Purification and Characterization of a Type II Casein Kinase from Drosophila melanogaster*

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A cyclic nucleotide-independent protein kinase has been isolated from Drosophila melanogaster by chromatography on phosphocellulose and hydroxylapatite followed by gel filtration and glycerol gradient sedimentation. As determined by sodium dodecyl sulfate gel electrophoresis, the purified enzyme is greater than 95% homogeneous and is composed of two distinct subunits, α and β, having Mₑ = 36,700 and 28,200, respectively. The native form of the enzyme is an α₂β₂ tetramer having a Stokes radius of 48 Å, a sedimentation coefficient of 6.4 S, and Mₛ ≈ 130,000. The purified kinase undergoes an autocatalytic reaction resulting in the specific phosphorylation of the β subunit, exhibits a low apparent Kₑ for both ATP and GTP as nucleoside triphosphate donor (17 and 66 μM, respectively), phosphorylates both casein and protamine but neither histones nor polyamines, modifies both serine and threonine residues in casein, and is strongly inhibited by heparin (I₅₀ = 21 ng/ml). These properties are remarkably similar to those of casein kinase II, an enzyme previously described in several mammalian and avian species. The strong similarities among the insect, avian, and mammalian enzymes suggest that casein kinase II has been highly conserved during evolution.

Enzyme activities which catalyze the phosphorylation of protein substrates have been described in a wide variety of eukaryotic organisms (1, 2). Many of these activities have been purified to homogeneity in recent years, and as a result at least 15 different types of protein kinase can now be distinguished on the basis of subunit composition, physical and kinetic properties, and substrate specificity (3). A few of these enzymes appear to be present in all eukaryotic organisms and in all tissues, suggesting that they play an essential role in the regulation of normal cell metabolism. The most thoroughly studied example is cAMP-dependent protein kinase. This enzyme has a broad but well-defined substrate specificity and has been shown to affect the activity of numerous enzymes and structural proteins (3–5). It is thought to mediate most and probably all of the effects of cAMP in eukaryotic cells (3) and, hence, to play a central role in integrating cellular responses to hormonal stimuli (5).

The casein kinases represent a second class of protein kinase which appears to be ubiquitous in eukaryotes (6). These enzymes are operationally defined as cyclic nucleotide-independent protein kinases which phosphorylate casein and protamine in vitro but not histones or protamine. Two distinct types are recognized. Type I, which has been described in mammals, birds, higher plants, and yeast, is a single polypeptide monomer with Mₑ = 30,000–40,000. Type II, so far reported only in mammals and birds, is an α₂β₂ tetramer with Mₛ ≈ 130,000. The type II enzyme has been purified to homogeneity from rat liver (7), Novikoff ascites tumor cells (8), rabbit reticulocytes (9), and calf thymus (10). Besides the obvious differences in subunit composition and molecular weight, the type I and type II activities can be distinguished on the basis of nucleotide triphosphate specificity, amino acid phosphorylated in casein, and sensitivity to heparin. Although the physiological functions of the casein kinases are unknown, their apparent ubiquity coupled with their broad substrate specificity (6) suggest that they play an important role in the control of cell metabolism.

We report here the purification of a cyclic nucleotide-independent protein kinase from Drosophila melanogaster. The enzyme can be obtained in milligram quantities and is essentially homogeneous. The subunit composition, physical and kinetic properties, substrate specificity, and other characteristics of the purified enzyme indicate that it is a type II casein kinase. The striking similarity between the insect enzyme and its mammalian and avian counterparts suggests that casein kinase II has been highly conserved since the divergence of these organisms.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Radiochemicals—ATP, ADP, cAMP, and cGMP were obtained from Sigma; GTP was from P-L Biochemicals, [γ-32P]-ATP (2000 Ci/mmol) was from Amersham, and [γ-32P]GTP (10–50 Ci/mmol) was from New England Nuclear.

Column Chromatography—Phosphocellulose P-11 was obtained from Whatman, and hydroxylapatite HTP and agarose A-1.5m were purchased from Bio-Rad.

Protein Substrates, Standards, and Enzymes—Hydrolyzed casein (5% solution, dephosphorylated by the method of Reimann et al. (11)) and phosphoargin from egg vitellogenin were obtained from Sigma. Protease was purchased from Calbiochem. Calf thymus histones were extracted and fractionated as described by Glover and Gorovsky (12). Marker proteins, thyroglobulin, apoferritin, catalase, fibrinogen, BSA, egg ovalbumin, and cytochrome c were all obtained from Sigma. Bacterial alkaline phosphatase was purchased from Worthington.

Protease Inhibitors—Sooyean trypsin inhibitor (Sigma type II-S), ovomucoid (Sigma type II-O), PMSF, and 1,10-phenanthroline were all obtained from Sigma. Analytical grade sodium metabisulfite

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1 The abbreviations used are: BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.
(Na₃S₂O₅) was purchased from Baker. Stock solutions of PMSF and sodium bialulfite were prepared as described by Banks et al. (13). Stock solutions of trypsin inhibitor and ovomucoid were prepared in water at 10 mg/ml and 100 mM 1,10-phenanthroline was prepared in 95% ethanol.

Thin Layer Chromatography—Precast cellulose MN 300 thin layer plates (20 cm x 20 cm x 0.1 mm) were purchased from Bruckmann. Phosphoserine and phosphothreonine were purchased from Sigma. O₂-phosphopyruvate, a gift of Dr. E. Kuczumski (Stanford University), was prepared by condensation of P₂O₅ with tyrosine (14). Nitroblue was obtained from Sigma.

Miscellaneous—Heparin from porcine intestinal mucosa was purchased from Sigma. Nitex screen was purchased from Tetko (Elmsford, NY).

Methods

Protein Kinase Assay—Routine kinase assays were performed at 25 °C in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl₂, 1 μM [γ-³²P]ATP (35 Ci/mmol, 1 μCi/reaction), with 1 mg/ml casein as acceptor in a total volume of 28 μl. Samples to be assayed were diluted in 20 mM Tris, pH 8.0, 0.5 mM EDTA, 200 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100 (standard diluent). Assays were initiated at 30-s intervals by the addition of enzyme. Reactions were stopped (at 30-s intervals) after 15 min by spotting the entire reaction volume on a 13-mm glass fiber filter and submerging the filter in 20% trichloroacetic acid, 0.1 M sodium pyrophosphate (1 ml/filter). Filters were washed for 10 min each in four changes of 20% trichloroacetic acid, 0.1 M sodium pyrophosphate, once in 70% ethanol, once in 95% ethanol, and air dried. Filters were counted in 0.2 ml of scintillation fluid (2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazoly)benzene in toluene) in a Beckman LS-230 liquid scintillation counter using an open window. Samples were diluted so that no more than 10% of the available ATP was utilized (0.1 μCi, 200,000 cpm).

Protein Determination—Protein concentration was estimated by the Coomassie blue dye-binding method of Bradford (15) with BSA as standard.

Embryo Collection—Mass cultures of D. melanogaster (Oregon R) were reared as described by Elgin and Miller (16). Embryos were collected for 12 h and aged for 6 h (6-18-h embryos), washed, and stored frozen at -70 °C without dechorionation as described by Elgin and Miller (16).

Dechorionation—Embryos were thawed and decchorionated as described by Elgin and Miller (17) except that 0.7% NaCl, 0.01% Triton X-100 was used for all washing and settling steps. From 100 to 500 g of decchorionated embryos (blotted damp but not dry) were used for routine enzyme preparations. The following description assumes 100 g of starting material which is equivalent to 200 ml of settled embryos.

Homogenization—Embryos were resuspended in 4 volumes (400 ml) of homogenization buffer (15 mM Tris, pH 7.9, 0.05 mM EDTA, 5 mM KCl, 0.5 mM MgCl₂, 0.35 mM sucrose, 0.5 mM dithiothreitol) containing 1 mM PMSF and 10 mM NaHSO₃ added before use as described by Banks et al. (13). Embryos were homogenized in 40-ml aliquots in a Dounce homogenizer (five strokes with a loose pestle, five with a tight pestle). The homogenate was filtered through a 75-μm Nitex screen. The retentate was rehomogenized in one additional volume (100 ml) and refiltered. The two filtrates were combined as Fraction I (homogenate).

Differential Centrifugation—Fraction I was centrifuged at 10,000 x g for 15 min to remove nuclei. The postnuclear supernatant was aspirated from between the overlying lipid layer and the loosely packed nuclear pellet and recentrifuged at 16,000 x g for 20 min. The postmitochondrial supernatant was aspirated as before and pooled as Fraction II.

PH Precipitation—The pH of Fraction II was reduced to 6.3 by the gradual addition (with constant stirring) of cold 1 M acetic acid at 0 °C. After 30 min of stirring at 0 °C, precipitated material was removed by centrifugation at 16,000 x g for 15 min. The pH of the supernatant was returned to its starting value with 1 M NaOH. The solution was then adjusted to 300 mM NaCl by the addition of 5 mM NaCl (Fraction III).

Phosphocellulose Chromatography—Fraction III was loaded onto a 100-ml column of phosphocellulose equilibrated in 30 mM Tris, pH 8.0, 0.5 mM EDTA, 350 mM NaCl, 10% glycerol, 0.1 mM PMSF, 0.5 mM dithiothreitol. The column was washed with 2 column volumes of starting buffer and eluted with a linear gradient (300 ml) of NaCl from 350 to 1000 mM. Activity eluted as a sharp peak at 600 mM NaCl (Fig. 1). Active fractions were pooled as Fraction IV. This fraction was stable and could be stored at 4 °C for at least 3 months without significant proteolysis or loss of activity.

Hydroxyapatite Chromatography—Fraction IV was loaded onto a 10-ml column of hydroxyapatite equilibrated in 30 mM Tris, pH 8.0, 0.5 mM EDTA, 600 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol. The column was washed with 3 column volumes of starting buffer, 3 volumes of this buffer without NaCl, and 3 volumes of 25 mM potassium phosphate, pH 7.5. Activity was eluted with a linear gradient (8 volumes) of potassium phosphate (pH 7.5) from 25 to 400 mM. Activity eluted at 100 mM phosphate (Fig. 2). Active fractions were pooled and precipitated by the addition of solid (NH₄)₂SO₄, to 60% saturation (36.1 g/100 ml of eluate). After 1 h of stirring at 0 °C, the precipitate was collected by centrifugation at 16,000 x g for 20 min and dissolved in 0.5 ml of 30 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol. The solution was clarified at 16,000 x g for 10 min and the supernatant was taken as Fraction V.

Exclusion Chromatography—Fraction V was applied to a column (0.8 cm inner diameter x 120 cm) of agarose A-1.5m equilibrated in 30 mM Tris, pH 8.0, 0.5 mM EDTA, 1 M NaCl, 10% glycerol, 0.5 mM dithiothreitol and eluted with the same buffer at a flow rate of 2 ml/h (4 ml/h/cm²). Activity eluted at a Kᵥ of 0.38 (Fig. 3). Active fractions were pooled and precipitated with (NH₄)₂SO₄ as described above. The precipitate was dissolved in 30 mM Tris, pH 8.0, 0.5 mM EDTA, 0.5% glycerol, 0.5 mM dithiothreitol and clarified at 16,000 x g for 10 min (Fraction VI). High salt was essential for proper chromatography on this resin. At 350 mM NaCl, the activity trailed and eluted near the included volume; at 50 mM, it was retained on the column.

Velocity Sedimentation—Fraction VI was layered onto a preformed linear 10-30% glycerol gradient in 30 mM Tris, pH 8.0, 0.5 mM EDTA, 1 M NaCl, 0.5 mM dithiothreitol and centrifuged in a Beckman

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Fig. 1 (left). Phosphocellulose chromatography. Fraction III was chromatographed on phosphocellulose P-11 as described under "Methods." Fraction IV was pooled as indicated by the horizontal bar.

Fig. 2 (center). Hydroxyapatite chromatography. Fraction IV was chromatographed on hydroxyapatite as described under "Methods." Fraction V was pooled as indicated by the horizontal bar.

Fig. 3 (right). Gel filtration. Fraction V was chromatographed on agarose A-1.5m as described under "Methods." Fraction VI was pooled as indicated by the horizontal bar. Blue dextran 2000 and phenol red, respectively, were used to estimate the excluded volume (Vₑ) and included volume (Vᵢ) of the column. Elution volumes of thyroglobulin (TG), apoferriatin (Apo), BSA, and cytochrome c (Cyt) were determined in a separate chromatographic run. Elution volumes (Vₑ) were converted to Kᵥ values according to the equation Kᵥ = (Vᵢ - Vₑ)/Vₑ.
SW 40 rotor at 40,000 rpm for 36-48 h at 4 °C. Fifty 0.25-ml fractions were collected from the bottom of the tube. Active fractions (Fig. 4) were pooled as Fraction VII and stored at 4 °C. The NaCl concentration in the gradient could not be reduced because low salt led to self-aggregation of the enzyme. For example, when the NaCl concentration was reduced to 0.1 M, the enzyme pellet (s_{20w} ≥ 12 S) self-aggregation at reduced ionic strength (<0.5 M NaCl) has previously been observed for casein kinase II from rabbit reticulocytes (9).

Alternate Sources—Purification of the kinase from Drosophila K, tissue culture cells (grown in suspension culture as described (18)) was identical except that sucrose was omitted and 0.05% Triton X-100 was included in the homogenization buffer. For purification of kinase from nuclei of either K, cells or embryos, washed nuclei were extracted twice with 30 mM Tris, pH 8.0, 0.5 mM EDTA, 0.35 M NaCl, 10% glycerol, 0.5 mM diithiothreitol. The combined extract was applied to a phosphocellulose column as described and the purification continued as usual.

Gel Electrophoresis—Electrophoresis in 10% acrylamide-SDS gels was as described by Laemmli (19). All samples for electrophoretic analysis were precipitated with 20% trichloroacetic acid, washed once with 10% trichloroacetic acid, once with 0.2% HCl in acetone, once in acetone, and dried under reduced pressure. Dried samples were dissolved in 40 mM Tris, pH 6.8, 1% SDS, 50 mM diithiothreitol, 8 M urea. Gels were routinely stained with Coomassie brilliant blue R-250 (0.07%) in 50% methanol, 5% acetic acid and destained by continuous diffusion in 7% methanol, 7% acetic acid. Amido black (0.1%) was occasionally substituted for Coomassie blue. Silver staining was by the method of Oakley et al. (20).

For quantitation, stained gels were scanned in a Quick Scan Jr. (Helena Laboratories). Gels containing 32P-labeled proteins were autoradiographed with Cronex X-ray film and a Lightening Plus intensifying screen (DuPont).

Acid Hydrolysis and Thin Layer Chromatography—Dried protein samples were dissolved in 6 M HCl and subjected to partial acid hydrolysis under reduced pressure at 105 °C for 1.5-4 h. Hydrolysates were recovered, mixed with standards (P-Ser, P-Thr, and P-Tyr), and analyzed by electrophoresis at pH 3.5 on cellulose thin layer plates as described by Hunter and Sefton (21). Standards were located by ninhydrin staining, and 32P was visualized by autoradiography.

RESULTS

Purification

Source—The Drosophila protein kinase was purified from four different sources: K, (tissue culture) cell nuclei, K, cell cytosol, embryo nuclei, and embryo cytosol. The chromatographic behavior of the activity and the final polypeptide composition of the purified enzyme were the same in each case, but the amount of enzyme obtained differed among the four sources. For both K, cells and embryos, the cytosol fraction yielded approximately 10-fold more enzyme than did the corresponding nuclear fraction. Embryos appeared to be a more economical source, 100 g of dechorionated embryos being equivalent to approximately 10 liters of K, cells at 107 cells/ml (both yielding 5-10 g of total protein in the homogenate and 0.5-1 mg of purified enzyme).

Purification—A typical purification from embryo cytosol utilizing casein as exogenous substrate is summarized in Table I. The experimental procedure is given under “Methods.” and typical elution profiles can be found in Figs. 1-4. Electrophoretic analysis of the fractions obtained during the purification (Fig. 5) revealed that the final product consisted of two major polypeptides (designated α and β) and a small number of minor contaminants. The two major polypeptides but not the contaminants precisely co-purified with the activity at each step in the purification. As estimated by quantitative densitometry of Coomassie blue-stained gels, α and β together constituted 95% of the protein in the purest fraction (Fraction VII). As shown in Table I, slightly more than 1000-fold purification was required to obtain enzyme of this purity. Hence, the enzyme was relatively abundant and represented approximately 0.1% of total protein in the homogenate.

Our standard purification procedure allowed recovery of approximately 10% of the available activity (Table I). Omission of the A-1.5 exclusion chromatography resulted in a 2-fold increase in yield with only a slight reduction in purity (90% homogeneous; see inset to Fig. 6, lane B).

Structural Studies

Physical Characterization—Physical parameters associated with the purified kinase are summarized in Table II. The Stokes radius and sedimentation coefficient were the same for enzyme activity present in crude homogenates from both K, cells and embryos and for various purified fractions including Fraction VII. The molecular weight and frictional ratio calculated from these data (22) indicated that in 1 M NaCl the native enzyme was an elongated structure with a mass of approximately 130,000 daltons.

Subunit Composition—The molecular weights of the α and β subunits are listed in Table II. The stoichiometry of the two
polypeptides, as estimated by quantitative densitometry of gels stained with Coomassie blue, was 1.084 (αβ, after correction for dye-binding on a weight basis (23)). A similar stoichiometry was obtained when gels were stained with Amido black. However, in silver-stained gels, a stoichiometry of 1:1.35 was observed. Our current model of the enzyme assumes an equimolar ratio of the two polypeptides. An αβ2 structure would have a molecular weight close to the value calculated from the hydrodynamic data (Table II).

Autophosphorylation—Casein kinase II from several species is known to undergo an autocatalytic reaction which results in the specific phosphorylation of the β subunit (6). Incubation of the Drosophila enzyme with \([γ^{32}P]ATP\) in the absence of added substrate resulted in the incorporation of approximately 1 mol of \(^{32}P\)/mol of enzyme (αβ2: tetramer) during a 30-min incubation. Analysis of the autophosphorylated enzyme by SDS gel electrophoresis and autoradiography (Fig. 6) revealed that >95% of the total incorporation was associated with the β subunit. Although we do not know for certain whether the β subunit is phosphorylated in vivo, partial phosphorylation of the enzyme could explain the heterogeneity of this subunit observed in SDS gels (e.g. Fig. 5) as well as the incorporation of less than 1 mol of phosphate/mol of β subunit during autophosphorylation.

![FIG. 5. SDS gel analysis of Fractions I-VII. A portion of each fraction obtained during the purification of casein kinase II was precipitated with trichloroacetic acid and electrophoresed in a 10% SDS gel. The gel was stained with Coomassie blue. M, molecular weight markers, 1 µg of band; H, Fraction I homogenate, 20 µg; Cyto, Fractions II and III, postnuclear and pH-precipitated postmitochondrial supernatants, respectively, 20 µg each; P, Fraction IV, phosphocellulose pool, 10 µg; HAP, Fraction V, hydroxylapatite pool, before and after (NH\(_4\))\(_2\)SO\(_4\) precipitation, 10 µg/lane; A-1.5m, Fraction VI, agarose A-1.5m pool, before and after (NH\(_4\))\(_2\)SO\(_4\) precipitation, 10 µg/lane; and G, Fraction VII, glycerol gradient pool, 10 µg. Marker proteins and their molecular masses in kilodaltons are BSA (68), pyruvate kinase (57), lactate dehydrogenase (35), lysozyme (14), and RNase A (13). α and β designate the polypeptide subunits of casein kinase II.](image-url)

![FIG. 6. Autophosphorylation. Casein kinase II was purified as described under "Methods" except that the gel filtration step and second (NH\(_4\))\(_2\)SO\(_4\) precipitation were omitted. The enzyme was diluted to 0.15 mg/ml (1.2 µM) in 50 mM Tris, pH 8.5, 10 mM MgCl\(_2\), 100 mM NaCl, 0.05% Triton X-100, 2% glycerol, divided into two aliquots, and incubated either with or without 100 µM \([γ^{32}P]ATP\) (1500 cpm/pmol) for 30 min at 25°C. Reactions were stopped by precipitation with 20% trichloroacetic acid and analyzed by electrophoresis in a 10% SDS gel. The gel was stained with Coomassie blue (A, B, C) and autoradiographed (D). A, molecular weight markers as in Fig. 5; B, casein kinase II incubated without ATP; C, casein kinase II incubated with ATP; D, autoradiograph of lane C. Solid line, densitometer scan of lane C. Dashed line, densitometer scan of lane D.](image-url)
Kinetic Analysis

All kinetic studies were carried out with casein as substrate. Fraction VII enzyme, at a final concentration of 0.13 μg/ml (10⁻⁹ M, assuming Mₛ = 130,000) was used throughout.

Temperature—The purified enzyme was stable in the standard diluent for at least 1 h of preincubation at any temperature between 0 and 25°C. Time-dependent irreversible denaturation was observed above 25°C. Denaturation was exponential, with the half-life of the activity being 40 min at 35°C and 1 min at 45°C. Over the range from 0 to 25°C, a plot of the logarithm of initial reaction velocity versus reciprocal of absolute temperature was linear. An energy of activation of 15,000 cal/mol was calculated from this plot (24).

pH—Optimum activity was observed in an alkaline pH range from 7.5 to 9.0 (Fig. 7A). Half-maximal activity was observed at pH 7.9. Neither transition appeared to be due to enzyme denaturation because preincubation of the enzyme at 25°C at any pH from 6.0 to 11.0 had no effect on activity. Irreversible denaturation was observed below pH 6, the half-life of the activity being 50 min at pH 5.5.

Ionic Requirements—The enzyme was stimulated by monovalent salt (NaCl or KCl) in the range 100–150 mM (Fig. 7B). In the standard reaction mix (50 mM Tris, 10 mM MgCl₂), this implied a total ionic strength of 0.18–0.23. As expected, the enzyme exhibited an absolute requirement for divalent cations. Activity was strongly stimulated by MgCl₂ up to a concentration of 10 mM (Fig. 7C). Stimulation by Mg²⁺ in excess of the amount necessary for formation of a stable Mg-ATP²⁻ complex suggests an additional function or functions for this metal ion. Beyond 10 mM MgCl₂, a gradual inhibition of activity was observed even when the total ionic strength was held constant by a compensating reduction in NaCl concentration.

Initial Velocity Studies—Fig. 8 (left) shows a plot of 1/v versus 1/[Mg·ATP²⁻] at different fixed concentrations of casein. A secondary replotted (inset) of the 1/v axis intercepts versus 1/[casein] was used to estimate a Kₘ for casein at saturating Mg·ATP²⁻ of 0.74 mg/ml. Fig. 8 (right) shows a similar plot of 1/v versus 1/[casein] at different fixed concentrations of Mg·ATP²⁻. The intercept replotted (inset) yielded a Kₘ for Mg·ATP²⁻ at saturating casein of 16 μM. Both replots gave an identical Vₘ₉₉ of 1.5 μmol/min/mg.

Both families of lines in Fig. 8 converge. This result is inconsistent with ping-pong mechanisms and implies that the reaction mechanism must be sequential (24). The convergence of the lines below the horizontal axis indicates that the binding of either substrate inhibits binding of the other (α > 1). Slope replots yielded estimates for α of 3.4 and 3.7. The kinetics of the enzyme is summarized in Table II where the kinetic constants have been interpreted assuming that the reaction mechanism is rapid equilibrium random Bi Bi (24, 25).

Product inhibition studies with Mg·ADP²⁻ revealed that...
Fig. 9. Identification of phosphorylated amino acids. Casein (2 mg/ml) was labeled in vitro for 30 min at 25 °C with 1.3 μg/ml (10−5 M) casein kinase II (Fraction VII) in 50 mM Tris, pH 8.5, 10 mM MgCl2, 125 mM NaCl, 0.1 mM [γ-32P]-ATP (1000 Ci/mmol). The reaction was stopped by precipitation with 20% trichloroacetic acid. Precipitated protein was dissolved in 6 M HCl and hydrolyzed at 105 °C for 1.5–4 h. Hydrolysates were recovered, mixed with standards (P-Ser, P-Thr, P-Tyr), and analyzed by thin layer electrophoresis at pH 3.5. An autoradiograph of the gel is shown. A, hydrolysis for 1.5 h; B, hydrolysis for 4 h. Dashed circles indicate the positions of the standards as visualized by ninhydrin staining. P, designates the mobility of inorganic phosphate. Other spots are small phosphopeptides which are formed as intermediates during hydrolysis.

Mg-ADP− is competitive with respect to Mg-ATP2−. This result confirms that the mechanism is not classical Ping Pong. The Ks determined from a replot of slope versus [Mg-ADP−] was 30 μM.

Substrate Specificity

Nucleoside Triphosphate Requirement—The purified enzyme was able to use Mg-GTP2− as efficiently as Mg-ATP2− as phosphate donor. At 4 mg/ml casein (approximately 5 times the Km) in the presence of 125 mM NaCl, the enzyme exhibited an apparent Km of 66 μM and 0.86 μmol/min/mg, respectively, compared to 17 μM and 1.2 μmol/min/mg, respectively, for Mg-ATP2− under the same conditions.

Protein Substrate—The purified kinase contained few if any endogenous substrates other than itself (see Fig. 6). Consequently, under standard assay conditions (10−3 M enzyme), very little phosphate was incorporated into acid-precipitable material in the absence of an added substrate. Of the purified proteins tested for their ability to serve as exogenous substrates, only casein and phosphorin proved to be good acceptors. An apparent Km and Vmax were determined for these two proteins at 125 mM NaCl and 65 μM ATP (5 times the Km). The apparent Km for phosphorin (0.02 mg/ml) was considerably lower than that observed for casein (0.5 mg/ml). However, the apparent Vmax with phosphorin as substrate was also lower (0.26 μmol/min/mg compared to 1.6 μmol/min/mg). Acid-denatured calf thymus histones, whether presented together or as individual purified fractions, were not substrates, nor was protamine. Any stimulation observed with such proteins was invariably the result of incorporation into higher molecular weight contaminants, as determined by gel electrophoresis. Basic ribosomal proteins present in intact Drosophila ribosomes were not recognized by the enzyme. In particular, the S6-like protein was not a substrate even if ribosomes were isolated from heat-shocked cells where this protein is completely dephosphorylated (18). Bovine serum albumin was not a substrate for the enzyme.

Amino Acid Substrate—The amino acid residue phosphorylated by the kinase was identified by thin layer chromatography of partial acid hydrolysates of protein labeled in vitro with [γ-32P]-ATP and Fraction VII enzyme. With casein as substrate (Fig. 9), both phosphoserine and phosphothreonine were observed. Assuming similar rates of release and destruction of the two modified residues during hydrolysis, serine was the predominant site of phosphorylation. No phosphorylation of tyrosine residues was observed.

Regulation

Cyclic Nucleotide Independence—Activity of the purified enzyme was unaffected by either cAMP or cGMP in the range of 0.1 to 100 μM with casein as substrate.

Heparin Sensitivity—Sensitivity to low concentrations of heparin (<1 μg/ml) is characteristic of type II casein kinase (6). Furthermore, because other kinases are relatively unaffected by this compound (26), heparin sensitivity is strongly diagnostic for this enzyme. As shown in Fig. 10, the Drosophila kinase was strongly inhibited by heparin. With casein as substrate, a heparin concentration of 21 ng/ml was sufficient to achieve 50% inhibition of the purified enzyme (present at 0.15 μg/ml). The molar ratio of heparin to catalytic sites (assuming 2 sites/tetramer) was only 1.51 under these conditions. Similar results were obtained with phosphorin as substrate.

Low concentrations of heparin were also found to inhibit autophosphorylation of the Drosophila enzyme (95% inhibition by 0.5 μg/ml heparin at 1.5 μg/ml kinase, no added substrate). This result demonstrated that the autophosphorylation reaction was catalyzed by a casein kinase II activity, presumably the enzyme itself, rather than by a contaminating protein kinase.

Discussion

We have described the purification of a protein kinase from D. melanogaster. This enzyme is by definition a casein kinase
because it is cyclic nucleotide-independent and phosphorylates casein and phosphoinositide but not histones or proteamines. In its other properties, it exhibits striking similarity to casein kinase II. These properties include its native molecular weight, an αβ2 subunit composition, autophosphorylation of the β subunit, use of either ATP or GTP as nucleoside triphosphate donor, chromatographic behavior on both phosphocellulose and hydroxylapatite, self-aggregation at low ionic strength, heparin sensitivity, and phosphorylation of both serine and threonine residues in casein. All of these properties distinguish the enzyme from casein kinase I, and at least two, heparin sensitivity and the low K_h for ATP, appear to be unique to casein kinase II. The combined results strongly suggest that the Drosophila enzyme is homologous to casein kinase II from mammalian and avian species (6) and imply a high degree of conservation of this enzyme since the divergence of these organisms.

The purified Drosophila enzyme appears to be essentially homogeneous by several criteria. First, it behaves as a homogeneous protein during velocity sedimentation (Fig. 4); second, it exhibits a specific activity of 1-2 μmol/min/mg, a value typical of purified casein kinase II from other organisms; and third, it has the expected αβ polypeptide composition. The relatively small size of the α polypeptide (36,700 daltons compared to 42,000-44,000 for the mammalian enzyme) as well as the heterogeneity of the β subunit might suggest that the enzyme is partially degraded. The α subunit of the mammalian enzyme is known to be protease sensitive, cleavage converting the α subunit into an α fragment of 37,000-40,000 daltons (6). However, we consider proteolysis unlikely for the following reasons: 1) the polypeptide composition of the enzyme was the same in five independent isolates; 2) K, cells and embryos, although they might be expected to contain different proteases, gave identical polypeptide patterns; 3) additional protease inhibitors (ovomucoid at 0.1 mg/ml, soybean trypsin inhibitor at 0.1 mg/ml, and 1,10-phenanthroline at 1 mM, in addition to the usual PMSF and NaHSO_4) yielded an identical pattern; and 4) incubation of the homogenate fraction at 25 °C for 4 h in the absence of any protease inhibitors resulted in no detectable changes in the pattern of whole embryo protein analyzed in SDS gels. Although we cannot conclusively rule out some cleavage near one or both ends of the β subunit, all of the heterogeneity in this polypeptide could be due to phosphorylation. Thus, the procedure described here appears to yield nearly homogeneous enzyme exhibiting little if any proteolytic degradation.

Mammalian and avian casein kinase II has been isolated from a variety of intracellular compartments including nuclei, membranes, ribosomes, mitochondria, and cytosol (6). As shown here, the Drosophila enzyme can be purified from either nuclei or cytosol. The failure to detect any differences between the nuclear and cytoplasmic enzymes suggests that they are in fact one and the same. For both K, cells and embryos, we consistently find 80-90% of casein kinase II in the cytosol fraction and 5-10% in nuclei. The activity associated with nuclei appears to be tightly bound and is efficiently released only upon extraction with 0.35 M NaCl. Unfortunately, the degree to which these cell fractionation results accurately reflect the intracellular distribution of the enzyme is unknown. Immunofluorescence and other techniques will be required to address this question.

In addition to casein kinase II described here, cAMP- and cGMP-dependent protein kinase activities have been reported in Drosophila (27). A cAMP-dependent enzyme has been partially purified (28), and the kinetic mechanism of this enzyme has been investigated in considerable detail (29). Casein kinase I, in spite of its widespread distribution among diverse phyla (6), has not yet been reported in Drosophila or in any other arthropod species. Throughout our purification procedure, casein kinase activity behaves as a single homogeneous species. The type I enzyme is unlikely to contaminate our purified type II preparation because the two enzymes differ markedly in size and, hence, should be readily resolved by both exclusion chromatography and glycerol gradient sedimentation if not by an earlier purification step. However, we have not yet made a systematic search for casein kinase I in Drosophila. Heparin inhibition of the type II enzyme should facilitate detection and subsequent purification of this activity if it exists.

The function of casein kinase II is presently unknown. Much work has been directed in recent years toward identification of the endogenous substrates of this enzyme. Substrates identified to date include translational initiation factors, acidic ribosomal proteins, proteins associated with messenger ribonucleoprotein particles, glyccogen synthase, membrane proteins, and nuclear proteins (6). Among the nuclear substrates are both acidic (30) and basic (31) non-histone chromosomal proteins, including RNA polymerase subunits (32) and high mobility group protein 17 (33). We have so far identified one endogenous substrate of the Drosophila enzyme: chromosomal protein D1 (34). D1 has been found in association with satellite sequences in Drosophila (35, 36) and is known to be phosphorylated in vivo (18). D1 is an excellent substrate for the Drosophila enzyme and was originally used as exogenous substrate for its purification. An analysis of endogenous phosphorylation by casein kinase II in Drosophila will be presented elsewhere. Purification of casein kinase II, analysis of its kinetics and regulation in vitro, and identification of its in vivo substrates should help to elucidate the physiological function of this enzyme. It is anticipated that by characterizing this activity in Drosophila it will be possible, in addition, to address functional questions by application of genetic techniques available in this organism.

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