**Inhibition of Casein Kinase II by Heparin**

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Casein kinase II, a cyclic nucleotide-independent pro-
tein kinase from rabbit reticulocytes, was shown to be
inhibited by heparin. Heparin specifically inhibited the
enzyme and had no effect on other protein kinases,
including casein kinase I, the type I and II cAMP-de-
pendent protein kinases, protease-activated kinase I,
and the hemin-controlled repressor. Heparan sulfate
was found to be 40-fold less effective than heparin
towards casein kinase II; other acid mucopolysaccha-
drides had little or no effect on the enzymatic activity.
Steady state studies revealed that heparan acted as a
competitive inhibitor with respect to the substrate,
casein. A value of 20 ng/ml or about 1.4 × 10^6 e.u./mg
of protein; 0.10 mM ATP (specific activity, 3.5 × 10^6
e.u./mg of protein); 0.10 mM ATP (specific activity, 100 to 300
cpm/mmol); 140 mM KCl; 10 mM MgCl2; 50 mM Tris-HCl, pH 7.0;
4.3 mg/ml of phosphorylated casein; heparin or other glycosaminogly-
cans in a volume of 0.07 ml. The reaction was initiated by addition of
substrates. Incubation was carried out at 30°C for 15 min, whereupon
the reaction was terminated as described previously (2). Under
the conditions of our assays, incorporation was linear for up to 60 min.
In comparing the relative effectiveness of the acid mucopolysaccharides,
the amount of material required to give 50% inhibition of the enzyme
(19) was determined by plotting the reciprocal of the remaining
activity versus the concentration of the inhibitor (19).

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard preparations of acid mucopolysaccharides
including heparin were obtained from Drs. Martin B. Mathews and
Joseph A. Cifonelli of the University of Chicago. Stock solutions of
the mucopolysaccharides were prepared gravimetrically from the dry
powders and stored at −70°C. Working solutions were prepared as
required by volumetric dilution. The tissue source and viscosity
average molecular weight (18) of the compounds were as follows:
heparin, bovine lung, 14,000; chondroitin 4-sulfate, sturgeon nato-
chord, 15,000; chondroitin 6-sulfate, sturgeon cranial cartilage, 29,000;
dermatan sulfate, hog mucosal tissue, 45,000; hyaluronic acid, human
umbilical cord, 230,000; heparan sulfate, bovine lung, and keratan
sulfate-2, human costal cartilage. (γ−32P)ATP was prepared as de-
scribed by Hathaway et al. (2) or purchased from ICN.

**Purification and Assay of Casein Kinase II**—Casein kinase II,
greater than 95% pure, was obtained from the postribosomal super-
natant as described previously (1, 2). The standard assay contained:
10 to 20 enzyme units of the casein kinase II (specific activity: 3.5 × 10^6
units/mg protein); 0.10 mM ATP (specific activity, 100 to 300
cpm/mmol); 140 mM KCl; 10 mM MgCl2; 50 mM Tris-HCl, pH 7.0;
4.3 mg/ml of phosphorylated casein; heparin or other glycosaminogly-
cans in a volume of 0.07 ml. The reaction was initiated by the addition of
substrates. Incubation was carried out at 30°C for 15 min, whereupon
the reaction was terminated as described previously (2). Under
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activity versus the concentration of the inhibitor (19).

**Purification and Assay of Other Protein Kinases**—The type I and
II cAMP-dependent protein kinases from reticulocytes were purified
and assayed with mixed histone IIA (Sigma) and phosphorylated
casein as described previously (20). Purified catalytic subunit of the
type II cAMP-dependent protein kinase from bovine cardiac muscle
was a gift of Dr. E. H. Fischer, University of Washington, Seattle.
Purification of casein kinase I was as described previously (1, 2) and
assays were identical with those for casein kinase II. Protease-acti-
vated kinase I was activated by limited trypsin digestion and assayed
with mixed histone IIA. The enzyme was purified as previously
described (21). The activity of the enzyme was examined at heparin
concentrations from 0 to 7 μg/ml, both when the protein kinase was
in an intact, inactive form and following activation. The protein
kinase was activated by brief digestion with trypsin (4 μg/ml) for 15
min in 10 mM Tris-HCl, pH 8.0. Protolysis was terminated by the
addition of soybean trypsin inhibitor (final concentration, 30 μg/ml).
The assay mixture for the protein kinase reaction (0.07 ml) contained
10 μg of activated or nonactivated enzyme, 50 mM Tris-HCl, pH 7.5;
30 mM 2-mercaptoethanol; 45 mM MgCl2; 0.4 μM (γ−32P)ATP (specific
activity, 100 to 300 cpm/mmol); 1 mg/ml of histone; and heparin.
The reaction was initiated by the addition of substrates and incubated
for 15 min at 30°C, whereupon the reaction was terminated as described
for the casein kinases (2). Results were the averages of triplicate
determinations. The hemin-controlled repressor was purified (22) and
assayed with initiation factor 2 by polycrylamide gel electrophoresis
in sodium dodecyl sulfate as described previously (2).

**Kinetics**—The inhibition of casein kinase II by heparin was deter-
mined as described above. Zero order kinetics with respect to sub-
strates were maintained throughout and rates were determined in
duplicate and averaged. Casein was varied in the assay at heparin
concentrations ranging from 0 to 350 ng/ml (0 to 25 mM). Double
reciprocal plots were fitted by an unweighted method of least squares
and apparent Kₐ values were obtained from the slopes and intercepts.
In these experiments, the ATP concentration was held constant at
0.10 mM, approximately 3 times Kₐ. In a second set of experiments,
the ATP concentration was varied from 0.05 to 0.50 mM while casein
was held constant at 2.5 mg/ml.

**Protein Determination**—Protein was determined by dye binding
according to the method of Bradford (23) with bovine y-globulin (Bio-
Rad) as a standard.
Inhibition of Casein Kinase II by Heparin

RESULTS

The activities of six different protein kinases from rabbit reticulocytes and the purified catalytic subunit of the type II cAMP-dependent protein kinase from bovine cardiac muscle were examined at increasing concentrations of heparin. The results, shown in Fig. 1, indicated that only casein kinase II was inhibited by heparin. In this experiment, 130 ng/ml was sufficient to inhibit casein kinase II 50%. In contrast, concentrations of heparin up to 15 μg/ml gave no inhibition with the other protein kinases. With the cAMP-dependent protein kinases, casein as well as histone was examined as substrate. This was in anticipation of possible interactions between the positively charged histone and the highly acidic heparin. No inhibition by heparin with either substrate was observed using the holoenzymes or the catalytic subunit. There was no effect of heparin on the inactive and trypsin-activated forms of protease-activated kinase I with histone as substrate. Furthermore, the activity of the hemin-controlled repressor in the phosphorylation of initiation factor 2 was unimpaired with a level of inhibitor up to 7 μg/ml.

Fig. 1. Effect of increasing concentrations of heparin on the activity of various protein kinases. Assays contained 0 to 7.0 μg/ml of heparin and 10 to 15 e.u. of each protein kinase, except for the hemin-controlled repressor, where 1 μg was added. Casein kinase I (A) and II (O) were assayed with dephosphorylated casein, 4.3 mg/ml. The type I (O) and type II (I) CAMP-dependent protein kinase from rabbit reticulocytes and the catalytic subunit of the type II enzyme from bovine cardiac muscle (A) were examined with histone, 4.3 mg/ml. Protease-activated kinase I (O) was activated by limited tryptic digestion and assayed with histone, 1.0 mg/ml, and the hemin-controlled repressor (O) was assayed with initiation factor 2, 44 μg/ml. When casein (4.3 mg/ml) was used as substrate in reactions with the cAMP-dependent protein kinases, data similar to that shown with histone were obtained.

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>I₀ (μg/ml)</th>
</tr>
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<tr>
<td>Heparin</td>
<td>0.13</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>5.4</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>43.0</td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>NI</td>
</tr>
<tr>
<td>Keratan sulfate-2</td>
<td>NI</td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>NI</td>
</tr>
<tr>
<td>Chondroitin 6-sulfate</td>
<td>NI</td>
</tr>
</tbody>
</table>

* NI, no inhibition observed.

To analyze the specificity of casein kinase II with respect to the nature of the mucopolysaccharide, chemically related glycosaminoglycans were tested. Table I shows the results obtained with heparin and standard preparations of six other mucopolysaccharides. Heparin proved to be 40 times more inhibitory than dermatan sulfate and 330 times more inhibitory than keratan sulfate. No inhibition was observed with hyaluronate, keratan sulfate-2, chondroitin 4-sulfate, and chondroitin 6-sulfate at 100 times the concentration observed to give 50% inhibition with heparin.

We investigated the nature of heparin inhibition of casein kinase II by varying the concentration of casein in the assay at different concentrations of inhibitor. Fig. 2A shows a double reciprocal plot of the data. Fig. 2B shows the same plot for data obtained in a similar experiment except that the concen-
Inhibition of Casein Kinase II by Heparin

Fig. 3. Determination of $K_{\text{apparent}}$. Values of $K_{\text{apparent}}$ were obtained from the slope and intercept of the lines shown in Fig. 2A by an unweighted method of least squares. The value of $K_{\text{apparent}}$ was obtained by a least squares fit of the data.


tation of ATP was varied. Comparison of the two sets of data revealed that heparin was acting as a competitive inhibitor of casein kinase II with respect to the substrate, casein, but was noncompetitive toward ATP. To obtain a value for $K_{\text{apparent}}$, the data depicted in Fig. 2A were fitted by the method of least squares and values of slope and intercept were obtained. A secondary plot was made of the resultant $K_{\text{apparent}}$ values versus heparin concentration as shown in Fig. 3. From the least squares fit, a value of 1.4 nM was obtained for the inhibitory constant.

In order to determine whether heparin was being phosphorylated by casein kinase II, reactions were carried out with the enzyme and heparin in the presence and absence of casein. Reactions were performed using standard assay conditions and were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (2). After staining the gels with toluidine blue (24) and Coomassie blue, autoradiography revealed that $^{32}P$ was incorporated only into casein. No incorporation was observed in the band which corresponded to heparin.

**DISCUSSION**

In our search for regulatory compounds for the casein kinases, an inhibitor was detected in some commercial preparations of reticulocyte lysate (25), in yeast tRNA, rat liver tRNA, and rabbit leukocytes, which specifically inhibited casein kinase II. The inhibitor from commercial preparations of reticulocyte lysate and from leukocytes was purified by phenol extraction, ethanol precipitation, gel filtration, and ion exchange chromatography on DEAE-cellulose and benzoylated DEAE-cellulose. The inhibitor was found to co-purify with a 5 S RNA and was inactivated by ribonuclease and micrococcal nuclease. On the basis of these observations, we concluded that the inhibitor was a RNA. However, further analysis by sucrose density gradient centrifugation revealed that 5 S RNA did not co-sediment with the inhibitory activity. At the same time, one of us observed that porcine mucosal heparin was a powerful inhibitor of casein kinase II. In fact, heparin exhibited the same properties as the unidentified inhibitor, including inhibition kinetics, co-purification, inactivation by ribonuclease (by binding to the nuclease), and specificity with respect to casein kinase II. We now believe that our original observations were the result of heparin which had been administered routinely as an anticoagulant to facilitate bleeding.

Heparin has been shown to alter the activity of a number of intracellular and extracellular mammalian enzymes including anti-thrombin III (8), DNA-dependent RNA polymerase (9), lipoprotein lipase (10), and ribonuclease (11). This has led to the suggestion that heparin should be viewed as a multifunctional molecule rather than one with limits narrowly assigned by virtue of its medical applications (16, 17). Our observations regarding the specific and sensitive nature of the interaction between heparin and casein kinase II support this suggestion. Heparin specifically inhibits casein kinase II and has no effect on the cAMP-dependent protein kinases or other cyclic nucleotide-dependent protein kinases from reticulocytes, including casein kinase I, protease-activated kinase I, and the heparin-controlled repressor. The other glycosaminoglycans examined showed reduced or no inhibitory activity towards the enzyme.

In heparin-containing cells such as liver, the casein kinases have been shown to be inactive until an initial chromatography step has been performed (26-28), and Mäenpää (29) has shown that heparin inhibits a phosphoinositol kinase purified from the liver of estrogen-treated roosters. These results suggest that heparin may function as a physiological regulator of casein kinase in some cell types.

Protein kinases have been shown to be regulated by cyclic nucleotides, hemin, calcium, double-stranded RNA, and proteolytic modification (30, 31). Interaction with heparin would represent another mechanism of regulation which has the potential of being a rapidly responsive control mechanism. Inhibition of casein kinase II would be expected to alter the subsequent modification of the four translational initiation factors which are substrates of the enzyme (2, 32, 33). The identification of an inhibitor specific for casein kinase II should allow experimental differentiation of the casein kinase activities in cell extracts and a clarification of the role of these enzymes in cellular control mechanisms.

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**REFERENCES**


With some preparations of casein, considerable upward curvature was noticed in these plots. We ascribed this to a substrate-heparin interaction which may be a function of variations in casein preparations.
Inhibition of Casein Kinase II by Heparin