Purification of a Yeast Protein Kinase Sharing Properties with Type I and Type II Casein Kinases

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ABSTRACT: Cyclic nucleotide independent protein kinases preferring casein as in vitro substrates were resolved into four distinct species. Only one of the enzymes (CKII) was retained by DEAE-cellulose, whereas the three other enzymes (CKI-1, CKI-2, and CKI-3) were absorbed to CM-Sephadex, eluted with 250 and 600 mM NaCl, and fractionated by heparin–Sepharose chromatography. The casein kinase CKI-3 eluting at the highest NaCl concentration (550 mM) was purified to electrophoretic homogeneity by fast protein liquid chromatography. CKI-1 and CKI-2 correspond to mammalian type I casein kinase, because they bind to CM-Sephadex, they are monomeric enzymes of molecular weights below 50 000, they accept ATP exclusively (CKI-1) or predominantly (CKI-2) as phosphate donor, and they are either completely or relatively heparin insensitive. CKII corresponds to type II casein kinase due to its chromatographic properties, complex quaternary structure, nucleotide specificity (both ATP and GTP are phosphate donors), and heparin sensitivity. CKI-3 shares the following properties with type I casein kinases: it is retained by CM-Sephadex but not by DEAE-cellulose, and it consists of a monomeric protein having a molecular weight of 38 000. On the other hand, CKI-3 accepts both ATP and GTP with equal efficiency, and it is heparin sensitive (50% inhibition at 0.3 μg/mL) like type II casein kinases. CKI-3 differs from the other three yeast casein kinases in requiring a low pH (5.5) and a high $MgCl_2$ concentration (50 mM) for optimal activity. All four casein kinases phosphorylate their own catalytic protein at serine and threonine residues.

Casein kinases are cyclic nucleotide independent protein phosphokinases (EC 2.7.1.37) preferring acidic proteins like casein as in vitro substrates. However, the phosphorylation of casein is of little physiological significance, since casein is absent from most cell types or organisms used as enzyme sources. More relevant to the possible in vivo functions of casein kinases is the observation that a variety of endogenous proteins including components of the translational apparatus, RNA polymerase, non-histone nuclear proteins, and glycogen synthetase are phosphorylated in vitro by these enzymes. Casein kinases are found in nuclei, cytoplasm, mitochondria, and plasma membrane fractions. They are generally divided into two classes according to their subunit structure, their chromatographic behavior, and their catalytic properties: type I casein kinases are monomeric enzymes with a molecular weight smaller than 50 000, they use only ATP as phosphate donor, and they are not retained by anion-exchange materials like DEAE-cellulose at neutral pH. In contrast, type II casein kinases are oligomeric enzymes with a molecular weight larger than 100 000, containing at least two different subunits, they accept ATP as well as GTP as phosphate donors, and they bind tightly to anion-exchange material (Hathaway & Traugh, 1982).

The yeast *Saccharomyces cerevisiae* has been shown to contain a monomeric, ATP-accepting type I casein kinase of molecular weight 43 000 (Lerch et al., 1975; Kudlicki et al., 1980), as well as a type II casein kinase containing two different subunits (molecular weights of 41 000 and 37 000) as an $\alpha_2\beta_2$ tetramer, and accepting both ATP and GTP as phosphate donor (Kudlicki et al., 1978, 1984). More recently, a second minor species of type I casein kinase (molecular weight 27 000) accepting only ATP was reported (Donella-Deana et al., 1985; Szyszka et al., 1985).

Here we describe a chromatographic procedure resolving yeast casein kinases into four different species, including a novel enzyme (CKI-3). This enzyme was purified to apparent homogeneity and shown to share properties with type I and type II casein kinases.

Materials and Methods

Commercial bakers' yeast was a product from Deutsche Hefewerke (Hamburg, FRG). [γ-^{32}P]ATP and [γ-^{32}P]GTP were purchased from Amersham-Buchler (Braunschweig, FRG). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight protein standards were obtained from Bio-Rad Laboratories (Richmond, CA). Polymin P, a poly(ethylenediamine) of average molecular weight 6000, is a product from BASF (Ludwigshafen, FRG). Partially dephosphorylated casein from bovine milk, histone II-A, the protease inhibitor aprotinin, heparin, and phosphoamino acids (*O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine) were purchased from Sigma (Deisenhofen, FRG). Nitrocellulose filters type SM (0.45 μm) were obtained from Sarorius (Göttingen, FRG). Centricon tubes are a product from Amicon Corp. (Danvers, MA). Chromatographic materials were purchased from the following suppliers: DEAE-cellulose DE 52 from Whatman (Maidstone, England), Ultrogel AcA 44 from LKB (Gräfelfing, FRG), and CM-Sephadex C50, Mono S, and Sepharose 4B from Deutsche Pharmacia (Freiburg, FRG). Sepharose 4B was the starting material for the preparation of heparin-bound Sepharose (Kohn & Wilcheck, 1982). For identification of phosphoamino acids, Silica-Rapid F254 plates from Woelm (Eschwege, FRG) were used.

Buffer Solutions. Buffer A was 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5; buffer B consisted of 30 mM sodium phosphate, pH 7.0; buffer C was composed of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5. All buffers contained 2 mM 2-mercaptoethanol, 10 μM phenylmethanesulfonyl fluoride

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(PMSF), and 5% w/v glycerol.

Assay of Protein Kinase Activities. Although individual casein kinases differ in their pH and Mg^{2+} optima, their activities were assayed routinely in 0.25 mL of the following standard mixture: 25 mM MES (pH 6.5), 50 mM Mg^{2+}, 2 mM 2-mercaptoethanol, 0.25 mg of casein, 0.05 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP (specific activity about 15 000 cpm nmol^{-1}), respectively, and varying amounts of casein kinase fractions. For kinetic studies using purified enzymes, the specific activity of nucleoside [γ-^{32}P]triphosphates was raised to 80 000 cpm nmol^{-1}. cAMP-dependent protein kinase was tested in the presence of cAMP with histone as substrate as described (Takai et al., 1973). Samples were incubated at 35 °C for 5 min. The reaction was stopped by addition of 0.7 mL of cold 10% trichloroacetic acid (TCA). The precipitation was collected on a nitrocellulose filter and washed 3 times with 5 mL of cold 5% TCA. After the filters were dried, the amount of retained radioactivity was counted in a scintillation counter.

One unit of the enzyme is defined as the amount of protein able to transfer 1 nmol of [^{32}P]P_i from [γ-^{32}P]ATP ([γ-^{32}P]GTP) to casein in 1 min at 35 °C under the conditions mentioned above (specific activity in units per milligram of protein).

Determination of Protein. During purification of the enzymes, protein concentration was determined by measuring the absorbances at 280 and 260 nm according to Warburg and Christian (1942). The concentration of isolated enzymes was determined according to Bradford (1976) using bovine serum albumin as standard.

Electrophoretic Methods. SDS–polyacrylamide slab gel electrophoresis was performed in 10% polyacrylamide gels as described by Weber and Osborne (1969). The standard proteins phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400) were used for molecular weight estimations, and aprotinin was added to all samples before heat treatment in order to prevent proteolysis.

Analysis of Phosphoamino Acids. Casein or enzyme protein was phosphorylated at standard conditions for 30 min at 35 °C with [γ-^{32}P]ATP or [γ-^{32}P]GTP (specific activity about 60 000 cpm nmol^{-1}) and hydrolyzed with HCl (De Paoli-Poach, 1981). In order to detect the acid-labile phosphotyrosine, protein was hydrolyzed by 5 N KOH (Martensen et al., 1983). Hydrolysates were analyzed by thin-layer electrophoresis on silica plates at 800 V, 2.5 h, using unlabeled phosphorylated serine, threonine, and tyrosine as standards (visualized by ninhydrin stain). Radioactive spots were identified after autoradiography using Cronex 4 X-ray films (Du Pont).

Isoelectric focusing was performed on LKB Ampholine PAG plates (pH range 3.5–9.5) according to Ranged-Aldao et al. (1979), using 1.0 M NaOH and 1.0 M H_3PO_4 as an anode or cathode electrode solution, respectively. During 1.5 h of the focusing procedure, the voltage was increased stepwise from 200 to 1500 V. For pH determination, gels were cut into 5-mm slices, which were minced with a glass pestle and suspended in 0.5 mL of distilled water. For determination of casein kinase activity, the minced slices were suspended in 0.1 mL of 25 mM MES, pH 6.5, for about 3 h at 0 °C and assayed in 50-μL aliquots at standard conditions.

Chromatographic Procedures. All steps were performed at 4 °C. The protease inhibitor PMSF (final concentration 10 μM) was added to all fractions immediately after elution. Commercial bakers' yeast (12 kg) was extracted by following the method of von der Haar (1979) up to the third step. The crude extract was treated with Polymin P to precipitate nucleic acids, and the supernatant protein was precipitated with 400 g/L ammonium sulfate (about 62% saturation) while continuously adding aqueous ammonia to keep the pH at 7.5. The precipitate was suspended in buffer B, dialyzed overnight against the same buffer, and, after dilution with an equal volume of buffer B, gently stirred for 30 min with 2.5 L of CM-Sephadex C50 equilibrated with buffer B.

After the supernatant was decanted, the slurry was poured into a 9 × 45 cm column and washed with 3000 mL of buffer B. The absorbed proteins weree then eluted in two steps with 3000 mL of buffer B containing 250 mM NaCl, followed by 3000 mL of the same buffer containing 600 mM NaCl. The active fractions (see Results) of the 250 mM NaCl eluate containing both histone and casein kinase were pooled, precipitated with ammonium sulfate (60% saturation), dissolved in a small volume of buffer A, and applied onto an UltroGel Aca 44 column (7 × 110 cm) which was equilibrated with buffer A containing 50 mM NaCl. Proteins were eluted with 4000 mL of buffer A containing 50 mM NaCl, resolving the early eluting histone kinase from a second peak containing casein kinase.

The active fractions of the latter peak were combined with the 600 mM NaCl eluate from CM-Sephadex containing only casein kinase activity, precipitated and dialyzed as described above, and applied onto a heparin-bound Sepharose 4B column (3 × 20 cm) equilibrated with buffer A. The column was washed with 500 mL of buffer A, indicating that all casein kinase activity was bound to the heparin matrix, and developed by a linear NaCl gradient (0–700 mM in buffer A, total volume 2500 mL). The fractions of the third casein kinase peak (CKI-3) were pooled, concentrated by ammonium sulfate precipitation (60% saturation), and, after dialysis against buffer A, applied onto a Mono S column of 8-mL bed volume. The column was equilibrated with 50 mL of buffer A (adjusted to pH 8.0) and developed with a linear gradient from 0 to 400 mM NaCl (total volume 60 mL) in the same buffer. Casein kinase activity was eluted with 290 mM NaCl. Rechromatography of the active fraction was performed on the same column, now equilibrated with buffer C (pH 6.5), using the same NaCl gradient in buffer C. Fractions containing the enzyme activity were concentrated by centrifugation using Centricon 30 tubes. To the remaining solution an equal volume of glycerol was added for storage at −20 °C. This solution was used for all further investigations described. Casein kinase CK-II was purified by DEAE-cellulose chromatography according to Kudlicki et al. (1984).

RESULTS

Fractionation of Protein Kinases. The first step of our rapid fractionation procedure (see Materials and Methods) is the precipitation of nucleic acids from a crude supernatant by Polymin P, whereby virtually all protein kinase activity remains soluble. Most of this activity is absorbed to CM-Sephadex, with the exception of CKII, which does not bind under the given conditions on this cation-exchange material.

Figure 1 shows the protein kinase pattern obtained by step elution from CM-Sephadex with 250 and 600 mM NaCl. The first elution step partially separates a cAMP-dependent histone kinase from casein kinase activity preferentially using ATP as phosphate donor, whereas the second step partially resolves two casein kinase fractions accepting either only ATP (fractions 180–200) or both ATP and GTP (fractions 210–240).

The histone and casein kinase activities of the first elution step were completely resolved by Ultrogel AcA 44 filtration,

Table I: Purification of Yeast Casein Kinase CKI-3[a]

step	volume (mL)	protein (mg)[b]	enzyme units with ATP	enzyme units with GTP[c]	sp act. (cpm nmol^{-1}) with ATP	sp act. (cpm nmol^{-1}) with GTP
(1) supernatant after Polymin P pptn	7600	186000	171000	43000 (25)	0.9	0.2
(2) ammonium sulfate ppt after dialysis	620	87400	187800	44800 (24)	2.1	0.5
(3) CM-Sephadex, 0.6 M NaCl eluate	820	710	44500	28600 (64)	62	40
(4) heparin–Sepharose, third peak	400	56	12300	11500 (94)	220	205
(5) Mono S chromatography at pH 8.0	8	2.8	8850	9070 (102)	3160	3240
(6) Mono S chromatography at pH 6.5	4	1.6	5500	5460 (99)	3460	3410

[a] Average of four purification procedures. [b] Protein was measured in steps 1–5 according to Warburg and Christian (1942) and in step 6 according to Bradford (1976). [c] The enrichment of GTP-accepting activity is indicated as the percentage of total (ATP-accepting) activity in parentheses.

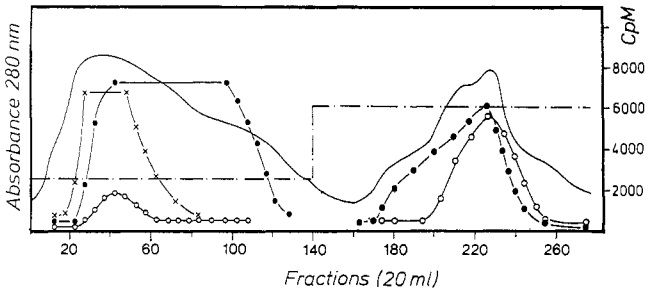

FIGURE 1: CM-Sephadex chromatography of protein kinases. Proteins were eluted in two steps with 250 and 600 mM NaCl (–·–). Absorption at 280 nm (—) was monitored continuously. Aliquots of 10 μL were assayed with histone (in the presence of cAMP) and [γ-^{32}P]ATP (×) and with casein and [γ-^{32}P]ATP (●) or [^{32}P]GTP (○).

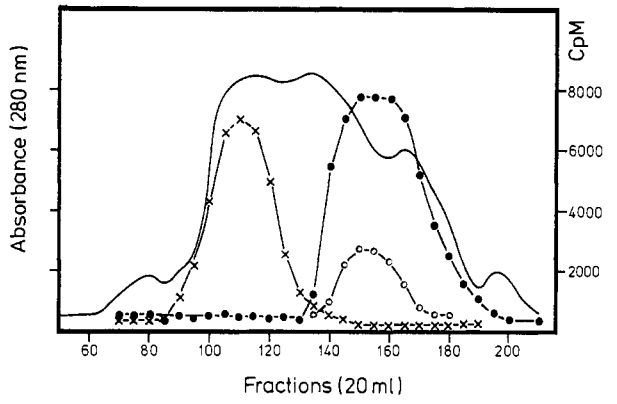

FIGURE 2: UltroGel AcA 44 filtration of the CM-Sephadex 250 mM NaCl eluate. A 7 × 110 cm column was developed with buffer A containing 50 mM NaCl. Absorption at 280 nm (—), cAMP-dependent histone kinase (×), and casein kinase with [γ-^{32}P]ATP (●) or [γ-^{32}P]GTP (○).

as shown in Figure 2. At the same time, the ratio between GTP- and ATP-accepting activities has slightly increased after the Ultrogel step, indicating that the recovery of CKI-2, which also uses GTP to some extent, was higher than that of CKI-1, which has no measurable GTP-accepting activity (see below).

The casein phosphorylating fractions of the Ultrogel filtrate (fractions 140–190 of Figure 2) were combined with the active fractions of the 600 mM NaCl elution step (see Figure 1) and resolved into three distinct casein kinase activities by affinity chromatography on heparin-bound Sepharose (Figure 3). The two casein kinase fractions derived from the two NaCl steps (Figure 1) were also separately analyzed by heparin–Sepharose chromatography, indicating that the 250 mM NaCl fraction contains mainly CKI-1 and CKI-2, whereas the 600 mM NaCl fraction is enriched in CKI-3.

CKI-3 was purified to electrophoretic homogeneity by two fast protein liquid (FPL) chromatography steps (Figure 4), and the enrichment of this enzyme following the above procedure is summarized in Table I. It should be emphasized

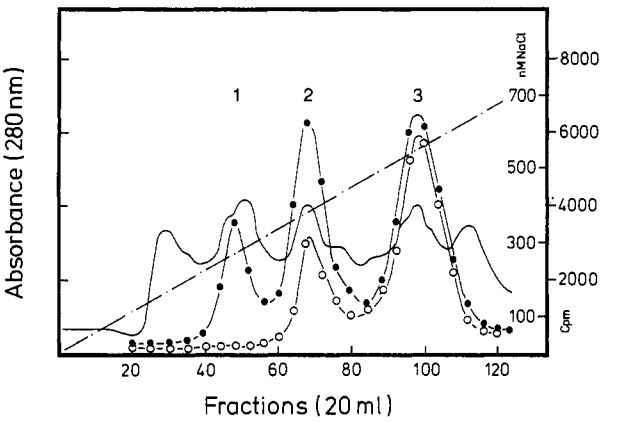

FIGURE 3: Heparin–Sepharose chromatography of casein kinase. Fractions 140–190 of the UltroGel eluate (Figure 2) and fractions 210–240 of the CM-Sephadex 600 mM NaCl eluate (Figure 1) were pooled and directly applied onto a 3 × 20 cm heparin–Sepharose column equilibrated in buffer A. The column was developed with a linear gradient (0–600 mM NaCl in buffer A). Absorption at 280 nm (—); casein kinase with [γ-^{32}P]ATP (●) or [γ-^{32}P]GTP (○).

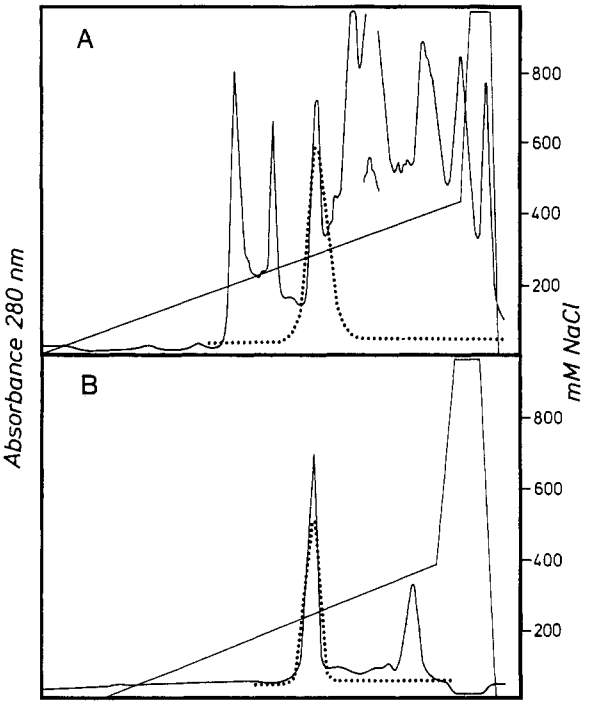

FIGURE 4: FPL chromatography of CKI-3 on Mono S at pH 8.0 (A) and 6.5 (B). Fractions 91–110 containing the third peak of the heparin–Sepharose eluate (Figure 3) were precipitated, dialyzed, and applied onto a Mono S column equilibrated with buffer A (pH 8.0). Proteins were eluted with a linear gradient (0–400 mM NaCl in the same buffer) and monitored at 280 nm (—). Fractions (2 mL) containing casein kinase activity with [γ-^{32}P]GTP (···) were pooled are rechromatographed on the same column equilibrated with buffer C, using the same salt gradient in buffer C.

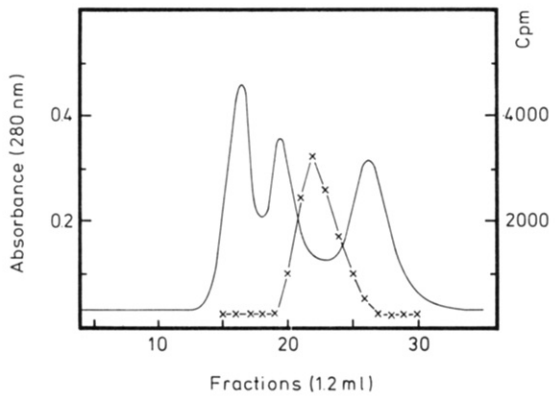

FIGURE 5: Gel filtration of CKI-3. An aliquot (45 μg) of the peak fraction eluting from the second Mono S column (Figure 4B) was applied onto a Bio-Gel P150 column (1 × 60 cm) together with the three marker proteins bovine serum albumin, M_r 68000 (1); ovalbumin, M_r 44000 (2); and myoglobin, M_r 17000 (3). Proteins were eluted with buffer A containing 50 mM NaCl, monitored at 280 nm (—), and assayed with casein and [γ-^{32}P]GTP (×).

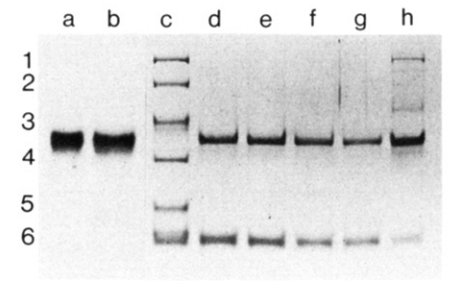

FIGURE 6: SDS–polyacrylamide gel electrophoresis of CKI-3. The peak fraction of Figure 5B was analyzed directly (lanes d and e) or after autophosphorylation with [γ-^{32}P]GTP (lanes a, b, f, and g). Lanes a and b show the autophosphorylated enzyme after autoradiography; all other lanes show Coomassie-stained protein bands. Lane h contains the peak fraction after the first Mono S chromatography (see Figure 4A). Lane c, size standards of molecular weights 92000 (1), 66000 (2), 45000 (3), 31000 (4), and 21000 (5). Band 6 is aprotinin (see Materials and Methods).

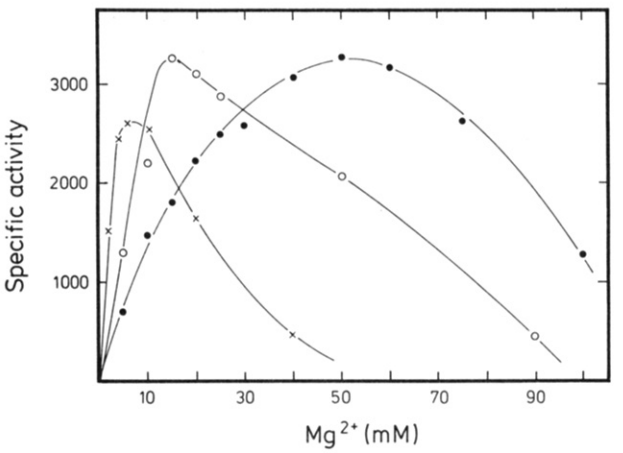

FIGURE 7: Correlation between the concentration of Mg^{2+} and the activity of CKI-1 (×), CKI-2 (○), and CKI-3 (●). Enzymes were assayed in presence of constant concentrations of ATP and casein and in the absence of monovalent cations.

that the ATP-dependent activities determined after steps 1 and 2 are produced by four different enzymes (CKI-1, CKI-2, CKI-3, and CKII), whereas the GTP-dependent activities after the first two steps are produced by two enzymes (CKI-3 and CKII), and after step 3 by CKI-3 alone, since CKII is not retained by CM-Sephadex. Therefore, the enrichment factor and overall yield of CKI-3 can only be roughly estimated as 15000 and 13%, respectively, by using the GTP-dependent activity data.

The fractionation procedure can be further simplified by eluting all protein kinase activity from CM-Sephadex in a single step (600 mM NaCl), followed by Ultrogel filtration. The two-step procedure described here was preferred because other enzymes like aminoacyl-tRNA synthetases were co-fractionated.

Properties of Casein Kinase CKI-3. (*A*) *Stability.* The pure enzyme can be stored at −20 °C in 50% glycerol solution containing 30 mM MES, pH 6.5, or 30 mM phosphate, pH 7.0, 5 mM β-mercaptoethanol, and 100 mM NaCl or $(NH_4)_2SO_4$. The protein concentration should be higher than 1 mg/mL. Under these conditions, the enzyme remains fully active for several months without detectable proteolytic degradation.

(*B*) *Structural Properties.* Figure 5 shows a symmetrical peak of CKI-3 activity (with GTP as phosphate donor) upon filtration on a Bio-Gel P120 column (1 × 60 cm), using buffer A + 50 mM NaCl. In comparison with three standard proteins of known molecular weights, and enzyme elutes as a protein of molecular weight of 38000. Polyacrylamide gel electrophoresis of CKI-3 under denaturating conditions (10% SDS) reveals a single protein band (Figure 6, lanes d–g) of the same apparent molecular weight (38000) as the native enzyme, indicating a monomeric structure of CKI-3. Electrofocusing experiments (see Materials and Methods) indicate that CKI-1 and CKI-2 are more basic (pI = 8.1 and 8.4) than CKI-3 (pI = 7.2).

(*C*) *Substrate Specificity and Catalytic Properties.* CKI-3 efficiently phosphorylates acidic proteins like casein and phosvitin, whereas basic proteins like histones and protamine were not accepted as substrates in the presence or absence of cAMP. Under optimal conditions (see below), the pure enzyme catalyzes the transfer of 3460 nmol of phosphate from GTP or ATP per milligram of protein during 1 min at 35 °C. It follows that 1 mol of enzyme transfers 2 mol of phosphate per second (k_{cat} = 2.4 s^{-1}) to partially dephosphorylated casein. The enzyme exhibits its highest activity in the presence of 2 mg/mL casein (apparent K_m = 0.6 mg/mL). No substrate inhibition up to 10 mg/mL casein was observed. With a constant concentration of casein, the K_m value for ATP and GTP could be determined as 15 and 10 μM, respectively. Both nucleoside triphosphates compete each other during phosphorylation of casein with a K_i value of 35 μM (ATP substrate, GTP competitive inhibitor) or 50 μM (vice versa).

(*D*) *Iron Requirements.* Figure 7 shows the Mg^{2+} requirement of three casein kinases in the absence of monovalent cations. CKI-3 requires the highest concentration (50 mM) for maximal activity, followed by CKI-2 (20 mM) and CKI-1 (10 mM). Replacement of Mg^{2+} by Mn^{2+} lowers the activity, and other cations (Ca^{2+}, Cd^{2+}, Cu^{2+}, and Zn^{2+}) completely suppress the activity of CKI-3.

The activities of CKI-1 and CKI-3 (measured at their optimal Mg^{2+} concentrations) are inhibited by Na^+ ions at concentrations above 30 mM, whereas CKI-2 is stimulated 2-fold by 50 mM NaCl (Figure 8). Similar results were obtained in the presence of a suboptimal concentration of Mg^{2+} (10 mM). Higher ionic strength caused by the addition of NaCl does not stimulate the enzymes; i.e., NaCl cannot replace Mg^{2+}. Furthermore, the same Mg^{2+} optimum of 50 mM was measured for CKI-3 in the presence of 50 mM NaCl. According to Figure 9, the three enzymes differ also in their pH optima.

As a result of the data of Figures 7–9, the optimal conditions for casein phosphorylation by CKI-3 are 50 mM MES, pH

Table II: Properties of Casein Kinases in *Saccharomyces cerevisiae*

	CKI-1	CKI-2	CKI-3	CKII[a]
sp act. (units/mg)	2750	3350	3460	nd[d]
ATP, K_m (M)	5.5×10^{-5}	2.2×10^{-5}	1.4×10^{-5}	0.7×10^{-5}
GTP, K_m (M)		12.0×10^{-5}	1.6×10^{-5}	5.5×10^{-5}
optima				
pH	8.0	6.5	5.5	7.5
Mg^{2+} (mM)	10	20	50	8
Na^+ (mM)	0	50	0	0
casein (mg/mL)	0.2	0.8	1.4	nd
inhibitors[b]				<5
heparin (5 μg/mL)	100	59	5	<5
pCMB (0.1 mM)	100	118	<10	nd
isoelectric point	8.1	8.4	7.2	nd
structure[c]	monomer	monomer	monomer	$\alpha_2\beta_2$
M_r	27000	42000	38000	41000 (α), 38000 (β)

[a] Data are taken from Kudlicki et al. (1984). [b] Data expressed as percent activity. [c] CKI-1, Donella-Deana et al. (1985); CKI-2, Lerch et al. (1975) [d] Not determined.

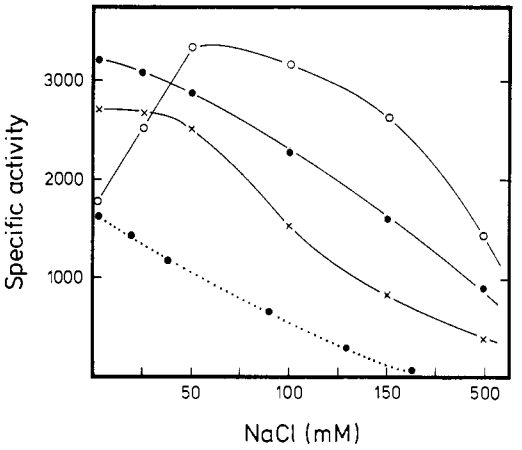

FIGURE 8: Influence of ionic strength on the activity of CKI-1 (×—×), CKI-2 (○—○), and CKI-3 (●—●). Enzymes were assayed in presence of their optimal Mg^{2+} concentrations, at their pH optima, and in the presence of constant concentrations of ATP and casein. The activity of CKI-3 was also measured at 10 mM Mg^{2+} (●···●).

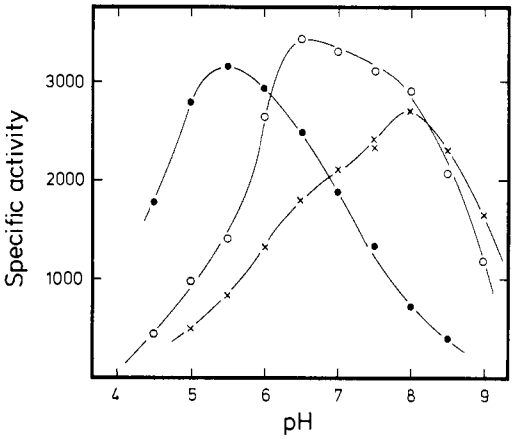

FIGURE 9: pH dependence of the activity of CKI-1 (×), CKI-2 (○), and CKI-3 (●). Enzymes were tested in 50 mM MES or Tris-HCl, respectively, in the presence of optimal concentrations of salts, ATP, and casein.

5.5, 50 mM Mg^{2+}, 5 mM mercaptoethanol, 2 mg/mL casein, and 0.1 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP. All kinetic data presented here were determined under these conditions.

(*E*) *Inhibitors.* The three casein kinases differ considerably in their heparin sensitivity: the most sensitive enzyme is CKI-3 (50% inhibition of 1 μg of enzyme by 0.3 μg/mL heparin). CKI-2 is about 20 times less sensitive (50% inhibition by 7 μg/mL), and CKI-1 is completely insensitive at heparin concentrations up to 30 μg/mL.

This differential sensitivity corresponds to the order of elution from heparin–Sephadex by a NaCl gradient (see Figure 3). Heparin competes with casein (K_i = 0.25 μg/mL) but not with ATP or GTP.

Another potent inhibitor of CKI-3 is the thiol reagent *p*-(chloromercuri)benzoate (pCMB). CKI-3 is almost completely inhibited at 10 μM pCMB, whereas the two other enzymes are insensitive. Higher concentrations of casein and/or GTP (ATP) appear to protect sensitive sulfhydryl groups of CKI-3, because inhibition is significantly reduced. Interestingly, CKI-2 is slightly but reproducibly stimulated at 5 μM pCMB.

(*F*) *Autophosphorylation and Phosphorylated Amino Acids.* In the absence of any protein substrate, the homogeneous enzyme CKI-3 phosphorylates it own protein, regardless of whether ATP or GTP is used as phosphate donor (see Figure 6, lanes a and b). At conditions optimal for casein phosphorylation, 1 nmol of enzyme autophosphorylates about two amino acid residues within 30 min at a considerably lower rate than that of casein phosphorylation. Autophosphorylation does not appear to change the casein phosphorylating activity of CKI-3.

The autophosphorylated enzyme as well as phosphorylated casein was hydrolyzed with HCl, and the hydrolysate was analyzed by electrophoresis as described under Materials and Methods. The hydrolysate of autophosphorylated enzyme contained both phosphoserine and phosphothreonine, and a very similar result was obtained with phosphorylated casein. It should be pointed out that the ratio of serine and threonine phosphorylation is difficult to quantify, since the phosphoamino acids are hydrolyzed at different rates by HCl. Phosphotyrosine could not be detected after alkaline hydrolysis of phosphorylated enzyme or casein.

Similar results were obtained with the three other casein kinases (CKI-1, CKI-2, and CKII): all yeast casein kinases described here phosphorylate both serine and threonine.

Discussion

Casein phosphorylating protein kinases from commercial bakers' yeast were fractionated and resolved into four major species, and there is no evidence for additional enzymes accepting casein as an in vitro substrate except for phosphorylase kinase which has a rather broad substrate spectrum (Pohlig et al., 1983).

The properties of the four casein kinases are summarized in Table II. The purification and properties of CKII (casein kinase II or CK 2) have already been reported (Kudlicki et al., 1984), and we have confirmed most of these data.

CKI-1 and CKI-2 appear to correspond to the previously reported enzymes casein kinase 1A or 1Aa (Donella-Deana et al., 1985; Szyszka et al., 1985) and casein kinase 1Ab or I (Lerch et al., 1975; Kudlicki et al., 1980), respectively, although not all parameters determined in this study were described by these authors. Except for the enzyme purified by Lerch et al. (1975), all other reported enzyme fractions had specific activities which are at least a magnitude lower than the values shown in Table II. A yeast protein kinase having the properties of CKI-3 has not yet been described, whereas enzymes resembling CKI-3 in structure and properties were isolated from wheat germ (Yan & Tao, 1982) and the slime mold *Dictyostelium discoideum* (Renart et al., 1984). The plant and slime mold enzymes, however, were classified as type II casein kinases by these authors, and it is not clear whether a complex oligomeric enzyme corresponding to yeast or mammalian type II casein kinase does exist in these organisms.

The data of Table II indicate that CKI-3 has three properties in common with the catalytic β-subunit of CKII: a molecular weight of 38 000, the utilization of both ATP and GTP as phosphate donors, and a pronounced heparin sensitivity.

Indeed, we have to consider the possibility that CKI-3 and the β-subunit of CKII are different forms of the same polypeptide: the quaternary structure (interaction with regulatory subunits), chromatographic behavior, and catalytic properties could depend on the state of posttranslational modifications such as protein phosphorylation. Alternatively, both catalytic entities (CKI-3 and CKII,β) may be coded by two related copies of the protein kinase gene family.

The multiplicity of casein kinases reported here raises the problem of classification and nomenclature. It is clear that properties like phosphate donor specificity (use of ATP alone or ATP + GTP), amino acid acceptor specificity (serine alone or serine + threonine), and heparin sensitivity cannot be used to classify the yeast casein kinases according to the categories type I and type II (Hathaway & Traugh, 1982), because CKI-3 combines properties of both types of mammalian kinases. However, we still adhere to the type I and II classification in order to avoid nomenclature confusion, by using only two criteria: chromatographic behavior and structural complexity.

In one point, our observations are at variance with published data (Lerch et al., 1975): we could not detect a casein kinase phosphorylating exclusively serine. All four enzymes, including CKI-2, produced both phosphoserine and phosphothreonine by autophosphorylation and casein phosphorylation. Since cAMP-dependent protein kinase and phosphorylase kinase also phosphorylate both amino acids (Takai et al., 1974; our unpublished observations), we doubt whether a major serine-specific protein kinase exists in yeast.

Our data suggest that the protein kinase pattern in yeast is more complex than previously assumed. In addition to the four casein kinases, cAMP-dependent protein kinase, and phosphorylase kinase resolved by our chromatographic procedure, yeast also contains a kinase specifically phosphorylating ribosomal proteins (Kudlicki et al., 1980), other serine- and threonine-phosphorylating protein kinases encoded by the cell cycle gene *CDC28* (Reed et al., 1985) and by a gene (*SNF1*) required for expression of glucose-repressible genes (Celenza & Carlson, 1986), and tyrosine-phosphorylating protein kinase activity (Schieven et al., 1986). Furthermore, a yeast gene has been cloned and sequenced in our laboratory which encodes a protein kinase (calculated molecular weight 46 000) structurally related to the catalytic subunit of cAMP-dependent protein kinase and functionally involved in the control of the cell cycle start (Lisziewicz et al., 1987).

Little is shown of the in vivo functions of protein kinases and how their activities are coordinated during the cell cycle. One obvious approach to answer these questions is the study of the in vitro phosphorylation of endogenous substrates by purified enzymes. We are also following a second line of research of which the yeast *Saccharomyces cerevisiae* is specially suited, namely, the isolation, sequence analysis, and manipulation of protein kinase genes.

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Registry No. ATP, 56-65-5; GTP, 86-01-1; heparin, 9005-49-6; casein kinase, 52660-18-1.

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