ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 305, No. 1, August 15, pp. 47–53, 1993

Isolation and Properties of YCK2, a *Saccharomyces cerevisiae* Homolog of Casein Kinase-1

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Received January 27, 1993, and in revised form April 25, 1993

A soluble fragment of YCK2, a casein kinase-1 isoform from *Saccharomyces cerevisiae*, has been purified and characterized *in vitro*. The procedure enriches enzyme activity to a final specific activity of 4.7 μmol min^{-1} mg^{-1} (when assayed with casein as substrate). Structural analysis reveals that the preparation arises from N-terminal modification and C-terminal proteolysis of the initially synthesized 546-residue protein, consisting of residues 2–495 ± 1. Kinetic analysis demonstrates that YCK2 is similar to casein kinase-1 isolated from other organisms in its inability to use GTP as nucleotide substrate, in its sensitivity to heparin and ribofuranosylbenzimidazole inhibitors, and in its peptide substrate selectivity. The enzyme is unusual, however, in that it is insensitive to the potent mammalian casein kinase-1 inhibitor *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide. © 1993 Academic Press, Inc.

Casein kinase-1 (CK1)[2] is a ubiquitous eukaryotic protein kinase that phosphorylates acidic substrate recognition sequences efficiently (1, 2). Once considered a single entity, it is now known to consist of subspecies that together constitute a distinct branch of the eukaryotic protein kinase family (3–5). Typically, members of the CK1 family are identified enzymologically by their monomeric quarternary structure, unusually high isoelectric point (p*I* ≥9.0), strict requirement for ATP as cosubstrate, moderate heparin sensitivity (IC_{50} ≈20 μg/ml), and use of phosphoserine as a determinant of substrate selectivity (6–8). In addition, these enzymes differ from known members of the eukaryotic protein kinase family at the level of primary structure. The principal differences appear in subdomain VIII, where the common peptide triplet Ala–Pro–Glu is replaced by Ser–Ile/Val–Asn, and in subdomain XI, where a conserved Arg is absent (4, 9). In the cAMP-dependent protein kinase, the third residue (Glu) of the triplet forms a salt bridge with the Arg residue of subdomain XI (10). Presumably, members of the CK1 family do not require this salt bridge for activity.

Like other eukaryotes, the yeast *Saccharomyces cerevisiae* contains CK1 activity (11). Fractionation of yeast extracts by cation-exchange chromatography suggests the activity is composed of at least three separate enzymes with molecular masses ranging from 27 to 43 kDa (12–16). A fourth form corresponds to the *HRR25* gene product and is predicted to have a molecular mass of 56 kDa (17). Recently, we purified a form of yeast CK1 (molecular mass of 56 kDa) and, after obtaining internal amino acid sequence data, cloned both its gene (termed *YCK2*) and that of a closely related and functionally equivalent homolog (termed *YCK1;* 4). Both YCK1 and YCK2 contain a consensus sequence for prenylation at their carboxyl termini (Gly–Cys–Cys; Ref. 18) and are probably substrates of the *S. cerevisiae* geranylgeranyl transferase-II catalytic β subunit, BET2 (19, 20). Normally, YCK2 has a molecular mass of 62 kDa and is associated exclusively with the particulate fraction of the cell (4). In cell lysates, however, YCK2 undergoes rapid proteolysis to yield the soluble 56-kDa form we isolated at the outset (4).

Here we present the isolation of the soluble, 56-kDa proteolysis product of YCK2 (termed yck2-56), describe its molecular properties, and compare them to those of

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[2] Abbreviations used: CK1, casein kinase-1; CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; DiBrRB, 5,6-dibromo-1-(β-D-ribofuranosyl)benzimidazole; 5′-DRB, 5,6-dichloro-5′-phosphate-1-(β-D-ribofuranosyl)benzimidazole; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N′*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; Kemptide, Leu–Arg–Arg–Ala–Ser–Leu–Gly; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Sp, phosphoserine.

0003-9861/93 $5.00

authentic mammalian[3] CK1. Because YCK1 shares >90% identity with YCK2 in its catalytic domain, we expect that the characteristics described herein for YCK2 will apply to YCK1 as well.

MATERIALS AND METHODS

Materials. Phosphocellulose was from Whatman (P11), whereas UltroGel AcA 44, TSKGel phenyl-5PW, and TSK 3000-SW media were from Pharmacia/LKB. Casein (5% solution; partially hydrolyzed and dephosphorylated), phosvitin, protamine, Kemptide, and bovine carbonic anhydrase II were from Sigma. Heparin from porcine intestine (sodium injection, USP) was from Elkins-Sinn, Inc. Calibration proteins for electrophoresis, chromatography, and sedimentation were from Sigma, Bio-Rad, and Boehringer Mannheim. DiBrRB, 5'-DRB, D-D-D-E-E-S-I-T-R-R, and Sp-Sp-Sp-E-E-S-I-T were the generous gifts of L. Pinna, Padova, Italy. CKI-7 was from Seikagaku America, Inc.

Buffers. The compositions of buffers used in purification of yck2-56 are described below. Homogenization buffer: 200 mM potassium phosphate, pH 7.0, 5 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM PMSF, 0.02% Triton X-100. Buffer A: 10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM PMSF, 0.02% Brij 35. Buffer B: 10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, 0.02% Brij 35. Buffer C: 10 mM Mops, pH 7.0, 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.02% Brij 35. Buffer D: 10 mM Mops, pH 7.0, 1 mM EGTA. Buffer E: 10 mM Hepes, pH 8.0, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 5% glycerol, 0.02% Brij 35.

CK1 purification. SP1 (*Matα ade8 his3 leu2 trp1 ura3;* Ref. 21) cells were grown overnight at 30°C in rich medium (YPD: 1% yeast extract, 2% Bacto-peptone, 2% glucose), harvested at stationary phase ($A_{660nm} \approx 12$) by centrifugation (15 min at 3000*g*; 4°C), washed with water, and stored at −70°C until used. This strain typically yields 10–13 g (wet wt) of cells per liter of culture.

All subsequent steps were carried out at 4°C. Frozen cells (100 g) were thawed and extracted as described previously (22). The crude extract was taken as Fraction 1.

Nucleic acids were cleared from the extract by adding polyethyleneimine (23) to 0.8% final concentration (from a 10% stock solution), stirring for 10 min, and centrifuging for 20 min at 20,000*g*. The clear amber supernatant was brought to 65% saturation with solid $(NH_4)_2SO_4$ (pH 7.8 was maintained by the gradual addition of 10 M NH_3), stirred for 30 min, and then centrifuged for 20 min at 20,000*g*. The resulting pellet was resuspended in 100 ml of Buffer A containing 50 mM NaCl, dialyzed against 2 liters of Buffer A containing 100 mM NaCl (4 h), adjusted to a conductance of 20 mS by dilution with water, and finally clarified by centrifugation (20 min at 20,000*g*). The resulting supernatant was taken as Fraction 2.

Fraction 2 was loaded onto a 100-ml (5 × 5-cm) phosphocellulose column equilibrated in Buffer B containing 200 mM NaCl. After the column was washed overnight with 10 bed volumes of the same solution, the activity was eluted from the column with Buffer B containing 550 mM NaCl. The protein peak was pooled and taken as Fraction 3 (phosphocellulose pool).

Fraction 3 was dialyzed 4 h against 1 liter of Buffer C containing 75 mM NaCl, adjusted to a conductance of 16 mS by dilution with 0.02% Brij 35, and loaded onto an 8-ml (1 × 10-cm) Mono-S HR 10/10 HPLC column equilibrated in Buffer C containing 100 mM NaCl and operated at a flow rate of 2 ml/min. The column was washed with 10 bed volumes of this same solution and eluted with a 130-ml gradient of increasing NaCl (120 to 400 mM) in Buffer C. Activity eluted as a sharp peak at 190 mM NaCl and was taken as Fraction 4 (first Mono-S pool).

[3] The classical 37-kDa form of mammalian CK1 consists of at least the α and β forms described in (3).

The Mono-S pool was made 1 M $(NH_4)_2SO_4$ (10 M NH_3 was added to maintain neutral pH) and loaded onto a 3.8-ml (8 × 75-mm) TSKGel phenyl-5PW HPLC column equilibrated in Buffer D containing 1 M $(NH_4)_2SO_4$ and operated at a flow rate of 0.5 ml/min. The column was washed with 5 bed volumes of this same buffer and then developed with a 25-ml linear gradient of decreasing $(NH_4)_2SO_4$ (from 1 to 0.3 M) in Buffer D. CK1 elutes as a sharp peak centered at 480 mM $(NH_4)_2SO_4$. The protein peak was taken as Fraction 5.

Fraction 5 was loaded directly onto a 190-ml (1.6 × 95-cm) column of UltroGel AcA 44 gel filtration medium equilibrated and run at 15 ml/h in Buffer B containing 150 mM NaCl. Activity eluted at V_e/V_o = 1.61 (K_{av} = 0.388) and was taken as Fraction 6 (gel filtration pool).

Fraction 6 was diluted with 0.02% Brij 35 until a conductance of 15 mS was attained and then applied directly to a 1-ml (5 × 55-mm) Mono-S HR 5/5 HPLC column equilibrated in Buffer E containing 100 mM NaCl and operated at 1 ml/min. The column was washed with 5 bed volumes of Buffer E containing 100 mM NaCl and developed with a 16-ml gradient of 100 to 350 mM NaCl in Buffer E. CK1 activity eluted as a sharp peak at 230 mM NaCl and was taken as Fraction 7.

The final pool was concentrated by dialysis against storage buffer (50% glycerol, 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.02% Brij 35, 1 mM dithiothreitol) and stored at −20°C. This procedure can be scaled up twofold by splitting the homogenate into two equal fractions and working them up in parallel until the gel-filtration step, whereupon the two fractions are combined, and the purification is continued as described above.

Analytical methods. The protein content of extracts and column fractions was estimated by the method of Bradford (24) using bovine serum albumin as standard.

Casein kinase activity was assayed as described previously (25). The standard reaction (40 μl) contained 25 mM Mes, pH 6.5, 50 mM NaCl, 15 mM $MgCl_2$, 2 mg/ml casein, 2 mM EGTA, and 100 μM [γ-^{32}P]ATP (100 to 400 cpm/pmol). For kinetic analyses, reaction conditions were varied by the addition of NaCl, heparin, DiBrRB, 5'-DRB, or CKI-7. To vary reaction pH, acetate, Mes, Hepes, Tris, and glycine buffers were substituted at 25 mM concentration. Initial velocity measurements were carried out in duplicate with either ATP (10, 16, 30, 47.5, and 100 μM) or casein (0.3, 0.48, 0.8, 1.2, and 1.6 mg/ml) as the varied substrate.

Phosphorylations of the synthetic peptides D-D-D-E-E-S-I-T-R-R and Sp-Sp-Sp-E-E-S-I-T were carried out under the conditions described above, but with the peptides substituting for casein. Initial velocity measurements were performed in duplicate with either D-D-D-E-E-S-I-T-R-R (140, 400, 600, and 1000 μM) or Sp-Sp-Sp-E-E-S-I-T (60, 80, 100, 140, and 220 μM) as the varied substrate. Phosphate incorporation was determined by the method of Racker and Sen (26). Assays for protamine and Kemptide phosphorylation were performed as described previously (27). All K_m and V_{max} values were estimated from the slopes and intercepts of Eadie–Hofstee plots (28).

The ionic strengths of buffers and column fractions were measured with a conductivity meter (Radiometer) following a 100-fold dilution of the sample in water.

SDS–polyacrylamide gels (9% acrylamide) were prepared, electrophoresed, and stained with Coomassie blue as described previously (25). Molecular mass markers included rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), bovine catalase (57.6 kDa), ovalbumin (42.7 kDa), and bovine carbonic anhydrase (29 kDa). Isoelectric focusing measurements (pH 3–10) were performed with a PhastSystem (Pharmacia/LKB) operated at 2000 V, 5 mA, and 3.5 W for 410 Vh at 15°C. Standards included soybean trypsin inhibitor (p*I* 4.55), bovine β-lactoglobulin A (p*I* 5.13), bovine erythrocyte carbonic anhydrase B (p*I* 5.85), human erythrocyte carbonic anhydrase B (p*I* 6.57), rabbit lactate dehydrogenase (p*I* 8.55), and bovine trypsinogen (p*I* 9.3). IEF gels were silver-stained as described previously (29).

Velocity sedimentation experiments were performed in linear 10–30% glycerol gradients containing 10 mM Mops, pH 7.0, 250 mM NaCl, 1 mM EGTA, 0.1% 2-mercaptoethanol, and 1 mM benzamidine. Standards included yeast alcohol dehydrogenase (7.4 S), bovine serum albumin

(4.3 S), ovalbumin (3.7 S), bovine erythrocyte carbonic anhydrase (3.2 S), and myoglobin (2.0 S). Samples (20 μg of each standard; 10 μg of CK1) were centrifuged for 40 h at 39,000 rpm at 4°C in a Beckman SW-41 rotor and then fractionated into 150-μl aliquots with a Buechler autofractionator. Fractions were assayed for CK1 activity to locate its position in the gradient and by SDS–polyacrylamide gel electrophoresis to locate the positions of the protein standards.

Analytical gel filtration was performed on a 15-ml (8 × 300-mm) TSK 3000-SW HPLC column equilibrated in Buffer B containing 150 mM NaCl and operated at 0.5 ml/min. Standards for the Stokes radius measurement (30) included yeast alcohol dehydrogenase (4.60 nm), bovine serum albumin (3.62 nm), ovalbumin (2.83 nm), and bovine erythrocyte carbonic anhydrase (2.01 nm). After collection of 250-μl fractions, elution of the standards and of CK1 was monitored as described above for velocity sedimentation. K_{av} values for standards were calculated from their elution volumes (V_e) by the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_o is the void volume (estimated by the elution of thyroglobulin) and V_t is the total column volume. Hydrodynamic parameters were calculated as described previously (25, 31).

Positive-ion matrix-assisted laser desorption mass spectrometry was performed as described previously (32), with multiply charged ions of bovine carbonic anhydrase II serving as mass calibrant. Purified yck2-56 was diluted into 30% acetonitrile, 0.1% trifluoroacetic acid (containing 25 mM cyano-4-hydroxycinnamic acid), and 1 pmol was analyzed. The resultant charge-to-mass ratios of ions M^{2+} through M^{5+} were used to calculate average mass ± standard deviation.

RESULTS

Extraction and stability. Although casein kinase activity extracted from yeast cells is stable for days when stored at 4°C, it is lost rapidly during subsequent purification. This instability stemmed from several sources. First, the enzyme is sensitive to the method of cell breakage; extensive proteolysis results when cells are homogenized with either a static (Aminco) or continuous (Stansted cell disrupter) French press. Proteolysis is minimized by homogenizing yeast in a bead mill, where rapid breakage and efficient cooling are available. Second, the recovery of CK1 activity is poor on most liquid chromatography resins. Because losses are reduced by nonionic detergents, Brij 35 was included during purification. Third, dilution of CK1 into buffers of low ionic strength consistently resulted in loss of enzyme activity. Therefore, solutions of CK1 were maintained at a conductance ≥12 mS. Finally, as with most protein kinases, yck2-56 loses activity when frozen. Nevertheless, cells stored at −70°C for 1 year still yield active enzyme.

Purification. The purification of yck2-56 from *S. cerevisiae* begins with the removal of nucleic acids with polyethyleneimine and the precipitation of activity with ammonium sulfate. Most casein kinase activity survives these precipitations, which yield a clear, concentrated protein solution suitable for chromatography.

Typical liquid chromatography elution profiles are presented in Fig. 1. The first two steps involve cation-exchange chromatography on phosphocellulose and Mono-S. On the basis of heparin sensitivity (33), these resins remove nearly all casein kinase-2 activity, which forms aggregates at low ionic strength (34) and as a result binds poorly to these cation exchangers. The remaining casein

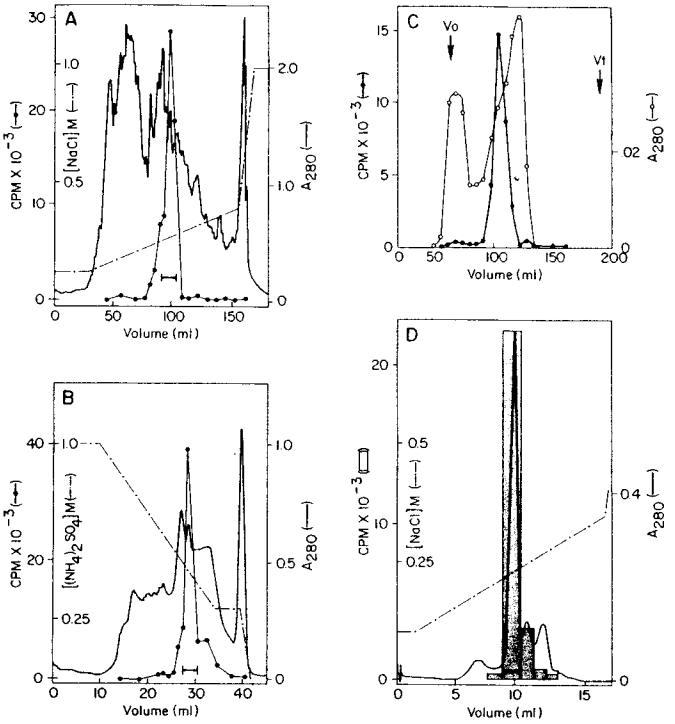

FIG. 1. Column chromatography profiles. Chromatography was performed as described under Materials and Methods. (A) Cation-exchange chromatography (Mono-S, pH 7.0) of Fraction 3. Fraction 4 was pooled as indicated by the horizontal bar. (B) Hydrophobic interaction chromatography (phenyl-5PW) of Fraction 4. Fraction 5 was pooled as indicated by the horizontal bar. (C) Gel-filtration chromatography of Fraction 5. Fraction 6 was pooled as indicated by the horizontal bar. The void volume (V_o) was estimated by elution of Blue Dextran 2000. V_t is the total column volume. (D) Cation-exchange chromatography (Mono-S, pH 8.0) of Fraction 6. Fraction 7 was pooled as indicated by the horizontal bar.

kinase activity elutes from Mono-S as a single, sharp peak that is insensitive to 1 μg/ml heparin. The third purification step employs hydrophobic-interaction chromatography on phenyl-5PW to achieve a sixfold enrichment of activity with excellent yield; the casein kinase activity again elutes as a single, sharp peak. The fourth step features gel-filtration chromatography. Although only a modest purification is achieved, this step efficiently removes high-molecular-weight contaminants that are difficult to eliminate using other methods. It also removes ammonium sulfate remaining from the previous step. The final chromatographic step returns to cation-exchange chromatography over Mono-S and yields a preparation with a high specific activity (discussed below). The concentrated pool is stable for at least 1 year when stored in 50% glycerol at −20°C. Analysis of the preparation by SDS–gel electrophoresis reveals that the purified enzyme consists of a single polypeptide with an apparent molecular mass (denatured) of ≈55 kDa (Fig. 2A). Additional analysis by isoelectric focusing confirms that the prepa-

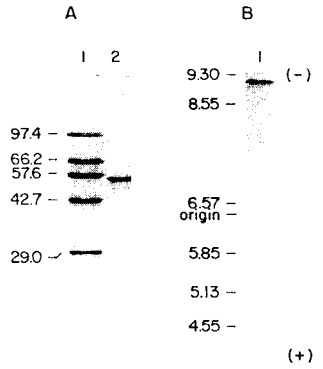

FIG. 2. Electrophoretic analysis of purified yck2-56. Electrophoresis was performed and migration calibrated relative to internal standards as described under Materials and Methods. (A) A Coomassie blue-stained, 9% SDS–polyacrylamide gel containing, in lane 1, molecular mass standards, and in lane 2, 2 μg of purified yck2-56. (B) A silver-stained, isoelectric focusing gel (pH 3–9) containing 200 ng of purified yck2-56 in lane 1. The origin is the site of sample application.

ration is highly purified and demonstrates that yck2-56 has a p*I* of approximately 9.0 (Fig. 2B).

The results of a typical purification of yck2-56 (scaled up to 200 g starting material) are summarized in Table I. The enzyme requires over 2000-fold enrichment to attain a high level of purity from starting extracts and is obtained in 5% yield.

Physical characterization. The physical properties of purified yck2-56 are summarized in Table II. The native molecular mass was determined from hydrodynamic data obtained from density gradient sedimentation and gel-filtration experiments (Fig. 3; Ref. 35). From the resulting values of Stokes radius and sedimentation coefficient, and assuming a partial specific volume of 0.735, we estimate that yck2-56 is a monomer with a molecular mass consistent with that determined from SDS gels. The observed frictional ratio (f/f_o) is high, however, and suggests that the enzyme is asymmetric and/or contains an unusually large amount of bound water.

Although purified yck2-56 migrates on SDS gels as an $\approx$55-kDa species (Fig. 2A), it is now clear that this form of the enzyme results from artifactual proteolysis of intact, 62-kDa YCK2 (4). To determine whether the site of proteolysis is near the N-terminus of YCK2, we subjected two separate preparations of yck2-56 to Edman degradation. Results from both preparations demonstrated that the N-terminus of YCK2 is blocked and that our purified preparations have not suffered N-terminal proteolysis (4). Because the deduced amino acid sequence of YCK2 begins Met–Ser– (4, 5), it is subject to cotranslational modification through removal of its N-terminal methionine (catalyzed by methionine aminopeptidase) followed by N^{α}-acetylation (catalyzed by N^{α}-acetyltransferase) of the penultimate serine residue (36). As described previously (4), Edman degradation performed on peptides prepared by partial proteolysis yielded amino acid sequences consistent with YCK2 and not YCK1.

The above result suggests that the site of YCK2 proteolysis must be near its C-terminus. To identify this site, purified yck2-56 was analyzed by matrix-assisted laser desorption mass spectrometry (Fig. 4). The measured mass of 56,510 $\pm$ 119 Da is most consistent with the mass of residues 2–495 of YCK2 plus an N-terminal acetate group (calculated mass = 56,510 Da). On the basis of these results, the site of YCK2 proteolysis lies within one or two residues of Lys^{495} in the sequence Gln^{491}–Gln–Lys–Ser–Lys–Gln–Phe^{497} (4).

Substrate selectivity. The kinetic properties of yck2-56 are summarized in Table III. When casein is used as protein substrate, the phosphotransferase reaction is optimal from pH 5.5 through pH 6.5 in buffers containing 50 to 75 mM NaCl (Fig. 5). Under these conditions, yck2-56 has a V_{max} of 4.7 μmol min^{-1} mg^{-1}, which corresponds to a k_{cat} of 4.3 s^{-1}. These values are typical of highly purified protein kinases. Conversely, neither protamine nor the cAMP-dependent protein kinase substrate Kemptide was phosphorylated when assayed at concentrations up to 200 μM.

TABLE I

Purification of yck2-56 from 200 g of Yeast Strain SP1

Fraction	Volume (ml)	Protein[a] (mg)	Activity[b] (nmol min^{-1})	Purification (fold)	Yield (%)
1. Extract	445	12,416	13,000	1	100
2. Precipitations[c]	400	6,520	9,080	1.3	70
3. Phosphocellulose	150	569	5,270	8.8	41
4. Mono-S (pH 7.0)	25	46	1,950	41	15
5. Phenyl-5PW HPLC	6	10	1,529	250	12
6. Gel filtration	18	3.3	1,284	372	10
7. Mono-S (pH 8.0)	1	0.28	676	2310	5

[a] Protein content was measured as described under Materials and Methods.

[b] Estimates of activity in Fractions 1 and 2 are unreliable owing to the presence of other kinases and phosphatases.

[c] 0.8% polyethyleneimine and 65% $(NH_4)SO_4$.

TABLE II

Physical Properties of yck2-56

Stokes radius	4.0 nm
Sedimentation coefficient ($s_{20,w}$)	3.3 S
Native molecular mass[a]	51,400 Da
Denatured molecular mass[b]	56,510 ± 119 Da
Quarternary structure	Monomer
Frictional ratio[a] $(f/f_0)_{obs}$	1.67
p*I*	9.0
Amino-terminus	Blocked

[a] Estimated from Stokes raduis, sedimentation coefficient, and partial specific volume (25, 35), neglecting any contribution from bound water.

[b] Mass ± SD was estimated by matrix-assisted laser desorption mass spectrometry.

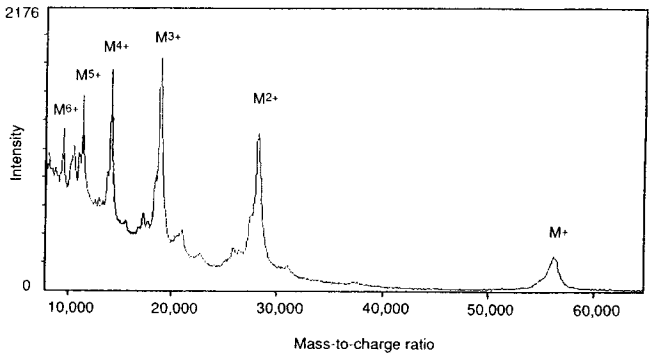

FIG. 4. Mass spectrum of purified yck2-56. Multiply charged species of yck2-56 in the mass-to-charge ratio range 7,810 to 64,917 are illustrated. A mass of 56,510 ± 119 Da was calculated from this data as described under Materials and Methods.

To determine whether it retained the same peptide substrate selectivity as mammalian CK1, yck2-56 was assayed with the synthetic peptides D-D-D-E-E-S-I-T-R-R and Sp-Sp-Sp-E-E-S-I-T (7). Both peptides were phosphorylated by yck2-56, with K_m values in the 0.2–1 mM range (Table III). The triply phosphorylated peptide was an excellent substrate for yck2-56, with a V_{max} of greater than 1 μmol min^{-1} mg^{-1} and a k_{cat}/K_m that was approximately an order of magnitude lower than that of partially dephosphorylated and hydrolyzed casein, the best substrate yet identified for CK1. Like its mammalian homolog, yck2-56 prefers a peptide substrate that contains phosphoserine residues N-terminal to a phosphorylatable serine.

To examine the nucleotide substrate selectivity of yck2-56, nonradioactive GTP was added to the standard reaction and allowed to compete as phosphoryl donor with 100 μM [γ-^{32}P]ATP. Because yck2-56 is not inhibited by the addition of up to 3 mM GTP, it appears to selectively use ATP as nucleotide substrate.

Inhibitors. Two classes of selective casein kinase inhibitor have been developed: the ribofuranosyl-benzimidazoles (7, 37) and the isoquinoline sulfonamides (38). Both are competitive inhibitors of nucleotide substrate. To further characterize its nucleotide binding site, yck2-56 was assayed in the presence of the ribofuranosyl-benzimidazoles DiBrRB and 5′-DRB and the isoquinoline sulfonamide CKI-7. The results are summarized in Table III. Both DiBr-RB and 5′-DRB are inhibitors of yck2-56, with K_i values of 30–70 μM. These values are approximately twice those observed with mammalian CK1. However, the lower affinity for ribofuranosyl-benzimidazoles observed with yck2-56 may simply reflect its lower affinity for adenine nucleotide relative to mammalian CK1 (27). Indeed, the K_i/K_{mATP} values observed for mammalian CK1 and yck2-56 inhibited with DiBrRB and 5′-DRB are re-

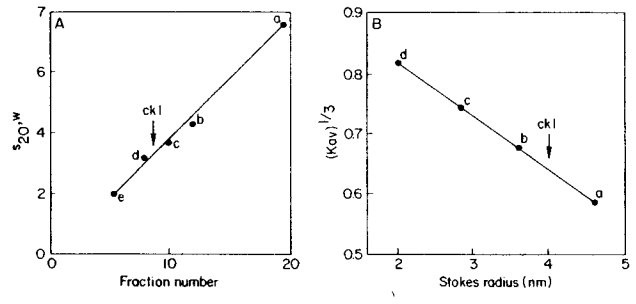

FIG. 3. Hydrodynamic measurements. Estimation of (A) the sedimentation coefficient (by density gradient centrifugation) and (B) the Stokes radius (by gel-filtration chromatography) of yck2-56 relative to internal standards. Standard proteins included (a) yeast alcohol dehydrogenase, (b) bovine serum albumin, (c) ovalbumin, (d) carbonic anhydrase, and (e) myoglobin. K_{av} values for the standards were calculated from their elution volumes (V_e) as described under Materials and Methods.

TABLE III

Kinetic Properties of Purified yck2-56

Substrate	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
D-D-D-E-E-S-I-T-R-R	0.35	0.32	940	340
Sp-Sp-Sp-E-E-S-I-T	1.3	1.2	200	5,900
Casein	4.7	4.3	≈54	80,000
ATP	—	—	24	—

	yck2-56			Mammalian[a]
Inhibitors	IC_{50}[b]	K_i[c] (μM)	K_i/K_{mATP}	K_i/K_{mATP}
diBrRB	43 μM	≈30	1.25	1.17
5′-DRB	98 μM	≈70	2.92	2.50
CKI-7	>500 μM	>350	>14.6	0.71
Heparin	68 μg/ml	—	—	—

[a] Data for mammalian CK1 were calculated from Refs. 27, 37, and 38.

[b] Assayed with 10 μM ATP and 2 mg/ml casein.

[c] Estimated from the approximate relationship: $IC_{50} = K_i(1 + S/K_m)$, assuming competitive inhibition with ATP.

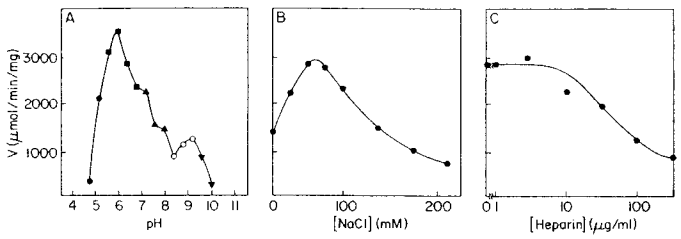

FIG. 5. Effects of pH, NaCl, and heparin on yck2-56 activity. The activity of yck2-56 was assayed under conditions described under Materials and Methods except as noted below. (A) Effect of pH. Reactions were buffered with 25 mM acetate (●), Mes (■), Hepes (▲), Tris (○), or glycine (▼). (B) Effect of NaCl. (C) Inhibition by heparin.

markably similar and suggest that the structural features responsible for ribofuranosyl-benzimidazole selectivity are conserved in evolutionarily distant YCK2 (Table III).

In contrast, the isoquinoline sulfonamide compound (CKI-7) inhibits yck2-56 weakly, with only 30% inhibition observed at the highest concentration assayed (500 μM). At these concentrations, CKI-7 cannot distinguish between yck2-56 and distantly related members of the protein kinase superfamily, such as protein kinase C (39). The diminished selectivity is reflected in the >20-fold larger K_i/K_{mATP} observed for yck2-56 relative to mammalian CK1 (Table III). Apparently, yck2-56 does not retain the structural features necessary for CKI-7 selectivity.

Unlike casein kinase-2, which is sensitive to very low concentrations of heparin (IC_{50} ≤0.15 μg/ml; Refs. 33, 40), mammalian CK1 is inhibited by much higher concentrations of this polyanion (IC_{50} = 24 μg/ml when assayed at 2 mg/ml casein substrate; Ref. 27). yck2-56 is sensitive to heparin as well and is inhibited with an IC_{50} of 68 μg/ml (Table III and Fig. 5C).

DISCUSSION

Normally, YCK2 is tightly associated with an insoluble component of the cell, and as a result is difficult to extract and purify in an active state (4). Proteolysis (catalyzed by an endogenous yeast protease), however, removes ≈50 C-terminal residues and releases the proteolysis product (yck2-56) as a soluble and active enzyme. We have taken advantage of this observation to purify yck2-56 and characterize its catalytic properties *in vitro*. Like CK1 isolated from other sources, yck2-56 is a monomer in solution, has an alkaline isoelectric point, and is constitutively active.

Qualitatively, the peptide substrate selectivity of yck2-56 follows the trend identified for mammalian CK1, in which the presence of phospho-Ser residues N-terminal to the phosphorylatable residue in a synthetic peptide increases the binding energy between enzyme and substrate. For mammalian CK1, this binding energy is realized equally well in both the enzyme-substrate and the transition state complexes and leads to a lower K_m with little change in V_{max} (7). Yck2-56 differs from mammalian CK1 in that it uses the additional binding energy both to lower K_m and to raise V_{max}. Apart from these quantitative aspects, the similarity in substrate selectivity between mammalian and yeast CK1 is consistent with their >50% identity in primary structure.

The nucleotide binding site of yck2-56 resembles that of its mammalian homolog in accepting ATP but not GTP as cosubstrate and in binding ribofuranosyl-benzimidazole inhibitors. Nonetheless, it is insensitive to CKI-7, the potent isoquinoline sulfonamide inhibitor of mammalian CK1 (38). As additional members of the CK1 family are characterized, it will be interesting to note their inhibitor sensitivity. The ribofuranosyl-benzimidazoles may emerge as broad-spectrum inhibitors of CK1 activity, whereas CKI-7 may selectively inhibit certain CK1 isozymes.

As described above, YCK2 resembles its mammalian homologs in catalytic activity. YCK1 and YCK2 are unique, however, in containing a consensus sequence for prenylation at their C-termini and in being associated exclusively with the particulate fraction of the cell (4). In mammalian cells, some CK1 activity is associated with particulate fractions, including nuclei (41, 42), and there is some evidence that these particulate forms are larger than the classical 37-kDa cytosolic form (2). An example is the Golgi-associated casein kinase isolated from lactating mammary glands (43). Because this enzyme is found specifically in mammary glands and functions to phosphorylate casein, the existence of a similar enzyme in yeast is unlikely. It is not clear which if any of the four known mammalian CK1 isozymes is the functional homolog of YCK2. On the basis of sequence similarity, mammalian CKI-γ is its closest homolog (60% identity in the catalytic region).

CK1 activity has been isolated from yeast previously (12–16). Fractionation of yeast extracts on cation-exchange resins revealed three low-abundance forms of the enzyme, with molecular masses of 27, 43, and 38 kDa. Larger forms, corresponding to intact YCK1, YCK2, HRR25, or our 56-kDa proteolysis product of YCK2, were not observed. Conversely, we saw little evidence of three major peaks of CK1 activity from cation-exchange resins, including phosphocellulose, Mono-S, or heparin–Sepharose. These discrepancies may result from differences in the way we and others grow and extract yeast. First, we prepare our enzyme from freshly grown, late-log-phase laboratory strains of yeast, whereas previous studies have relied on commercial yeast cakes as starting material. The quality of some yeast enzyme preparations, such as topoisomerase II, varies greatly between the two sources (44). Second, we find that YCK2 undergoes extensive proteolysis during homogenization and subsequent handling, with forms smaller than 56 kDa predominating when a French press replaces the bead-mill during homogenization. As observed for other yeast proteins (45),

the relatively high temperature produced in the French press may be responsible for exacerbating proteolysis.

In summary, the product of the *YCK2* gene is enzymologically similar to the classical 37-kDa form isolated from mammalian sources. It is unique in being insensitive to the inhibitor CKI-7, and in being associated exclusively with the particulate fraction of yeast. The purified preparation of yck2-56 described here will be used to produce the immunological reagents necessary to localize YCK2 within the cell.

ACKNOWLEDGMENTS

We thank Professor Lorenzo Pinna for generously supplying synthetic peptide substrates and inhibitors, Georgia Binns and Dan Marshak for conducting amino acid sequence analyses, Jim Duffy, Phil Renna, and Michael Ockler for art work and photography, and Scott Patterson for helpful suggestions. This work was supported by NIH Grants GM 42816 and GM 44806 (J.K.).

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