

Protein Kinase Activity in Commercially Available Crystalline Yeast Alcohol Dehydrogenase

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Commercially available crystalline yeast alcohol dehydrogenase contained protein kinase activity. Casein and phosvitin were readily phosphorylated, but whole calf thymus histone was not. The protein kinase activity was inhibited by KCl, was not stimulated by cyclic AMP and could be separated from the alcohol dehydrogenase activity by sucrose density centrifugation.

Protein kinases have been found in a wide variety of organisms (Kuo & Greengard, 1969). Although the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinases have been studied most extensively, the existence of cyclic AMP-independent protein kinases has been reported in mammalian tissues (Chen & Walsh, 1971) and in yeast (Sy & Rickter, 1972).

During sucrose-gradient studies of human lymphocyte protein kinases it was found that the inclusion of yeast alcohol dehydrogenase as an internal molecular-weight marker enhanced the casein kinase activity. This paper reports the presence of protein kinase activity in commercially available crystalline yeast alcohol dehydrogenase preparations.

Materials and Methods

Substrates

[γ -³²P]ATP was prepared as described by Glynn & Chappell (1964). Dephosphorylated casein was prepared by the method of Reimann *et al.* (1971) and dialysed against 0.2M-KCl and then against 10mM-Hepes [2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid] buffer, pH 7.5.

Casein was purchased from the British Drug Houses Ltd. (Poole, Dorset, U.K.). Cytochrome *c*, catalase, ATP, NAD⁺, NADH, cyclic AMP, whole thymus histone and bovine serum albumin (Cohn fraction V) were purchased from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Yeast alcohol dehydrogenase

Yeast alcohol dehydrogenase preparations were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. (15418, No. 7152404), Sigma

Chemical Co. (340-27, lot 125B-1800) and Calbiochem (Australia) Pty. Ltd. (Sydney, N.S.W., Australia) (126783, lot 100311 and 12676, lot 901432).

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Assay of alcohol dehydrogenase activity

The assay procedure was based on that described by Kagi & Vallee (1960). Reactions, carried out in a 1cm-light-path cuvette, contained 265 μ mol of sodium pyrophosphate buffer, pH 8.8, 5.7 μ mol of NAD⁺, 0.1ml of 95% (v/v) ethanol and enzyme in a final volume of 3.0ml. Enzyme was diluted to 10-20 μ g/ml in 0.1M-sodium phosphate buffer (pH 7.5) immediately before assaying. Incubations were carried out at 25°C and initial velocities were measured by following the rate of change in E_{340} with a Unicam SP. 800 recording spectrophotometer.

Assay of protein kinase activity

Assays contained 5 μ mol of Hepes (Na⁺ salt; pH 7.5), 1 μ mol of MgCl₂, 2.86nmol of [γ -³²P]ATP (specific radioactivity greater than 37.3mCi/mmol), 0.57mg of dephosphorylated casein and yeast alcohol dehydrogenase (30-300 μ g of protein) in a final volume of 100 μ l. Assays (done in duplicate) were carried out at 37°C for 10min, and terminated by adding 25 μ l of a solution of bovine serum albumin in 100mM-EDTA (50mg/ml); portions (100 μ l) were pipetted on to 2.3cm diam. discs of Whatman no. 50 paper and dried at room temperature. The paper discs were washed as described by Murray & Froscio

(1971), dried and counted for radioactivity in a solution of 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in sulphur-free toluene by using a Packard Tri-Carb liquid-scintillation spectrometer.

Sucrose-gradient centrifugation

Sucrose gradients [5–20% (w/v); 4.5 ml], containing 4.5 mM-Hepes buffer, pH 7.5, 7 mM-2-mercaptoethanol and 150 mM-KCl were centrifuged at 4°C for 10 h at 280000g_{av.} by using a 6 × 5 ml Ti rotor in an MSE 'Superspeed' 65 centrifuge. Fractions (0.18 ml) were collected and assayed for casein kinase activities as described above except that the incubation time was 20 min. Assays for alcohol dehydrogenase activity of the sucrose-gradient fractions contained 60 μmol of Hepes (Na⁺ salt; pH 7.5), 12.5 μmol of acetaldehyde and 18 μmol of NADH in a final volume of 3 ml. Incubations were carried out at 25°C in a cuvette and initial velocities were measured by following the rate of change in E₃₄₀ with a Unicam SP.800 recording spectrophotometer. Horse heart cytochrome *c* (6 mg) and catalase (0.2 mg) were included in the gradients as standards for molecular-weight estimations.

Results

Protein kinase activity

The protein kinase activity was separated from the alcohol dehydrogenase activity by sucrose-density centrifugation (Fig. 1). The protein kinase activity sedimented as a single peak with a molecular weight of approximately 35000.

Yeast alcohol dehydrogenase and casein kinase activities could also be separated by gel chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden). After gel chromatography only 1.6% of the total casein kinase activity was associated with the alcohol dehydrogenase activity (Fig. 2). The casein kinase activity was more sensitive to dilution than was that of alcohol dehydrogenase. An alcohol dehydrogenase preparation (0.0825 mg/ml) stored at 2°C for 17 h lost 70% of its casein kinase activity and 13% of its alcohol dehydrogenase activity compared with a more concentrated preparation (10 mg/ml) stored under the same conditions.

A number of commercially available yeast alcohol dehydrogenase preparations were assayed for protein kinase activity. The protein kinase and alcohol dehydrogenase activities are shown in Table 1. All the crystalline preparations of alcohol dehydrogenase as well as the freeze-dried powder contained protein kinase activity. The Boehringer alcohol dehydrogenase suspended in (NH₄)₂SO₄ had negligible casein kinase activity (0.33 pmol/min per mg) when assayed

directly. After removal of the (NH₄)₂SO₄ by dialysis against 6 mM-Hepes buffer, containing 6 mM-2-mercaptoethanol (pH 7.5), this preparation contained relatively high casein kinase activity.

Substrate specificity

The effect of different protein substrates on the activity of the protein kinase in both the presence and the absence of 2 μM-cyclic AMP and 150 mM-KCl is shown in Table 2. Dephosphorylated casein was the most effective substrate and phosphorylation of whole calf thymus histone was very low. Cyclic AMP did not significantly stimulate phosphorylation of any of the substrates tested. Previous experiments (B. E. Kemp, unpublished work) with lymphocyte casein kinase had shown that the activity was maximally stimulated by 150 mM-KCl; however, the yeast protein kinase activity was inhibited by 150 mM-KCl.

Characterization of the phosphorylated product

In reaction mixtures containing casein most of the radioactivity of the phosphorylated product was alkali-labile (97%; 0.1 M-NaOH, 100°C, 15 min) and stable in acid (87%; 0.1 M-HCl, 100°C, 15 min). After

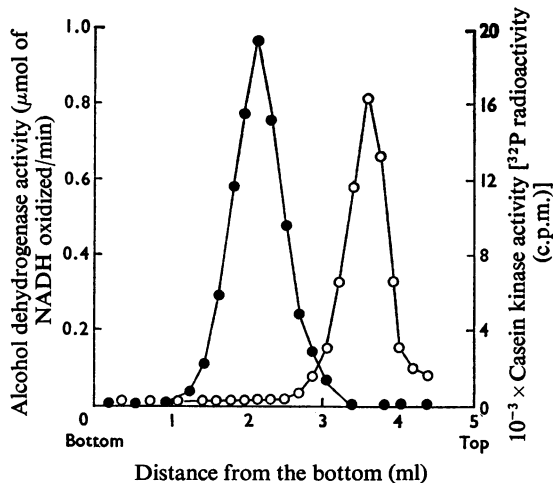


Fig. 1. Sucrose gradient of yeast alcohol dehydrogenase

Sucrose gradients (5–20%, w/v) containing 4.5 mM-Hepes buffer, pH 7.5, 7 mM-2-mercaptoethanol and 150 mM-KCl were loaded with 0.1 ml of alcohol dehydrogenase (Calbiochem, freeze-dried powder, 12676; 10 mg/ml) in the same buffer and centrifuged for 10 h at 4°C. Fractions were collected and assayed for casein kinase (○) and alcohol dehydrogenase (●) activities (see the Materials and Methods section).

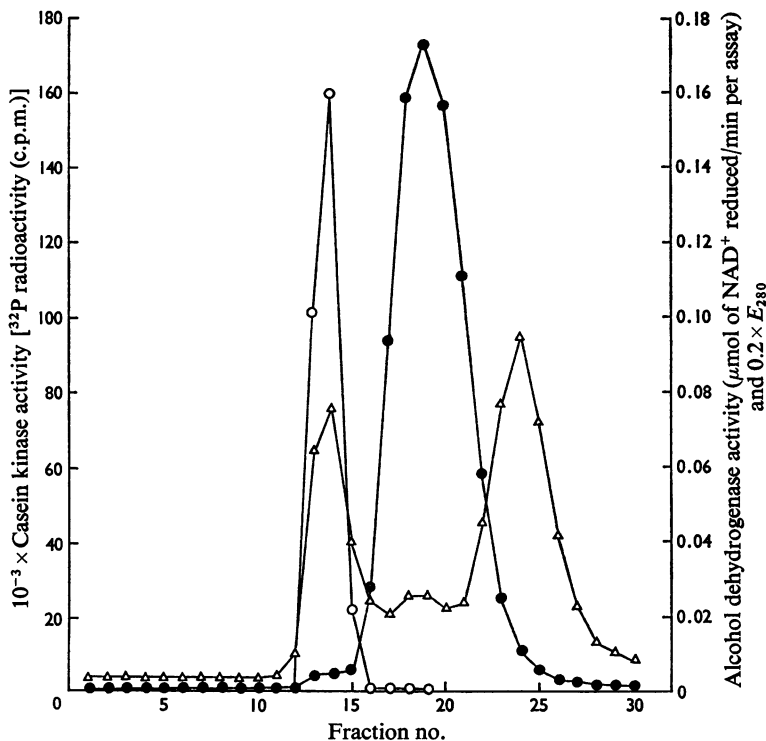


Fig. 2. Gel chromatography of yeast alcohol dehydrogenase

Yeast alcohol dehydrogenase (Calbiochem, 126783, 6 mg) and cytochrome *c* (Sigma, horse heart, no detectable casein kinase activity, 7.5 mg) were run on a column (1.5 × 27 cm) of Sephadex G-75; with 10 mM-Hepes buffer, pH 7.5, containing 100 mM-KCl and 5.7 mM-2-mercaptoethanol as solvent. The column was run at 4°C and fractions (1.1 ml) were collected and assayed for casein kinase (●) and alcohol dehydrogenase (○) activities (see the Materials and Methods section). △ is E₂₈₀.

Table 1. Comparison of the protein kinase activity of some commercially available yeast alcohol dehydrogenase preparations

For details see the text.

Enzyme preparation	Alcohol dehydrogenase activity (μmol/min per mg of protein)	Casein kinase activity (pmol/min per mg of protein)
Boehringer [crystalline enzyme, (NH ₄) ₂ SO ₄ suspension]	423	933*
Sigma (crystalline enzyme)	52	370
Calbiochem (freeze-dried powder, 12676)	247	269
Calbiochem (crystalline enzyme, 126783)	144	1362

* Casein kinase activity determined after dialysis.

partial acid hydrolysis (2M-HCl, 100°C, 4h) and electrophoresis (4h, 40V/cm) in formic acid-acetic acid-water (1:4:45, by vol.; pH 2) 45% of the ³²P was not hydrolysed and remained at the origin and

17% migrated with the *O*-phosphoserine marker. Only a small amount of radioactivity (1%) was detected in the *O*-phosphothreonine position.

Radioactivity (15%) was detected between the

Table 2. *Substrate specificity*

Assay conditions were as described in Materials and Methods section, except that the concentration of protein substrate in the assay mixture was 1 mg/ml. The yeast alcohol dehydrogenase used was from Calbiochem (126783, lot no. 100311; crystalline enzyme).

Substrate	Substance added ...	Protein kinase activity (pmol of P _i transferred/min per mg of protein)		
		—	2 μM-cyclic AMP	150 mM-KCl
Whole calf thymus histone		2.6	3.0	1.14
Dephosphorylated casein		624	634	169
Phosvitin		442	406	132

O-phosphoserine and P_i markers on the electrophoretogram. Since casein was used as the substrate, it seems likely that the radioactivity with greater anionic mobility represents di- and tri-serine phosphate residues (see Williams & Sanger, 1959).

There was no detectable endogenous phosphorylation of the alcohol dehydrogenase preparation in the absence of added protein; boiled alcohol dehydrogenase had no detectable protein kinase activity.

Discussion

A protein kinase in yeast was reported by Rabinowitz & Lipman (1960). The protein kinase activity present in crystalline alcohol dehydrogenase preparations has two features in common with the protein kinase studied by these workers. Both preparations phosphorylate phosvitin and both are inhibited by salts. In the present studies the protein kinase activity was not stimulated by cyclic AMP, in agreement with recent observations by Sy & Rickter (1972) on partially purified yeast protein kinase.

Since the molecular-weight estimate of the yeast casein kinase activity (35000) corresponded closely to the molecular weight of the yeast alcohol dehydrogenase subunit (37000), an attempt was made to examine whether the protein kinase activity was associated with the subunits of alcohol dehydrogenase. Treatment of yeast alcohol dehydrogenase with 1,10-phenanthroline dissociates the enzyme into its subunits (Kagi & Vallee, 1960). Under the conditions described by these authors (10 mM-phenanthroline, 75 h, 2°C) phenanthroline treatment of the dehydrogenase preparation did not increase the casein kinase activity even though alcohol dehydrogenase activity was abolished. Thus it is unlikely that the casein kinase activity measured is an activity of the alcohol dehydrogenase subunit, and it presumably represents a separate enzyme activity.

The pyruvate dehydrogenase complex found in ox kidney mitochondria contains a protein kinase and a phosphatase (Linn *et al.*, 1969). The pyruvate dehydrogenase-associated protein kinase is active at low Mg²⁺ concentrations. In the case of the yeast alcohol dehydrogenase activity there was no detectable endogenous phosphorylation over a range of Mg²⁺ concentration of 0.01–10 mM.

In view of the possible use of crystalline yeast alcohol dehydrogenase in enzyme coupled reactions or as an internal marker in sucrose-gradient studies, the presence of protein kinase activity should be recognized.

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