes in the P/N ratio and the secondary structure of phosvitin. The phosphate content of phosvitin decreased, the γ-random coil and β-turn gradually transformed into α-helices, and the secondary structure of protein tended to become more orderly.

Table 1. Phosvitin secondary structure analysis during incubation

<table>
<thead>
<tr>
<th>Day</th>
<th>β-sheet (%)</th>
<th>γ-Random coil (%)</th>
<th>α-Helix (%)</th>
<th>β-Turn (%)</th>
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<td>9</td>
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<tr>
<td>10</td>
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<td>7.09</td>
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<td>20</td>
<td>21.46</td>
<td>0.00</td>
<td>29.81</td>
<td>48.73</td>
</tr>
</tbody>
</table>

1Fourier transform infrared spectroscopy data was pretreated by Fourier self-deconvolution, second derivative, and band curve-fitting. Then the relative contents of different secondary structures were determined according to area under the curve after band curve-fitting. Two replicates were performed. Phosvitin secondary structure analysis on d 9 to 14, 16, 18, and 20 were listed.

Figure 4. Changes in the alkaline phosphatase (ALP) activity of chicken embryo tibias during incubation. The ALP activity on d 12 to 21 was measured.
to become more orderly; these changes mainly occurred on d 13 to 16. Bone formation of the chicken embryos occurred primarily on d 14 to 18, whereas ALP activity and body length growth increased substantially. All of these findings demonstrate that the P in phosvitin is involved in chicken embryo bone formation through dephosphorylation.

Our research revealed that ALP activity and P/N ratio exhibit the same trend. Alkaline phosphatase can produce free phosphate through the decomposition of phosphate compounds. The questions of whether ALP plays a role in the process of phosvitin dephosphorylation and how that process occurs require further study. Our research demonstrates that the P in phosvitin is involved in chicken embryo bone formation through dephosphorylation. Thus, would injecting inorganic P into an embryo also promote bone formation? Does the chicken embryo have special access to P using phosvitin? These questions also merit further study.

**REFERENCES**


