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Expression, Purification, Crystallization, and Preliminary X-ray Analysis of Casein Kinase-1 from *Schizosaccharomyces pombe**

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The catalytic domain of *Schizosaccharomyces pombe* casein kinase-1 (the product of the *cki1* gene) has been overexpressed in *Escherichia coli*, purified by chromatographic methods, characterized *in vitro*, and crystallized in the presence and absence of nucleotide substrate. The best crystals belong to the trigonal space group $P3_121$ or its enantiomorph, have unit cell parameters $a = b = 79$ Å, $c = 121$ Å, and diffract x-rays to 2.0-Å resolution. Kinetic characterization of the purified catalytic domain and other C-terminal deletion mutants of Cki1 suggests that it is subject to two forms of regulation. One mechanism involves autophosphorylation, and results in a 4-fold decrease in the affinity for protein substrate. In contrast, truncation of intact Cki1 results in a 3-fold activation in its catalytic rate. This activation may arise from the removal of an inhibitory domain present in the intact enzyme.

Casein kinase-1 (CK1)[1] is a ubiquitous eukaryotic protein kinase active in the regulation of DNA repair pathways and cell morphology (Pinna, 1990; Tuazon and Traugh, 1991; DeMaggio *et al.*, 1992; Robinson *et al.*, 1993). Once considered a single entity, it is now known to consist of subspecies that together comprise a distinct branch of the eukaryotic protein kinase family (Rowles *et al.*, 1991; Wang *et al.*, 1992; Robinson *et al.*, 1992). Family members identified to date consist of a highly conserved, ≈290 residue N-terminal catalytic domain, joined to a C-terminal region that is not conserved between family members and that varies in size from 40 to 180 amino acids.

The CK1 catalytic domain differs from that of most other protein kinases both structurally and enzymologically. First, it contains neither the peptide triplet Ala-Pro-Glu in subdomain VIII nor the Arg residue in subdomain XI that, in other protein kinases, interact to form a salt bridge (Knighton *et al.*, 1991a; De Bondt *et al.*, 1993; Hanks and Quinn, 1991). The conservation of this feature throughout the CK1 family suggests it contributes to an intrinsic biochemical property of CK1 family members, such as their ability to interact with substrates. Second, CK1 is unusual in that its substrate selectivity appears to be directed toward phosphate groups rather than unmodified amino acids. Although CK1 can phosphorylate synthetic peptide substrates containing glutamic or aspartic acids at positions −3 or −4 (relative to the position of a phosphorylatable Ser or Thr residue), substitution of those carboxylic acid residues with phosphoserine yields a dramatically superior substrate (Flotow *et al.*, 1990; Meggio *et al.*, 1991; Perich *et al.*, 1992; Umpress *et al.*, 1992). How CK1 recognizes phosphopeptides is unclear and may differ substantially from the well characterized interaction between the cAMP-dependent protein kinase and its inhibitor protein (Knighton *et al.*, 1991b). Finally, CK1 catalytic domains interact selectively with two classes of inhibitor: the ribofuranosyl benzimidazoles (Meggio *et al.*, 1990; Meggio *et al.*, 1991) and the isoquinoline sulfonamides (Chijwa *et al.*, 1989). Although both classes are competitive inhibitors of nucleotide substrate, the structural basis of their selectivity for CK1 over other protein kinases is not clear.

In contrast to the catalytic domain, little is known about the function of the C-terminal regions of CK1 homologs. Because they vary greatly in length and amino acid composition, these regions may promote differential subcellular localization of each isoform or direct their interaction with specific regulatory molecules (Wang *et al.*, 1992).

To learn more about the unusual structural features of CK1, and the biological function of its individual isoforms, we turned to the lower eukaryote *Schizosaccharomyces pombe* to develop a system in which we could combine genetic and enzymological approaches. This organism contains four CK1 homologs encoded by *cki1*$^+$, *cki2*$^+$, *hhp1*$^+$, and *hhp2*$^+$.[2,3] We have demonstrated that Cki1, the largest fission yeast CK1 homolog at 446 amino acids, can serve as a representative member of the CK1 family in that it retains the enzymological features of CK1, including substrate and inhibitor selectivities.[2] We plan to establish the structural basis for this unusual ligand selectivity by determining the three-dimensional structure of Cki1. Toward that end, we define here the catalytic core of recombinant Cki1 through C-terminal truncation analysis, and describe its crystallization in the presence and absence of nucleotide substrate.

EXPERIMENTAL PROCEDURES

Materials—Casein (5% solution; partially hydrolyzed and dephosphorylated) for protein kinase assays and calibration proteins for electrophoresis and chromatography were from Sigma. Ni^{2+}-nitrolotriacetate-agarose was from Qiagen (Chatsworth, CA). The pET-15b expression vector was from Novagen (Madison, WI). Polyethylene glycol 4000 and 8000 for crystallization were from Fluka.

Cki1 Mutagenesis—A *cki1* cDNA that was modified by polymerase chain reaction (to introduce useful restriction sites) and isolated in phagemid vector pT7B (Carmel and Kuret, 1992) was prepared for mutagenesis by the method of Kunkel *et al.* (1987) as described previously (Kuret *et al.*, 1988). All C-terminal deletions of the *cki1* coding

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[1] The abbreviations used are: CK1, casein kinase-1; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

[2] P. C. Wang, A. Vancura, A. Desai, G. Carmel, and J. Kuret, unpublished data.

[3] N. Dhillon and M. Hoekstra, unpublished data.

sequence were prepared by loopout mutagenesis (Eghtedarzadeh and Henikoff, 1986) and were confirmed by DNA sequence analysis.

Escherichia coli Expression—The cDNAs for wild-type *cki1* as well as deletion mutants *cki1*Δ390 and *cki1*Δ348 were isolated as *NdeI/Bam*HI fragments and ligated into the *NdeI/Bam*HI sites of expression vector pET-15b. Unlike pT7B, this derivative of the T7 expression system (Studier *et al.*, 1990) drives the overproduction of proteins fused to an N-terminal, 20-residue peptide (Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-) that allows affinity purification on immobilized nickel columns (Hochuli *et al.*, 1987). The final constructs (pET-15b/*cki1*, pET-15b/*cki1*Δ390, pET-15b/*cki1*Δ348, and pT7B/*cki1*Δ298) were transformed into BL21(DE3) cells to create the strains used for CK1 overproduction.

BL21(DE3) cells harboring any of the pET-15b- or pT7B-based plasmids described above were grown in Luria broth medium containing ampicillin (200 μg/ml) at 37 °C to an $A_{600\ nm}$ of 1, at which point isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 3 h of induction, cells were harvested by centrifugation (20 min at 3000 × *g*; 4 °C), washed with STE (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA), and stored at −70 °C until used. A 3-liter growth typically yields 8 g (wet weight) of cells.

Purification of Polyhistidine-tagged Cki1 Mutants—All steps were carried out at 4 °C. Frozen cells were thawed, resuspended in 5 volumes of lysis buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, 5 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, aprotinin, and pepstatin), and ruptured by two passes through a French press operated at 1000 p.s.i. The resultant homogenate was sonicated briefly (≈15 s) to shear nucleic acids, made 0.1% Brij 35, and centrifuged (100,000 × *g* × 1 h) to yield a clear supernatant (crude extract).

Following filtration through a 0.45-μm filter, the extract was loaded directly onto a 4-ml Ni^{2+}-nitrolotriacetate-agarose column pre-equilibrated in lysis buffer containing 0.1% Brij 35. The column was washed with 200 ml (50 bed volumes) of lysis buffer, and developed with sequential 20-ml steps of lysis buffer containing 0.05% Brij 35 and 10, 20, and 40 mM imidazole. Fractions containing casein kinase activity (eluting at 40 mM imidazole) were pooled, brought 75% saturation with solid $(NH_4)_2SO_4$, stirred 20 min, then centrifuged 20 min at 27,000 × *g*. The resultant pellet was resuspended in Buffer A (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.1 mM EGTA) to a final volume of 4 ml, and loaded directly onto a 180-ml column (1.6 × 91 cm) of Sephacryl S-100 HR gel filtration medium equilibrated and run in Buffer A at 30 ml/h. Fractions containing casein kinase activity were pooled, diluted 3-fold with 0.02% Brij 35 (to reduce the NaCl concentration to 50 mM), and loaded onto a MonoQ 5/5 HPLC column equilibrated in Buffer B (10 mM Tris-HCl, pH 7.5, 0.02% Brij 35, 0.1% 2-mercaptoethanol) containing 50 mM NaCl. The column was washed with 5 bed volumes of this buffer and developed with a 20-ml linear gradient of increasing NaCl (from 50 to 400 mM). Fractions containing casein kinase activity were pooled, concentrated by dialysis against storage buffer (50% glycerol, 10 mM MOPS, pH 7.0, 150 mM NaCl, 0.1 mM EGTA, 0.02% Brij 35, 1 mM dithiothreitol), and stored at −20 °C.

Purification of Cki1Δ298—Nonfusion Cki1Δ298 was expressed in BL21(DE3) cells as described above and was purified through the first two steps of the procedure developed for nonfusion, full-length Cki1.[2] However, instead of proceeding onto hydrophobic interaction chromatography, Fraction 2, the product of Polymin P and $(NH_4)_2SO_4$ precipitations, was loaded directly onto a 350-ml (2.2 × 91 cm) column of Sephacryl S-100 HR gel filtration medium equilibrated and run in Buffer A at 50 ml/h. Cki1Δ298 eluted at V_e/V_o = 1.41 (K_d = 0.293), and was taken as Fraction 3 (gel filtration pool).

Fraction 3 was dialyzed overnight against 1 liter of dialysis buffer (10 mM MOPS, pH 7.0, 50 mM NaCl, 0.1 mM EGTA, 0.02% Brij 35). In the morning, a fine white precipitate was removed by centrifugation (20 min at 27,000 × *g*) and the resulting supernatant loaded onto a 5 ml (1.2 × 5 cm) Sepharose-Q fast flow column equilibrated in dialysis buffer. Protein that did not bind to the column was identified and pooled by optical density and taken as Fraction 4.

Fraction 4 was loaded onto an 8 ml (8 × 75-mm) MonoS high performance liquid chromatography column equilibrated in Buffer C (10 mM MOPS, pH 7.0, 0.1 mM EGTA, 0.1% 2-mercaptoethanol) containing 50 mM NaCl. The column was washed with 2 bed volumes of Buffer C and developed with a 150-ml linear gradient of increasing NaCl (from 50 to 250 mM). Cki1Δ298 elutes as a series of sharp peaks between 150 and 200 mM NaCl. Individual protein peaks were pooled and concentrated by centrifugal filtration (Centricon-30; Amicon, MA). These concentrates were made 1 mM dithiothreitol, adjusted to 10 mg/ml protein, and stored at 4 °C for up to 1 month.

Analytical Methods—The concentrations of purified Cki1 preparations were quantified spectrophotometrically using absorbance coefficients calculated from amino acid content (Perkins, 1986).

Casein kinase activity was assayed as described previously (Vancura *et al.*, 1993). 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 (100–400 cpm/pmol). Initial velocity measurements were carried out in duplicate under these conditions with casein (0.6, 1.0, 2.0, and 4.0 mg/ml) as the varied substrate. K_m, V_{max}, and their standard errors were calculated as described by Wilkinson (1961).

SDS-polyacrylamide gels (10% acrylamide) were prepared, electrophoresed, and stained with Coomassie Blue as described previously (Vancura *et al.*, 1993). Molecular mass markers included bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), rabbit glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), and bovine trypsinogen (24 kDa).

Amino acid sequence data was obtained from 10-μg samples as described previously (Kuret and Pflugrath, 1991). Protein-bound phosphate was determined in triplicate as described by Buss and Stull (1983).

Mass Spectrometry—Positive ion electrospray mass spectrometry was performed on a triple quadrapole instrument (Finnigan MAT TSQ-700). Purified protein samples were precipitated with 6% trichloroacetic acid, washed twice with neat acetone, dried under vacuum, and stored at −20 °C until they were analyzed. Dried samples were dissolved in Buffer D (50% methanol, 5% acetic acid, ≈1% hexafluoroisopropanol), brought to ≈10 μM final concentration, and applied to the spectrometer (1 μl/min) with a Harvard syringe pump. Data from over the mass-to-charge range 400–2000 (16 × 5-s scans) was used to calculate mass and its standard deviation. Spectra were calibrated with equine myoglobin (16,951.5 Da; Beavis and Chait (1990)) and deconvoluted as described by Mann *et al.* (1989).

Crystallography—Crystallization conditions were sought for 10 mg/ml Cki1Δ298 (peak II) in the presence and absence of 6 mM ATP, 1.5 mM MgCl at 16 °C using the sparse matrix screening method of Jancarik and Kim (1991). Hanging drops (10 μl) containing equal volumes of protein or protein/nucleotide solution and precipitant were mixed, placed on siliconized microscope coverslips, and equilibrated over 1 ml of precipitant solution in 24-well tissue culture plates (ICN). The growth of large single crystals was optimized by varying precipitant concentration and pH. For data collection, single Cki1Δ298 crystals were harvested and mounted as described previously (Kuret and Pflugrath, 1991).

All x-ray diffraction data were collected on beamline X12-C at the National Synchrotron Light Source (Brookhaven National Laboratory) on a FAST television area detector as described by Kumar *et al.* (1992). The resultant data sets were scaled and merged with the programs FS and PROTEIN (Weissman, 1982; Steigemann, 1974). Precession photography and crystal density measurements were performed as in Kuret and Pflugrath (1991).

Nomenclature—Cki1 truncation mutants are designated by the symbol Δ followed by a number that indicates the point of C-terminal deletion. Proteins that are fused to an N-terminal polyhistidine tag are indicated by the letter h. Thus, the enzyme Cki1Δ390h consists of polyhistidine-tagged Cki1 residues 1–390.

RESULTS AND DISCUSSION

C-terminal Deletions of Cki1—On the basis of its primary structure and subcellular location, Cki1 is the fission yeast homolog of the *YCK* gene products from *Saccharomyces cerevisiae*. Its structural organization is shown schematically in Fig. 1*A*. Like Yck1p and Yck2p, it consists of a typical CK1 catalytic core (residues 8–298) followed by a 12-residue segment (residues 299–310) that is conserved among Yckp and Cki proteins but not other forms of CK1. This in turn is connected to a putative C-terminal prenylation site (residues 396–439) via a hydrophilic, 85-residue segment that is rich in Pro and Ser residues and that is predicted to contain the most flexible region of the molecule. We have referred to this region as the hydrophilic tether, because it links the catalytic domain to the C-terminal localization signal (Wang *et al.*, 1992). Like all forms of CK1 isolated to date, Cki1 is constitutively active *in vitro*.

To better define the amino acid residues that comprise the catalytic core of Cki1, a series of three C-terminal truncation

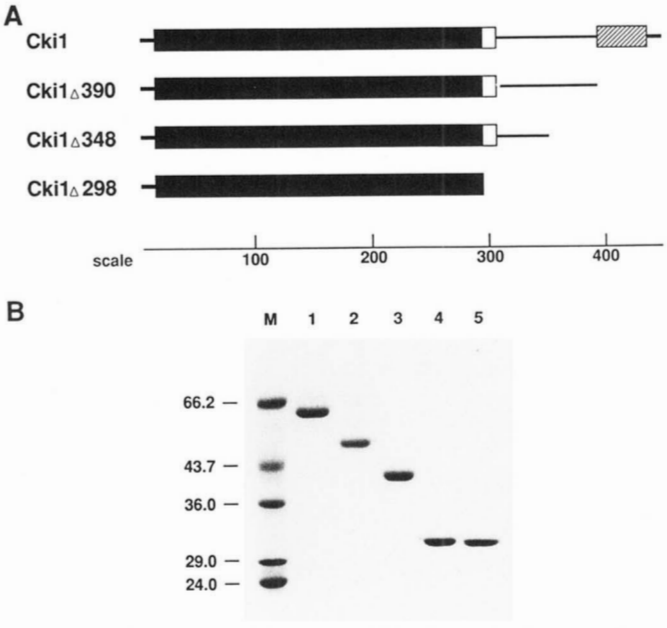

FIG. 1. **Design and purification of Cki1 C-terminal truncations.** *Panel A,* schematic diagram of four forms of Cki1 created by C-terminal truncation. The size of each mutant is shown alongside a scale reflecting protein length in amino acid residues. Also shown is the relative positions of the highly conserved, ≈290-residue catalytic domain (*black*), a 12-residue segment that is conserved among the *YCK* and *cki* gene products (*white*), and the 51-residue region that includes a putative prenylation site (*shaded*). *Panel B,* this Coomassie Blue-stained 10% SDS-polyacrylamide gel contains in *lane M*, molecular mass standards; *lane 1*, Cki1h; *lane 2*, Cki1Δ390h; *lane 3*, Cki1Δ348h; *lane 4*, Cki1Δ298 (peak 1); and *lane 5*, Cki1Δ298 (peak 2). Approximately 0.5 μg of each protein was loaded on this gel.

TABLE I
The effect of C-terminal truncation on the kinase activity of Cki1

Enzyme	k_{cat}[a]	K_m[a]	k_{cat}/K_m[a,b]
	s^{-1}	mg/ml	$\mu M^{-1}\ min^{-1}$
Cki1h	7.37 ± 0.73	5.81 ± 0.81	1.71 ± 0.29
Cki1Δ390h	7.26 ± 0.59	3.90 ± 0.53	2.52 ± 0.35
Cki1Δ348h	20.1 ± 1.6	7.40 ± 0.84	3.67 ± 0.42
Cki1Δ298 (peak 1)	16.5 ± 1.7	4.90 ± 0.83	4.54 ± 0.77
Cki1Δ298 (peak 2)	18.7 ± 0.8	1.55 ± 0.22	16.3 ± 2.4

[a] Values ± S.E. of the estimate.
[b] Calculated assuming the molecular mass of casein is 22.5 kDa.

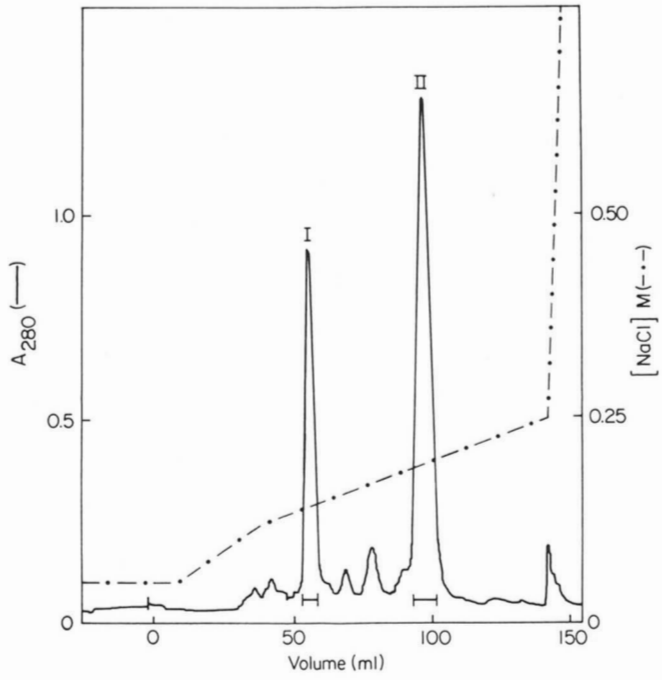

FIG. 2. **Cation exchange chromatography resolves multiple forms of recombinant Cki1Δ298.** Fraction 4 (MonoQ flow-through) was chromatographed on MonoS as described under "Experimental Procedures." A_{280} (——) and the salt gradient (- -) are shown *versus* elution volume. Major peaks I and II were pooled as indicated by the *horizontal bars*.

mutants were prepared by oligonucleotide-directed mutagenesis. These are illustrated in Fig. 1*A*. The first truncation, Cki1Δ390, eliminates the C-terminal 56 residues of Cki1 that may be responsible for localization of Cki1 to the plasma membrane. The second truncation, Cki1Δ348, eliminates the C-terminal 98 residues of Cki1, including approximately half of the tether region. The final truncation, Cki1Δ298, contains only those residues that are conserved in all known forms of CK1. To determine the effect of truncation on catalytic efficiency, each truncation mutant was expressed in *E. coli* and purified as described below.

Overexpression and Purification of Cki1 Truncation Mutants—Unlike most protein kinases, Cki1 expresses solubly in *E. coli*, where it can accumulate to 3–6% of the total soluble protein.[2] Nonetheless, its purification is tedious, in part because it elutes broadly from ion-exchange chromatography columns. As described below, this behavior probably results from extensive autophosphorylation of the enzyme. The problem was overcome by expressing Cki1 tagged at its N terminus with polyhistidine, which allows rapid and efficient purification of the resultant fusion protein after affinity chromatography over a nickel-chelate column. Because the site of fusion is well away from the active site of Cki1, addition of the polyhistidine tag should not affect enzyme activity *in vitro*. To confirm this hypothesis, kinetic constants for purified recombinant Cki1h were estimated by assaying its phosphotransferase activity at various concentrations of protein substrate as described under "Experimental Procedures." The results, summarized in Table I, show that Cki1h is a very active casein kinase, with apparent V_{max} and K_m values that are within the standard error of those measured for nonfusion recombinant Cki1.[2] Analysis of the preparation by automated Edman degradation gave the N-terminal sequence Gly-Ser-Ser-, which corresponds to the polyhistidine tag sequence minus its initiating formylmethionine residue. Thus, the final preparation consists of Cki1 fused to a 2,032-Da nonadecapeptide (the polyhistidine tag). We conclude that addition of this polyhistidine tag to Cki1 confers rapid affinity purification without affecting Cki1 catalytic activity, and that it is possible to directly compare the activity of polyhistidine-tagged Cki1 mutants to that of nonfusion Cki1.

In addition to full-length Cki1, truncation mutants Cki1Δ390 and Cki1Δ348 were expressed as polyhistidine tag fusions and purified to near homogeneity. As shown in Fig. 1*B*, the migration of Cki1h and Cki1Δ390h during SDS-polyacrylamide gel electrophoresis is 16–18% slower than predicted from the calculated molecular masses of these fusion proteins. The anomaly does not result from the polyhistidine tag epitope, because a similarly slow migration was observed with nonfusion Cki1.[2] Cki1Δ348h migrates with an apparent mass of 45 kDa, which is ≈8% greater than the calculated mass. As described below, Cki1Δ298 migrates within 5% of its calculated molecular mass. Thus, the region of Cki1 that is responsible for anomalous migration on polyacrylamide gels lies between residues 298 and 390, and corresponds to the proline-rich tether region.

Preparation of Cki1Δ298 for Structural Studies—Because we planned to crystallize the catalytic core of Cki1, and wanted to avoid potential complications arising from the presence of the

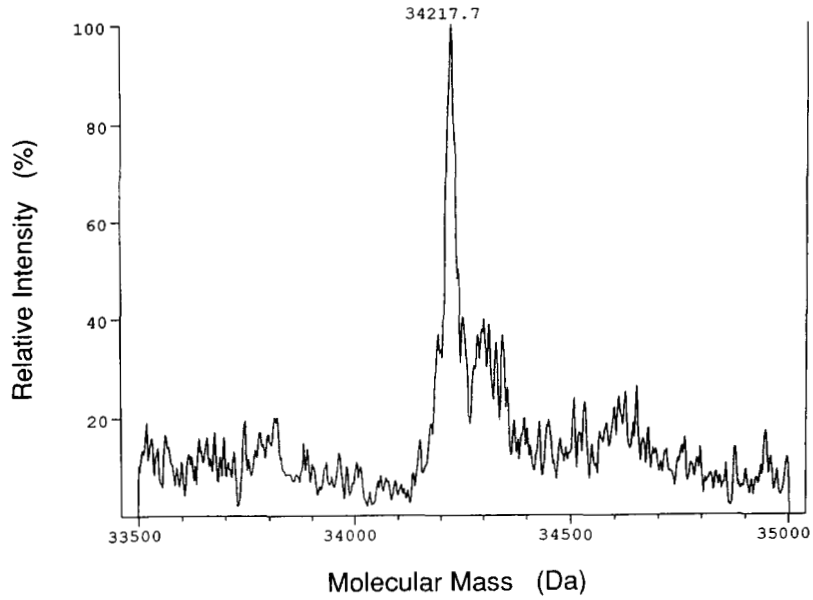

FIG. 3. **Mass spectrum of Cki1Δ298.** Peak II enzyme was subjected to mass spectrometry as described under "Experimental Procedures," using equine myoglobin as mass standard.

polyhistidine tag, one mutant, Cki1Δ298, was purified by conventional methods (described under "Experimental Procedures"). The purification begins with the removal of nucleic acids with polyethyleneimine and precipitation of casein kinase activity with $(NH_4)_2SO_4$. Gel filtration chromatography resolves a single peak of casein kinase activity, the migration of which is consistent with a monomeric, globular protein of ≈35 kDa. Remaining impurities are removed by passing the gel filtration pool through an anion exchange column, to which Cki1Δ298 does not adsorb. Although Cki1Δ298 is monodisperse through most of the procedure, a final cation exchange step resolves the enzyme into two major (peak I and peak II) and several minor species (Fig. 2). Each peak of protein contains CK1 activity and, upon electrophoretic analysis, migrates with a single polypeptide of 33 kDa molecular mass (Fig. 1*B*). A protein of this mass is identifiable throughout the purification and is consistent with that of Cki1Δ298.

Physical Characterization of Cki1Δ298—Analysis of major peaks I and II by automated Edman degradation gave identical amino acid sequences corresponding to residues 2 through 8 of Cki1: Ser-Gly-Gln-Asn-Asn-Val-Val-. Thus, the initiator formylmethionine of Cki1Δ298 is absent in both peaks I and II, and the differential migration of Cki1Δ298 peaks I and II seen during cation exchange chromatography is not the result of N-terminal modification.

To determine the structural relationship between peaks I and II, samples of each were subjected to electrospray mass spectrometry as described under "Experimental Procedures." The mass spectrum of peak II enzyme shows the preparation is composed principally of a single species of molecular mass 34,217.7 ± 19.6 Da (Fig. 3). This value is within 1 S.D. of the predicted mass of Cki1 residues 2–298 (34,200.0 Da), and suggests the preparation consists of unmodified protein. It is possible, however, that the ≈18 Da difference between the measured and calculated masses is significant and results from oxidation of one of the 7 methionine residues in Cki1Δ298 to the sulfoxide during enzyme preparation or storage (Allen, 1981). In contrast, the mass spectrum of peak I enzyme is complex and suggests it consists of a heterogeneous mixture. The masses of its two most abundant components are summarized in Table II, along with the absolute mass difference between each of them and peak II enzyme. The results show that each form of peak I enzyme is larger than peak II by a multiple of ≈80 Da, or the mass of a single phosphate group, and that peak I consists mostly of mono- (≈60%) and tri- (≈30%) phosphorylated Cki1Δ298. We conclude that the two peaks of Cki1Δ298 are related through the presence (peak I) or absence (peak II) of multiple phosphate groups. This conclusion was confirmed by assaying both peaks for phosphate content as described under "Experimental Procedures." While peak II contains trace amounts of covalently bound phosphate, peak I contains on average 1.5 mol of phosphate/mol of protein. The addition of negatively charged phosphate is consistent with the earlier elution of peak I relative to peak II during cation exchange chromatography.

Because Cki1Δ298 is expressed solubly in *E. coli*, and is active catalytically, the multiple forms of Cki1Δ298 observed probably result from autophosphorylation occurring *in vivo* during bacterial expression. Recombinant Cki1h, Cki1Δ390h, and Cki1Δ348h also contain covalently bound phosphate, and exhibit average stoichiometries of 9.1, 4.8, and 4.0 mol of phosphate/mol of enzyme, respectively. We suspect that the

TABLE II
Mass spectroscopic analysis of molecular mass and phosphate content of Cki1Δ298

Mass of Cki1Δ298[a]			
Peak I	Peak II	Net difference[b]	Phosphate[c]
Da			*mol/mol*
34,298.6 ± 13.6	34,217.7 ± 19.6	80.9 ± 23.9	1.01
34,458.8 ± 15.0		241.1 ± 24.7	3.01

[a] Mass ± S.D. of the estimate was estimated by mass spectrometry as described under "Experimental Procedures."
[b] Absolute mass difference between peak I and peak II forms of Cki1Δ298.
[c] Deduced phosphate content in mol of phosphate/mol of protein assuming 80.0 Da/phosphate.

TABLE III
Properties of yeast Cki1Δ298 crystals

Crystal	Apo	Binary 1	Binary 2
Unit cell dimensions (Å)	128 × 114 × 46	63 × 65 × 86	79 × 79 × 121
Space group	$P2_12_12_1$	$P2_12_12_1$	$P3_121$[a]
Molecules/unit cell	8	4	6
Molecules/asymmetric unit	2	1	1
Packing density (Å³/Da)[b]	2.47	2.59	2.74
Solvent content[b]	50%	52%	55%
Resolution limit (Å)	3.2	2.7	2.0

[a] Enantiomorph is possible.
[b] Calculated as described by Matthews (1968).

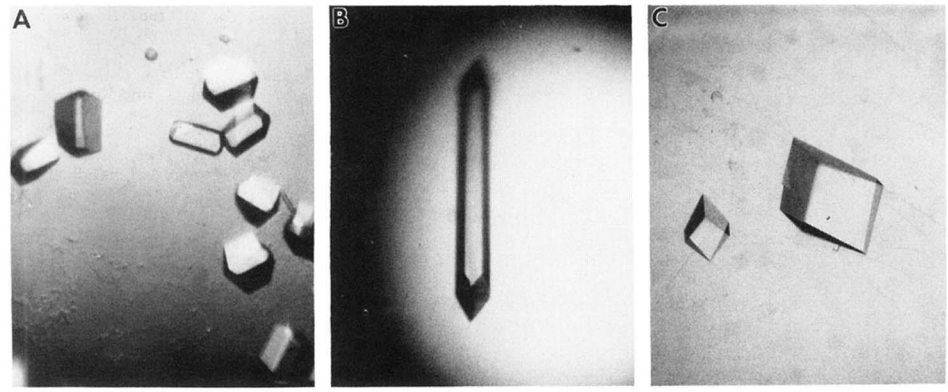

FIG. 4. **Photomicrographs of Cki1Δ298 crystals.** *A*, orthorhombic crystals of apo Cki1Δ (maximum dimension is 0.3 mm). *B*, orthorhombic binary form (maximum dimension is 1 mm). *C*, trigonal binary form (maximum dimension of larger crystal is 0.8 mm).

broad elution pattern observed during ion-exchange chromatography of these three enzymes results from the copurification of multiply phosphorylated forms.

Kinetic Properties of CK1 Truncation Mutants—To determine the effect of Cki1 C-terminal deletion on catalytic efficiency, kinetic constants were estimated for each of the Cki1 truncations by measuring phosphotransferase activity at varying concentrations of casein as described under "Experimental Procedures." Results are presented in Table I. Full-length recombinant Cki1h phosphorylates casein with a V_{max} of 8.41 ± 0.83 μmol of mg^{-1} min^{-1} (k_{cat} = 7.37 s^{-1}) and a K_m of 5.81 ± 0.81 mg/ml (k_{cat}/K_m = 1.71 μM^{-1} min^{-1}). Comparison of these kinetic values with those of the truncation mutants shows that, although Cki1 is constitutively active, deletion of its C-terminal region results in a modest increase in its k_{cat}/K_m for casein. The increase in k_{cat}/K_m comes in part through a ≈3-fold increase in catalytic rate. Indeed, the turnover numbers observed for Cki1Δ348h and Cki1Δ298h (16–20 s^{-1}) are the highest reported to date for any CK1 homolog. Examination of the data in Table I points to the removal of residues located between Ser^{348} and Gly^{390} as being responsible for the increase in catalytic rate.

It will be of interest to determine whether this activation phenomenon is a general feature of the CK1 family. Like other protein kinases, CK1 may bind a pseudosubstrate or other motif that inhibits the catalytic site until removed by proteolysis *in vitro* or through interaction with an activator *in vivo* (*e.g.* Knighton *et al.* (1992)). Evidence for the latter mechanism has been presented for the δ form of mammalian CK1 (CKIδ; Graves *et al.* (1993)). This 428-residue CK1-homolog increases its k_{cat}/K_m for peptide substrate 5-fold in response to heparin. Deletion of the C-terminal region of CKIδ eliminates heparin-mediated activation.

The catalyic activities of Cki1Δ298 peak I and peak II enzymes point to another potential mechanism of regulation. Peak I enzyme phosphorylates casein with a V_{max} of 28.9 ± 3.0 μmol of mg^{-1} min^{-1} (k_{cat} = 16.5 s^{-1}) and a K_m of 4.90 ± 0.83 mg/ml (k_{cat}/K_m = 4.54 μM^{-1} min^{-1}). Under identical conditions, peak II phosphorylates casein with a V_{max} of 32.8 ± 1.3 μmol of mg^{-1} min^{-1} (k_{cat} = 18.7 s^{-1}) and a K_m of 1.55 ± 0.22 mg/ml (k_{cat}/K_m = 16.3 μM^{-1} min^{-1}). Thus, while its catalytic rate remains essentially unchanged, autophosphorylation of Cki1Δ298 results in ≈4-fold increase in its apparent K_m for peptide substrate. As shown in Table I, the combination of truncation and dephosphorylation of Cki1 results in an overall activation of nearly 10-fold.

Crystallization of Cki1Δ298—Because of its ease of preparation, high specific activity, and absence of post-translational modification, Cki1Δ298 peak II was selected for crystallization trials. Crystallization conditions for Cki1Δ298 (peak II) were sought in the presence and absence of MgATP as described under "Experimental Procedures." Results are summarized in Table III and illustrated in Fig. 4. We note that it is not possible

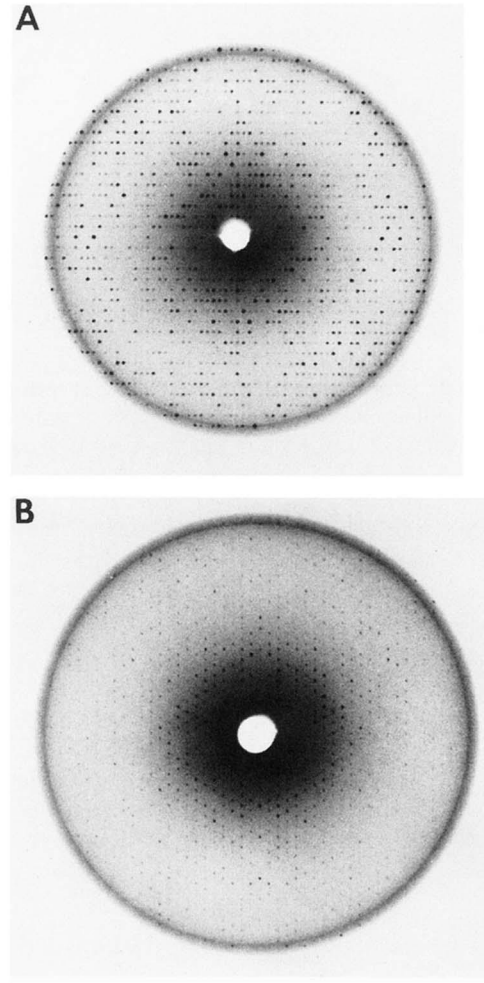

FIG. 5. **Screened precession photographs obtained from a trigonal crystal of Cki1Δ298 at μ = 12° (20-h exposure).** *A*, the 0*kl* zone: reflections along 00*l* (*i.e.* the horizontal axis) appear only when *l* = 3*n*. *B*, the *hk*1 zone: upper level 3*m* symmetry confirms the space group is $P3_121$ or its enantiomorph.

to crystallize Cki1Δ298 peak I under either the conditions described below or by seeding with existing crystals of the peak II enzyme.

In the absence of substrate, Cki1Δ298 crystallizes in 8.5% (w/v) polyethylene glycol 4000, 8.5% (v/v) isopropyl alcohol over pH range 8.5–9.5. The crystals grow as rhombohedral prisms up to 0.3 × 0.3 × 0.3 mm in size and diffract to 3.2-Å resolution. Assignment to space group $P2_12_12_1$ was made on the basis of systematic absence of reflections along the *h*00, 0*k*0, and 00*l* axes.

In the presence of MgATP, Cki1Δ298 crystallizes in two different crystal forms. The first form (binary 1) emerged from 3%

(w/v) polyethylene glycol 8000, 20 mM $(NH_4)_2SO_4$ at neutral pH. Although crystals grown under these conditions are frequently twinned, single rods that have dimensions 1.0 × 0.1 × 0.1-mm and that diffract to 2.7-Å resolution are obtainable. Again, assignment to space group $P2_12_12_1$ was made on the basis of systematic absences in the diffraction data. The second binary form emerged from 1.55 M $(NH_4)_2SO_4$, 50 mM sodium citrate, pH 5.6. These crystals grow to dimensions 0.8 × 0.5 × 0.5-mm and diffract to ≈2.0-Å resolution. Assignment to space group $P3_121$ (or its enantiomorph) was made on the basis of precession photography (Fig. 5). Although the $0kl$ zone shows reflections along the $00l$ axis when $l = 3n$ (where n is an integer), which is consistent with a hexagonal crystal system, the upper level $3m$ symmetry in the $hk1$ zone confirms $P3_121$ or its enantiomorph as the true space group. Six trigonal crystals were used to collect a native data set that is 94.2% complete between 20.0 and 3.07 Å, 90.1% between 3.07 and 2.44 Å, 76.2% between 2.44 and 2.13 Å, and 33.6% between 2.13 and 1.94 Å. A total of 142,178 measurements with 24,212 unique reflections (to 1.94-Å resolution) were merged with an overall error (R-merge) of 7.92% with $F/\sigma(F) > 1$, where F is the structure factor.

From the molecular mass of Cki1Δ298 and unit cell parameters summarized in Table III, we calculate packing densities of 2.47–2.74 $Å^3$/Da and solvent contents of 50–55% for the three crystal forms described above. These values, which were confirmed by crystal density measurements, are typical of protein crystals (Matthews, 1968).

Because CK1 is so distantly related to other protein kinases in primary structure (Wang *et al.*, 1992), successful use of the molecular replacement technique for phase determination (Aguilar *et al.*, 1993) using established structures as search models may be difficult. Therefore, we plan to solve the phase problem by multiple isomorphous replacement of the trigonal crystals, and a search for useful heavy atom derivatives is underway.

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