Chromatin-associated Protein Phosphokinases of Rat Ventral Prostate

CHARACTERISTICS AND EFFECTS OF ANDROGENIC STATUS*

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Protein phosphokinase activity endogenous to rat ventral prostate chromatin was assayed by using dephosphophosvitin as an exogenous substrate. For maximal activity of the kinase reaction, the presence of 200 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol was essential. Two apparent pH optima were observed, a broad one between pH 7 and 7.4, and one at pH 7.89. At pH 7.4 the apparent Kₘ for 31% dephosphophosvitin was 0.3 mg per ml. With respect to ATP, two apparent Kₘ values (0.04 and 0.41 mM) were found. The kinase activity was minimal toward exogenous histones when used as substrates (3% for lysine-rich and 0.3% for arginine-rich (f₁) histones, compared with dephosphophosvitin controls). The protein phosphokinases were not significantly stimulated by cyclic adenosine 3':5'-monophosphate (cyclic AMP) when histones were used as substrate. With dephosphophosvitin as substrate, cyclic AMP produced a small inhibition (5 to 15%).

Orchiectomy of adult rats resulted in a rapid decline in the chromatin-associated protein phosphokinase activity assayed using optimal experimental conditions described above. At 9 hours postorchiectomy, a 30% decline in the activity was observed; this was further reduced to about 50% of the control by 18 hours. This decrease in the kinase activity (e.g. at 9 hours postorchiectomy) appears to precede measurable changes in the protein and RNA complements of chromatin. Testosterone replacement following orchiectomy abolished this decline in the chromatin-associated activity. The chromatin-associated protein phosphokinase activity toward lysine-rich and arginine-rich histones was also sensitive to androgenic status of the animals and declined rapidly postorchiectomy. The results suggest the presence of multiple and androgen-sensitive protein phosphokinases associated with rat ventral prostate chromatin, which may modulate the phosphorylation of nuclear nonhistone phosphoproteins (AHMED, K. (1971) Biochim. Biophys. Acta 243, 38; AHMED, K., AND ISHIDA, H. (1971) Mol. Pharmacol. 7, 323) with changing gene action mediated by testosterone in this target tissue.

In previous work (2, 3) we reported that rat ventral prostate nuclei in vitro could incorporate ³²P from [γ-³²P]ATP into nonhistone (acidic) and histone phosphoproteins, the former being the predominantly labeled fraction. Further, this phosphorylation of prostate nuclear proteins was sensitive to the androgenic status of the animal. We also demonstrated (2, 3) that the stimulated rates of phosphorylation of nuclear nonhistone phosphoproteins, observed in vitro, correlated with increased gene activity in the prostate, in response to the hormonal treatment of orchietomized rats. Concurrent with these observations, Reddi et al. (4) reported that rat ventral prostate cytosol cyclic adenosine 3':5'-monophosphate-stimulated protein phosphokinase was unaffected by the androgenic status of the animals. On the other hand, our results (3) obtained from working with purified prostatic nuclei, did indicate that the androgen-mediated changes in the nuclear phosphoprotein phosphorylation could possibly be explained in terms of early in vivo alterations in the activity of protein phosphokinase(s) rather than the levels of phosphate acceptor proteins endogenous to the nucleus. Hence, the present work was undertaken to establish the presence of androgen-sensitive protein phosphokinase(s) associated with prostatic nuclei and to delineate their general properties. For this purpose, we chose to utilize an exogenous phosphate acceptor protein substrate added in saturating concentrations; partially dephosphorylated egg yolk phosphoprotein, phosvitin, was found suitable for such a study (5).
The possibility that chromosomal proteins and their post-synthetic modifications may have a regulatory role in the control of gene action has aroused a considerable interest in the study of these proteins and the enzymes involved in their modification (2-22). The nuclear nonhistone acid proteins and phosphoproteins derived from them are tissue-specific, and have been shown to stimulate the DNA-dependent RNA polymerase reaction in vitro (23-25). Recent reports on the protein phosphokinases associated with rat liver nuclei suggest a multiplicity (26-28) and tissue-specific variations (28) of these enzymes. Further, it appears that specific temporal changes in the activity of chromatin-associated protein phosphokinase(s) do take place in the prereplicative phase of the cell cycle (29). Thus, studies of protein phosphokinases associated with various eukaryotic nuclei are of importance to understand the factors which may modulate phosphorylation of the various chromosomal proteins.

Rat ventral prostate is an excellent model for such a study for several reasons. The strict control of the development and activity of this tissue by androgens is amply documented (30-35). Gene activity in rat ventral prostate can be physiologically controlled by deprivation or administration of testosterone. One of the earliest effects of administration of testosterone to orchiectomized rats is an increase in the activity of nuclear DNA-dependent RNA polymerase activity (31-35) which, as mentioned before, correlates temporally with the increased phosphorylation of nuclear nonhistone phosphoproteins (2, 3). Thus, the present studies are of interest not only for the reasons given above, but also for their possible relevance to the eventual understanding of the molecular mechanism of action of testosterone in target tissues such as the ventral prostate.

EXPERIMENTAL PROCEDURES

Materials

Animals-Male Sprague-Dawley rats weighing 255 to 325 g (from ARS/Sprague-Dawley Co., Madison, Wis.) were used as the source of ventral prostate. They were given standard laboratory diet and water ad libitum, while being maintained on a constant photoperiod of 14 hours of light and 10 hours of dark.

Chemicals-Testosterone propionate was obtained from Sigma Chemical Co., St. Louis, Mo. Arginine-rich (\(\epsilon\)) and lysine-rich histones were purchased from Worthington Biochemical Corp., Freehold, N. J. All other reagents used were of the highest purity available.

Methods

Preparation of Chromatin-The preparation and properties of purified rat ventral prostate nuclei have been described earlier (2, 3) except for the modification that the original tissue homogenate (2) was prepared with 12 strokes (up and down counted as one stroke) in a glass homogenizer equipped with a Teflon pestle rotating at 280 rpm. Purified nuclei (from 12 to 24 normal rats or 24 to 36 orchietomized rats) were used as the source of water-soluble chromatin prepared by adapting the method of Ueda et al. (36). The ultraviolet absorption spectrum characteristics of these preparations have been described previously (5); the chemical composition is given in Table II. The chromatin preparation (as HzO solution, generally 1.5 mg/ml of original tissue wet weight) was stored in small aliquots at -20°. Freezing the chromatin solution caused gelation, but it was dispersed easily on gentle homogenization. The protein phosphokinase activity associated with the preparation was not significantly changed over a period of about 2 weeks. Longer storage at -20° resulted in a gradual loss of activity, and hence, each preparation was tested within 1 or 2 weeks after isolation.

Preparation of Dephosphophosvitin-Phosvitin was prepared according to the procedure of Joubert and Cook (37). It was partially dephosphorylated with the aid of a beef spleen phosphatase isolated and used according to the procedures detailed by Revel (38). The dephosphophosvitin was purified by the method of Mano and Lipmann (39). The exact level of dephosphorylation was measured by chemical analysis of the P, content in the dephosphophosvitin preparation (39). In general, attempt was made to achieve a 30% to 32% dephosphorylation of phosvitin for use as phosphate acceptor in the kinase assays.

Protein Phosphokinase Assay The method was based on assaying the transfer of 32P from \([\gamma-32P]ATP\) into the dephosphophosvitin substrate. The standard reaction medium in a final volume of 1 ml, contained 5 mm MgCl₂, 200 mm NaCl, 1 mm dithiothreitol, 30 mm \(\mathrm{Na}_2\mathrm{HPO}_4, \mathrm{pH} 7.4, \text{at } 37^\circ\), 5 mm ATP containing trace amounts of \([\gamma-32P]ATP\), and 2 mg of 30% dephosphophosvitin. The specific radioactivity of the ATP mixture in the reaction was generally 3000 dpm/nmol of ATP. The reaction was initiated by the addition of an amount of chromatin containing 5 to 70 mg of DNA, and was carried out for 30 min at 37°. The reaction was terminated by the addition of 1 ml of 30% (w/v) trichloroacetic acid containing 2 mm P, and 5 mm NaCl. Sufficient carrier phosphosvitin to act as a coacceptor was added to each tube to a final amount of 5 mg. The tubes were allowed to stand on ice for 30 min, and the precipitated protein was washed twice with 5 ml each of 15% (w/v) trichloroacetic acid containing 1 mm P, and 1 mm NaCl, ATP, followed by four washes with 5 ml each of 15% trichloroacetic acid containing 1 mm P, and 1.5 mm NaCl. Finally, the residue was hydrolyzed in 1 N NaOH for 17 hours at 37°, and radioactivity of the Pi released was measured as described previously (2).

The results, expressed as nanomoles of 32P per mg of chromatin DNA or protein, were calculated from the specific radioactivity of ATP in the reaction. The incorporation of 32P into chromatin protein in the above assay was also determined by omitting dephosphophosvitin from the reaction medium. This endogenous radioactivity in chromatin was subtracted from values obtained in the presence of dephosphophosvitin substrate. Corrections were also applied for zero time control and background radioactivity. During the course of the reaction, the activity of ALV did not change significantly. When histones were used as substrates, special care was needed to estimate the nonenzymic binding of radioactivity to the lysine-rich and arginine-rich histones. For this, it was found necessary to also include controls comprising of the complete reaction medium minus the source of enzyme, and these controls were incubated for the same period of time as the experimental tubes. Finally, in the first two washes of the precipitated histone proteins, the residue was suspended in 0.5 ml of 1 N NaOH (at 4°) each time, followed by the addition of 0.5 ml of n HCl, and the trichloroacetic acid medium as described under the legends for the respective tables. This problem of nonspecific binding of radioactivity was not encountered with dephosphophosvitin as substrate.

Other Methods-Protein content of dephosphophosvitin solutions was determined according to the method of Lowry et al. (41) using desicated phosvitin as standard; however, in all other protein analyses, bovine serum albumin was used as standard. DNA and RNA were estimated according to the methods of Burton (42) and Ceriotti (43), respectively, by using appropriate standards. The procedures for the preparation and purification of [\(\gamma-32P\)]ATP (44), orchietomy of rats and their testosterone treatment (2, 3) have been described earlier.

RESULTS

General Properties and Time Course Studies-Under standard experimental conditions, described under "Methods," rat ventral prostate water soluble chromatin was capable of transferring 32P from \([\gamma-32P]ATP\) into dephosphophosvitin at a linear rate over a period of 60 min studied. Linear rates were also obtained when chromatin preparations from 72-hour orchietomized and testosterone-treated orchietomized rats were used in a similar assay. The data given in Table I depict the general properties of the prostatic chromatin-associated protein phosphokinase activity. It is apparent that Mg\(^{2+}\) is essential for the reaction, and an appropriate salt concentration (NaCl or KCl) is needed to achieve maximal rates of the reaction. Likewise, 1 mm dithiothreitol was found to optimally stimulate the activity. Histones were not good substrates for the chromatin-associated phosphokinase activity. Compared with dephosphophosvitin as substrate, some activity was observed toward lysine-rich histone (about 3%), but only a minimal activity was apparent with arginine-rich (\(\epsilon\)) fraction.
followed by another increase in activity at pH 7.89. At pH values higher than 8.2 a rapid reduction in the kinase activity was noted.

Effects of Various Cations—As mentioned earlier, Mg$^{2+}$ was essential for the kinase reaction; the result in Fig. 2A demonstrates the optimal concentration of this cation to be 4 to 5 mM. In Fig. 2B the effect of substituting the divalent cations such as Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, or Ca$^{2+}$ for Mg$^{2+}$ in the protein phosphokinase assay is demonstrated. Mn$^{2+}$ at low concentrations (up to 0.5 mM) can effectively substitute for Mg$^{2+}$; however, at concentrations above 0.5 mM it appears to have an inhibitory effect. Zn$^{2+}$, Cu$^{2+}$, or Ca$^{2+}$ are essentially incapable of replacing Mg$^{2+}$ in the chromatin-associated kinase reaction. It may be recalled that these divalent cations can substitute for Mg$^{2+}$ in the cytosol protein phosphokinase reaction (45). Fig. 2C shows the effect of the monovalent cation composition on the rate of reaction. It is apparent that the presence of 200 mM NaCl (or KCl) is essential to achieve maximal reaction rates. In the absence of any monovalent cations in the reaction, there was an almost 50% reduction in the protein phosphokinase activity, as also the concentrations of NaCl above 200 mM became inhibitory.

Effect of Varying Dephosphophosvitin—Varying the substrate dephosphophosvitin over a range from 0.1 to 2.0 mg/ml in the kinase reaction gave the typical Michaelis-Menten-type kinetics. The Lineweaver-Burk (46) plot gave an apparent $K_m$ value of 0.3 mg/ml for 31% dephosphophosvitin. The apparent $K_m$ value for dephosphophosvitin can vary depending upon the level of dephosphorylation of phosvitin. However, if the amount of dephosphophosvitin in the reaction is added at a suitable concentration (depending upon the apparent $K_m$ value), maximal rates of the chromatin-associated kinase activity can be determined.

Effect of Varying ATP—The data on the kinetics of the effect of ATP on the kinase reaction, plotted according to the method of Lineweaver and Burk (46), are depicted in Fig. 3B. Two apparent $K_m$ values for ATP (i.e. 0.044 and 0.41 mM) were obtained. Further, the break in the Lineweaver and Burk plot as shown in Fig. 3B was also observed when the amount of dephosphophosvitin in the reaction was reduced from 2.0 to 0.5 mg/ml. It may be noted that the concentration of ATP in the standard reaction medium was maintained at 3 mM to take into account the higher value for the apparent $K_m$ for ATP.

Effect of Orchiectomy and Testosterone Replacement—The effect of orchiectomy on chromatin-associated protein phosphokinase activity was studied by using the standard experimental conditions established above. The results plotted in Fig. 4 demonstrate that orchiectomy of adult rats resulted in a rapid decline in the chromatin-associated protein phosphokinase activity. This effect was apparent when the data were plotted based either on the amount of chromatin DNA or chromatin protein present in the assay. As early as 9 hours postorchietomy a significant decline (about 30%) in the chromatin-associated kinase activity (per unit of DNA) was observed, and by 18 hours the activity had declined to some 50% of the control values. There was essentially a complete loss of this activity in chromatin derived from animals castrated for a period of 120 to 136 hours. Further, the protein phosphokinase activity of chromatin preparations from 18- and 48-hour orchiectomized rats was also tested at pH 7.89; the result obtained was similar to that observed in experiments per-

### Table I

<table>
<thead>
<tr>
<th>Reaction medium</th>
<th>Protein phosphokinase activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>Omit Mg$^{2+}$</td>
<td>0</td>
</tr>
<tr>
<td>Omit Na$^+$</td>
<td>42</td>
</tr>
<tr>
<td>Omit dithiothreitol</td>
<td>61</td>
</tr>
<tr>
<td>Omit phosvitin</td>
<td>4</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>Plus 5 mM cyclic AMP</td>
<td>95</td>
</tr>
<tr>
<td><strong>Experiment C</strong></td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>100</td>
</tr>
<tr>
<td>Omit phosvitin and add</td>
<td></td>
</tr>
<tr>
<td>a. Lysine-rich histone</td>
<td>3.0</td>
</tr>
<tr>
<td>b. Lysine-rich histone plus 5 mM cyclic AMP</td>
<td>3.2</td>
</tr>
<tr>
<td>c. Arginine-rich histone (f)</td>
<td>0.30</td>
</tr>
<tr>
<td>d. Arginine-rich histone plus 5 mM cyclic AMP</td>
<td>0.33</td>
</tr>
</tbody>
</table>

(0.3%) as substrate. No significant stimulation by cyclic AMP was detected. Under the experimental conditions, incorporation of $^{32}$P into endogenous proteins of chromatin was also noted. The nature of these phosphoproteins and the characteristics of their phosphorylation are currently under investigation in this laboratory.

Effect of pH—The results in Fig. 1 show the complex nature of the pH versus protein phosphokinase activity with prostatic chromatin. The kinase activity increased rapidly as the pH was increased from 6.45 to 7.0, remained at a high level between 7.0 and 7.45, but then declined significantly at pH 7.65. This was

The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate.

K. Ahmed and M. J. Wilson, unpublished data.
The pH values indicated on the abscissa refer to the final pH of the reaction medium measured at 37°C, and were achieved by adding 30 mM imidazole-HCl (pH 6.45 to 7.20), 30 mM Tris-HCl (pH 7.2 to 8.22), or 30 mM NaHCO₃-Na₂CO₃ (pH 8.22 to 9.0) in the reaction medium. Chromatin (10 µg of DNA) was added to initiate the reaction, the duration of which was 30 min. The result shown is a plot of the mean of three experiments using three different preparations of chromatin. Precisely the same changes in phosphokinase activity versus pH were observed with all three preparations of chromatin. The range of values obtained is indicated in the figure. All other details were the same as described under “Methods.”

**Fig. 2.** (right). A, effect of varying Mg²⁺ on chromatin-associated protein phosphokinase activity. All the details were the same as described under “Methods” except that the concentration of Mg²⁺ was varied as shown. To obtain the value for zero Mg²⁺, 20 mM EDTA (Tris salt, pH 7.4) was added in the appropriate tube. Chromatin (10 µg of DNA) was incubated at 37°C for 30 min. B, effect of various divalent cations on chromatin-associated protein phosphokinase activity. Divalent cations as indicated were substituted for Mg²⁺ at the concentrations shown. Chromatin (20 µg of DNA) was incubated at 37°C for 30 min. All other details were the same as described under “Methods.” C, effect of monovalent cations on chromatin-associated protein phosphokinase activity. All the details were the same as described under “Methods,” except that the concentration of NaCl (or KCl) was varied as shown. Chromatin (10 µg of DNA) was incubated at 37°C for 30 min.

**Fig. 3.** A, effect of varying dephosphophosvitin on chromatin-associated protein phosphokinase activity. The concentration of 31% dephosphophosvitin was varied as shown while keeping all other conditions the same as described under “Methods.” Chromatin (20 µg of DNA) was incubated at 37°C for 15 min. The inset shows the result plotted according to the method of Lineweaver and Burk (46); the apparent Kₘ for dephosphophosvitin obtained was 0.3 mg/ml. B, effect of varying concentration of ATP on chromatin-associated protein phosphokinase activity. The concentration of ATP in the reaction was varied as shown. Chromatin (10 µg of DNA) was incubated at 37°C for 30 min. All other conditions were the same as described under “Methods.” The result is plotted according to Lineweaver and Burk (46). The apparent Kₘ values for ATP, obtained from the figure, were 0.044 mM and 0.41 mM.

formed at pH 7.4 (data not given). It is of interest to note also that the rapid effect of androgen withdrawal from adult rats, on the chromatin-associated protein phosphokinase activity is of the same order, or somewhat greater, than that observed on the decline of the RNA polymerase activity of prostatic nuclei from castrated rats (34). Further, it is shown in Fig. 4 that when protein kinase activity of chromatin preparations from orchietomized rats with and without testosterone treatment were compared, a significant increase in the activity (expressed either on the basis of chromatin DNA or protein) was observed.
The values of chromatin-associated protein phosphokinase activity at 0, 9, and 18 hours postorchiectomy are the means ± SEM obtained from five experiments using five different preparations of chromatin in each case. All other values recorded are means of two experiments in each case. Inset, effect of testosterone on ventral prostate chromatin-associated protein phosphokinase activity. Testosterone propionate dissolved in 0.2 ml of sesame oil, 0, or filled bars, refer to protein phosphokinase activity per mg of chromatin protein, whereas 0, or empty bars, refer to protein phosphokinase activity per mg of chromatin DNA. Chromatin (20 μg of DNA or 40 μg of protein) was incubated at 37°C for 15 to 30 min depending upon the activity.

suggesting a possible activation in vivo of the prostatic chromatin-associated kinases by testosterone. The effect of a single injection, over a time course, on the chromatin-associated protein phosphokinases is being currently investigated. These androgen-sensitive changes were not observed in chromatin preparations derived from livers of rats under varying androgenic status, suggesting a target-tissue specificity of these actions of testosterone (data not given).

The rapid initial decline in the chromatin-associated protein kinases following orchiectomy becomes apparent prior to the emergence of significant changes in the chemical composition of chromatin. Thus, as shown in Table II, there was no detectable change in the protein complement of DNA at 9 hours postorchiectomy, even though the kinase activity showed an average of 30% reduction. A small decline in the chemical composition of chromatin was observed at 18 hours postorchiectomy; however, the reduction in the associated phosphokinase activity was considerably greater than that in the protein complement itself.

The data shown in Table III clearly illustrate that histone phosphokinase activity, associated with rat ventral prostate chromatin, is sensitive to the androgenic status of the animal. At 18 hours postorchiectomy a significant decline in activity toward both the lysine-rich and the arginine-rich (f2) histones was apparent. By 48 hours postorchiectomy, this effect was even greater.

**DISCUSSION**

From the foregoing it is clear that rat ventral prostate chromatin possesses protein phosphokinases, the activity of which is remarkably dependent upon the androgenic status of the animal. This chromatin preparation also phosphorylates lysine-rich and arginine-rich histones although it is much less active toward these substrates than toward dephosphophosvitin. The chromatin-associated histone phosphokinase activity seems to be distinctly different from the cytosol enzyme in that it is sensitive to the androgenic status of the animal, and is not activated by cyclic AMP. The stimulation of the cytosol protein phosphokinases from various tissues by cyclic AMP is well known (4, 47-51).

The lack of any significant effects of cyclic AMP on chromatin-associated protein phosphokinases tested in the presence of dephosphophosvitin or histones is commensurate with our previous work on the phosphorylation of rat ventral prostate nuclear phosphoproteins in vitro. Other investigators have reported variable effects of cyclic AMP on individual nuclear proteins and associated phosphokinases of liver (28, 52). Therefore, at present we cannot rule out the possibility that cyclic AMP may have stimulatory effect on some of the enzymes associated with prostatic chromatin while having an equal inhibitory effect on the others, so that, fortuitously, we have observed no effect. This seems unlikely, although, to be the case when histones were used as substrate in this study. Tissue-specific variations in these responses also cannot be ruled out at the present.

The present work, although not demonstrating directly, is strongly suggestive that the protein phosphokinase activity associated with the prostatic chromatin may be due to the presence of multiple enzymes. This is indicated by the general kinetic properties of the chromatin-associated enzyme system, e.g. the activity profile with respect to pH, requirement for ATP, and nature of the substrate. Kish and Kleinsmith (28) have shown the presence of several protein phosphokinases associated with liver and kidney nuclei. Preliminary observations on the separation of chromatin-associated protein phos-
phosphokinases indicate a similar situation in the prostate.*

Taken together with our previous work (2, 3, 5, 20, 29), the present observations strongly indicate that endogenous protein phosphokinases are involved in the phosphorylation of nuclear non-histone acidic proteins in vitro in cell-free systems in response to in vivo manipulations of the androgenic status of the animal. Since it has been postulated that nuclear non-histone acidic phosphoproteins may have a positive regulatory role in the control of transcription (23), it is tempting to postulate that the modulation of chromatin-associated protein phosphokinases of prostate may control the phosphorylation of these proteins during the course of androgen-stimulated gene action. The mechanism by which testosterone affects the androgen-sensitive chromatin-associated protein phosphokinases remains to be established.

Acknowledgment—We wish to express our gratitude to Mr. Alan Davis for his excellent assistance during the course of this work.

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