# PHOSPHORYLATED SITES OF CALF THYMUS H2B HISTONE BY ADENOSINE 3':5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM BOVINE CEREBELLUM*

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SUMMARY: Phosphorylated sites of calf thymus H2B histone were studied with adenosine 3':5'-monophosphate-dependent protein kinase partially purified from bovine cerebellum. Amino acid analysis of the phosphopeptides which were obtained by proteolytic digestion revealed that the enzyme had the ability to phosphorylate Ser-32 as well as Ser-36. The evidence together with the previous results which were obtained with silkworm enzyme (Hashimoto,E., Takeda,M., Nishizuka,Y., Hamana,K. and Iwai,K. (1975) Biochem. Biophys. Res. Commun., 66, 547-555) seems to suggest that this class of enzymes lacks tissue- as well as species-specificities in their catalytic properties.

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It has been reported that adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase phosphorylates five major fractions of calf thymus histone (1). Recently, the primary sequences surrounding the phosphorylated sites have been elucidated for H1, H2A, and H2B histones (2-5). Shlyapnikov et al. (3) have reported that pig brain enzyme is capable of phosphorylating Ser-14 and Ser-36 of H2B histone. However, a preceding report from our laboratories has described that the enzyme which is partially purified from silkworm pupae (Bombyx mori) phosphorylates Ser-32 as well as Ser-36 (5). Judging from the ability to activate phos-

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phorylase kinase (6-8), it is suggested that cyclic AMP-dependent protein kinases obtained from various sources do not show species- nor tissue-specificity, at least in their functional properties. The experiments described here were designed to show the lack of species-specificity of this class of enzymes in the phosphorylation of H2B histone. The evidence obtained in this study together with that previously described (5) will indicate that the same specific sites in H2B histone, Ser-32 and Ser-36, are phosphorylated by cyclic AMP-dependent protein kinases from bovine cerebellum and silkworm. Cyclic AMP-dependent protein kinase will be tentatively referred to hereafter as protein kinase A.

H2B histone was prepared by the method of Oliver et al. (9). Protein kinase A was prepared from bovine cerebellum by the method described by Takai et al. (10) except that Sephadex G-150 was used instead of Sephadex G-200 and gel filtration was repeated twice. The enzyme fraction with specific activity of about 24 units per mg of protein was employed for the present studies.[1/] The purified preparation was free of endogenous phosphate acceptor under the conditions employed for the present study.

H2B histone (200 mg) was phosphorylated by cerebellar protein kinase A (3.2 mg) in the reaction mixture (300 ml) which contained 20 mM Tris-HCl at pH 7.5, 5 mM magnesium acetate, 100 μM [$\gamma$-$^{32}$P]ATP ($11.2 \times 10^6$ cpm/μmole), and 1 μM cyclic AMP. After 2 hours at 30° the reaction nearly leveled off, and about 0.9 mole of phosphate was incorporated per mole of the histone as judged by the acid-precipitable radioactivity. The radioactive H2B histone (186 mg protein, 10.5 μmoles $^{32}$P) was isolated from the reaction mixture

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1/ One unit of enzyme was defined as that amount of enzyme which incorporated 1 nmole of phosphate from ATP into acid-precipitable material per min under the conditions described previously (10).

as described previously (5), and finally washed once with acetone-HCl (0.5 ml 36% HCl in 100 ml acetone) and once with acetone. The dried histone was suspended in 36 ml of 0.1 M ammonium carbonate buffer at pH 8.5, and then digested with trypsin (Worthington, Code TRTPCK) (trypsin:histone = 1:50 (w/w)) for 4 hours at 37°. After the mixture was lyophilized, the radioactive tryptic phosphopeptides were taken up with 3 ml of 50% (w/v) acetic acid and subjected to gel filtration on a Sephadex G-15 column. As shown in Fig. 1, the

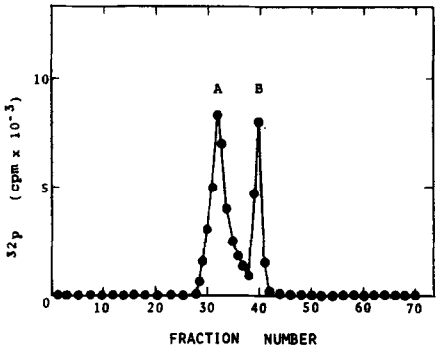


Fig. 1. Sephadex G-15 gel filtration of tryptic phosphopeptides from H2B histone which was phosphorylated by cerebellar protein kinase A. A Sephadex G-15 column (112 x 1.7 cm) was equilibrated with 50% (w/v) acetic acid. Elution was performed downward with the same acetic acid solution at a flow rate of 11 ml per hour. Fractions of 3 ml each were collected, and a 5 µl aliquot of each fraction was determined for the radioactivity with a Nuclear-Chicago Geiger Muller gas flow counter, model 4338.

radioactive tryptic phosphopeptides were resolved into two fractions, A and B (7.13 µmoles $^{32}P$ in A; 2.86 µmoles $^{32}P$ in B). Upon chromatography on an SP-Sephadex column, the fraction A was resolved further into three major ($A'_4$, $A_4$ and $A_7$) and four minor peaks ($A_1$, $A_3$, $A_5$ and $A_6$)[2/] and the fraction B was into one major ($B_1$) and

2/ When these radioactive phosphopeptides are compared with those obtained from H2B histone phosphorylated by silkworm protein kinase A (see Fig. 2 in Ref. 5), peak 2 is not detected and peak 4 is resolved into two peaks, $A_4$ and $A'_4$.

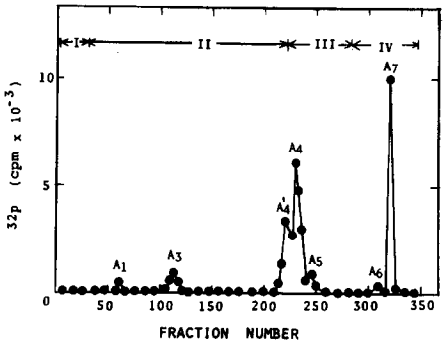


Fig. 2. SP-Sephadex column chromatography of tryptic phosphopeptide A. Peak A in Fig. 1 (7.13 µmoles $^{32}P$) was lyophilized and taken up with 5 ml of 0.1 M pyridine-acetic acid buffer at pH 3.1 and applied to an SP-Sephadex column (50 x 2.2 cm) equilibrated with the same buffer. Elution was performed downward at a flow rate of 16 ml per hour with the following buffers; (I) 140 ml of 0.1 M pyridine-acetic acid buffer at pH 3.1; (II) a linear gradient (total volume, 1,000 ml) formed from 0.1 M at pH 3.1 and 0.25 M at pH 3.25 pyridine-acetic acid buffer; (III) 250 ml of 0.25 M pyridine-acetic acid buffer at pH 3.25; (IV) a linear gradient (total volume, 500 ml) formed from 0.25 M pyridine-acetic acid buffer at pH 3.25 and 8.25 M pyridine. Fractions of 5 ml each were collected and a 50 µl aliquot of each fraction was determined for the radioactivity with a Packard Tri-Carb liquid scintillation spectrometer, model 3320, with Cerenkov radiation (11).

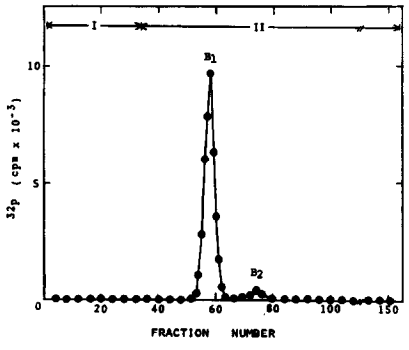


Fig. 3. SP-Sephadex column chromatography of tryptic phosphopeptide B. Peak B in Fig. 1 (2.86 µmoles $^{32}P$) was applied to an SP-Sephadex column (30 x 2.2 cm) equilibrated with 0.1 M pyridine-acetic acid buffer at pH 3.1. Elution was performed downward at a flow rate of 33 ml per hour with the following buffers; (I) 165 ml of 0.1 M pyridine-acetic acid buffer at pH 3.1; (II) a linear gradient (total volume 600 ml) formed from 0.1 M at pH 3.1 and 0.25 M at pH 3.25 pyridine-acetic acid buffer. Fractions of 5 ml each were collected and the radioactivity was measured under the conditions specified in the legend to Fig. 2.

one minor ($B_2$) peaks as shown in Figs. 2 and 3. An aliquot of each major fraction (950 nmoles $^{32}P$ in $A'_4$; 2.06 μmoles $^{32}P$ in $A_4$; 900 nmoles $^{32}P$ in $A_7$; 2.19 μmoles $^{32}P$ in $B_1$) was lyophilized and further purified by the chromatography on a QAE-Sephadex column under the conditions specified earlier (5). Peaks $A_4$, $A_7$ and $B_1$ were eluted as a single peak at about 0.4 M, 0.06 M and 0.3 M ammonium bicarbonate buffer at pH 8.5, respectively. Peak $A'_4$ was eluted at about 0.55 M with a 700 ml-linear concentration gradient (500 mM to 1 M) of the same buffer. After gel filtration on a Sephadex G-10 column each phosphopeptide was hydrolyzed and analyzed for amino acid composition as described previously (5). The results shown in Table I together with the known primary structure of H2B histone (12,13) indicate that the amino acid sequences of $A_4$, $A_7$ and $B_1$ were Lys-Glu-$Ser^{36}$-Tyr-$Ser^{38}$-Val-Tyr-Val-Tyr-Lys, Lys-Arg-$Ser^{32}$-Arg and $Ser^{32}$-Arg, respectively. The phosphopeptide $A_4$ contained two moles of serine (Ser-36 and Ser-38) and one mole of phosphate per mole of peptide. When the latter phosphopeptide was digested further with chymotrypsin under the conditions described previously (5), a phosphopeptide with the sequence of Lys-Glu-$Ser^{36}$-Tyr ($A_{4C}$ in Table I) was quantitatively produced. It was concluded, therefore, that one of the main phosphorylated sites in H2B histone was Ser-36. Another main phosphorylated site was identified as Ser-32 from the amino acid sequence of phosphopeptide $A_7$ and that of phosphopeptide $B_1$. The phosphopeptide $A_7$ seemed to be a product of incomplete digestion. The phosphopeptide $A'_4$ showed an amino acid sequence of $Ser^{32}$-Arg-Lys-Glu-$Ser^{36}$-Tyr-$Ser^{38}$-Val-Tyr-Val-Tyr-Lys and contained two moles of phosphate per each mole of the peptide. Therefore, this peptide was identified to be another product of incomplete tryptic digestion. Approximately 50% and 35% of the initial total acid-precipitable

Table I

*Amino acid compositions of tryptic and chymotryptic phosphopeptides from H2B histone phosphorylated by cerebellar protein kinase A*[a/]

| Amino acid | Phosphopeptide | | | | |
|---|---|---|---|---|---|
| | A'4 | $A_4$ | $A_{4C}$ | $A_7$ | $B_1$ |
| Lys | 1.8 | 1.9 | 0.9 | 0.9 | |
| Arg | 0.9 | | | 2.1 | 1.1 |
| Ser[b/] | 3.1 | 2.1 | 1.1 | 1.1 | 0.9 |
| Glu | 1.3 | 1.1 | 1.0 | | |
| Val | 2.0 | 1.9 | | | |
| Tyr | 2.9 | 3.0 | 1.0 | | |
| $^{32}P$[c/] | 1.9 | 1.2 | 1.2 | 1.2 | 1.2 |

a/ The data are presented in molar ratios.
b/ Corrected for 15% loss during hydrolysis.
c/ Calculated from radioactivity.

radioactivity were calculated to be incorporated into Ser-32 and Ser-36, respectively.

It has been proposed that Ser-36 is a possible candidate for the site in H2B histone which may be phosphorylated by protein kinases A obtained from pig brain (3) and human tonsillar lymphocytes (4). In confirmation of these observations, the preceding report (5) has presented evidence that Ser-36 is one of the major sites for silkworm protein kinase A by isolating phosphotetrapeptide Lys-Glu-$\overset{36}{\text{Ser}}$(P)-Tyr. In addition, the silkworm enzyme has been shown to be capable of phosphorylating Ser-32 in the same

histone (5). The experimental results presented in this paper now provide evidence that protein kinase A from bovine cerebellum has the capacity to phosphorylate Ser-32 as well as Ser-36. Using pig brain enzyme, Shlyapnikov et al. (3) have isolated Lys-Gly-$Ser^{14}$(P)-Lys as one of the major tryptic phosphopeptides. However, this phosphopeptide has not been detected as a major product in the present study. The experimental results seem to be compatible with the supposition that protein kinases A from various vertebral and nonvertebral organisms lack species- as well as tissue-specificities in their catalytic and functional properties.

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