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The protein kinase 60S is a free catalytic CK2α′ subunit and forms an inactive complex with superoxide dismutase SOD1☆

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Abstract

The 60S ribosomes from *Saccharomyces cerevisiae* contain a set of acidic P-proteins playing an important role in the ribosome function. Reversible phosphorylation of those proteins is a mechanism regulating translational activity of ribosomes. The key role in regulation of this process is played by specific, second messenger-independent protein kinases. The PK60S kinase was one of the enzymes phosphorylating P-proteins. The enzyme has been purified from yeast and characterised. Pure enzyme has properties similar to those reported for casein kinase type 2. Peptide mass fingerprinting (PMF) has identified the PK60S as a catalytic α′ subunit of casein kinase type 2 (CK2α′). Protein kinase activity is inhibited by SOD1 and by highly specific CK2 inhibitor—4,5,6,7-tetrabromo-benzotriazole (TBBt). The possible mechanism of regulation of CK2α′ activity in stress conditions, by superoxide dismutase in regulation of 80S-ribosome activity, is discussed.

Keywords: Protein kinases; CK2α′; P1/P2 proteins; Ribosome phosphorylation; SOD1; Yeast

Reversible phosphorylation of proteins is the most significant factor in control of cell function. This reaction regulates a number of important cell processes, including cell metabolism, response to external stimuli, cell cycle, transcription, and translation [1,2].

A complex of acidic ribosomal proteins (P-proteins) forming universally conserved lateral stalk on the 60S ribosomal subunit is one of the main structures controlled by phosphorylation. In eukaryotes the stalk structure is formed by protein P0, homologous to prokaryotic L10 and a set of P1 and P2 proteins, homologous to bacterial L7/L12 [3–5]. The eukaryotic P-protein family, formed by two or more polypeptides, shows a conserved amino acid sequence [6]. The P-protein family according to its sequence homology can be divided into two subgroups called P1 and P2 [7]. The number of proteins in each subgroup varies with the organism from one in human [8], rat [9], *Artemia salina* [10], and *Drosophila melanogaster* [11,12], two in yeast [13–16], and more in *Trypanosoma cruzi* [17]. In baker's yeast, the two P1 proteins have been called P1A and P1B and the two P2 proteins P2A and P2B [18,19]. One of the most interesting features of eukaryotic P proteins is their capability to be phosphorylated. In yeast, they are phosphorylated when bound to the ribosome [19] but are also found as a cytoplasmic pool of non-phosphorylated proteins [20] and, during the translation, exchange between proteins present on the ribosome and cytoplasm takes place. The free cytoplasmic P-proteins have been shown to participate in the process of exchange with the ribosomes in yeast [21], plants [22], and mammals [23]. This exchange seems to be due to extending of

☆ *Abbreviations:* CK1, casein kinase type 1; CKI1, yeast casein kinase type 1 homolog 1; CK2, protein kinase type 2 known as casein kinase type 2; CKA1 and CKA2, α and α′ subunit of CK2; PK60S, protein kinase 60S ribosomal subunit; PKA and PKC, protein kinase type A and type C, respectively; CaMPK, Ca^{2+} and calmodulin-dependent protein kinase; SOD1, Cu–Zn superoxide dismutase; rYP2A, yeast recombinant acidic ribosomal P2A protein; TBBt, 4,5,6,7-tetrabromo-benzotriazole.

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phosphorylation of ribosomal stalk proteins [24,25], and changes in a stalk conformation and composition [3]. These can affect expressions of specific proteins by influence on the initiation and elongation factors and thereby the efficiency of translation of individual mRNAs [5,26–30].

Five different second messenger-independent protein kinases phosphorylating in vitro P-proteins have been isolated from yeast, namely protein kinase 60S (PK60S) [31], RAP kinases I–III [32–34], and CK2 (casein kinase type 2) [35]. Except for CK2, they are monomeric proteins and utilise only ATP as phosphate donor, while CK2 has an oligomeric structure ($\alpha\alpha'\beta_2$ or $\alpha_2\beta_2$) and uses ATP and GTP [36–38]. All enzymes phosphorylate P1/P2 proteins and other ribosomal stalk protein P0 at the equivalent position in the amino acid sequence, namely at the serine residue closest to the C-terminal end of polypeptides [25,39].

One of the enzymes described above, the protein kinase 60S, was isolated from a cytosolic fraction of yeast cells [31]. This protein kinase was characterised as monomeric 71 kDa protein utilising only ATP ($K_m \approx 13\,\mu M$) as a phosphate donor, not sensitive to second messengers—modulators of PKA, PKC, and CaMPK, and not sensitive to heparin and polyamines—modulators of CK2. Earlier results from the same laboratory have demonstrated the presence of a specific acidic ribosomal protein kinase activity in a similar yeast protein fraction [40]. The enzyme, named protein kinase 1B, was shown to have a molecular weight of ~39 kDa which is much lower than that of PK60S. A similar protein kinase (ARP-K) phosphorylating P-proteins was purified and characterised from maize axes [41]. The ARP-K was shown as a single 38 kDa protein enzyme with properties similar to those reported for CK2.

The yeast casein kinase type 2 is the second enzyme phosphorylating ribosomal P-proteins studied to a greater extent. This enzyme differs from kinases isolated from animal sources. The sequences of the yeast catalytic α and α′ subunits (44 and 39 kDa, respectively) and regulatory β and β′ subunits (41 and 32 kDa) have been determined [36]. The amino acid sequences of the yeast $\alpha\alpha'\beta\beta'$ holoenzyme are homologous to CK2 from other organisms. The β subunit stabilises the α subunit against thermal inactivation and stimulates the kinase activity against most of the protein substrates [36,37]. The regulatory subunit contains an N-terminal autophosphorylation site, an internal acidic region, and a C- terminal Zn^{2+}-binding motif engaged in dimerisation of the CK2β [37,42].

CK2 has been established as messenger-independent because small signalling molecules such as cyclic-nucleotides, lipids, or Ca^{2+} do not affect its activity [43]. However, a number of external small particles have been identified as CK2 regulators. Between them are 2,3-bisphospho glycerate, glycosoaminoglycans, halogenated benzimidazole ribosides, and halogenated 2-azabenzimidazoles identified as potential inhibitors of the CK2 [37,38,44]. One of them, TBBt, was shown to inhibit both CK2 and PK60S kinases phosphorylating acidic ribosomal proteins [45]. Basic compounds such as basic proteins or polyamines are stimulators of CK2 activity. Both oligoamines and polyanionic compounds similar to heparin were shown to interact with CK2 holoenzyme through its β regulatory subunit [37,46]. An increase of CK2 activity has been reported as a response to a number of hormones promoting growth, development, and transformation [36,37,47].

In our previous work, we have shown that in *Saccharomyces cerevisiae* state of phosphorylation of P-proteins can be regulated by direct interaction of superoxide dismutase (SOD1) with protein kinase 60S [48]. This paper is a continuation of our studies on the phosphorylation of yeast ribosomal stalk proteins. We report here the purification of protein kinase known as PK60S and identification of this enzyme as free catalytic α′ subunit of CK2. Possible role of the SOD1 protein in regulation of CK2α′ in stress conditions is discussed.

Materials and methods

Strains and growth conditions. Yeast strain *S. cerevisiae* W303 (a/α; *leu 2*, *trp 1*, *ura 3*, *his 3*, *ade 2*, and *can 1*) was cultivated under aerobic conditions in YPD medium (yeast extract, peptone, and glucose) to the exponential growth phase at 28 °C. *S. cerevisiae* $CKA1_\Delta$ (gene YIL035c), $CKA2_\Delta$ (gene YOR061w), and $CK11_\Delta$ (gene YHR135C) single deletion strains (Y01428, Y01837, and Y01963, respectively) were from EUROSCARF collection centre.

Protein kinases. Protein kinases purified almost to homogeneity: CK1 [49] and CK2 [50] were obtained from yeast. Protein kinase activities were tested under standard conditions either with exogenous casein and bovine calmodulin or endogenous protein substrates (80S ribosomes, rYP2A, and rYP2B) as previously described [48].

Synthetic peptide substrates (0.5 mM) were phosphorylated by incubation in a medium (50 μl of final volume) containing 50 mM Tris–HCl buffer, pH 7.5, 10 mM Mg^{2+}, and 20 μM [γ-^{32}P]ATP (specific radioactivity 500–1000 cpm/pmol) unless otherwise indicated. The reaction was started with the addition of 0.2 μg of appropriate protein kinase and stopped by addition of 0.25 M EDTA and cooling in ice. Phosphorylation was quantitated by the phosphocellulose paper procedure [51].

Protein kinase CK2α′ (former PK60S [31] or protein kinase 1B [40]) was purified from a ribosome-free extract (S-100 fraction). A 2600-mg sample of S-100 fraction was loaded onto 225 ml DEAE-cellulose equilibrated with 20 mM Tris–HCl buffer, pH 7.5, 6 mM 2-mercaptoethanol, 1 mM EDTA, and 0.5 mM of phenyl-methylsulfonyl fluoride (buffer A). The flow-through column fractions were then chromatographed on phosphocellulose. These protein fractions contain activities of: the casein kinase type 1, a ribosome-specific kinase activity [40], the SOD1 protein [48], and a protein phosphatase type 2A active with P-proteins [52]. From this column the P-proteins-specific protein kinase was eluted with 600 mM NaCl in buffer A and pooled. Dialysed overnight in buffer A active fractions were then chromatographed on α-casein–agarose. The kinase was recovered in a 40-ml linear gradient of NaCl over 250–600 mM in buffer A (Fig. 1A). Fractions containing P protein-specific kinase activity were concentrated, dialysed against buffer A, and applied to a SOD1-peptide

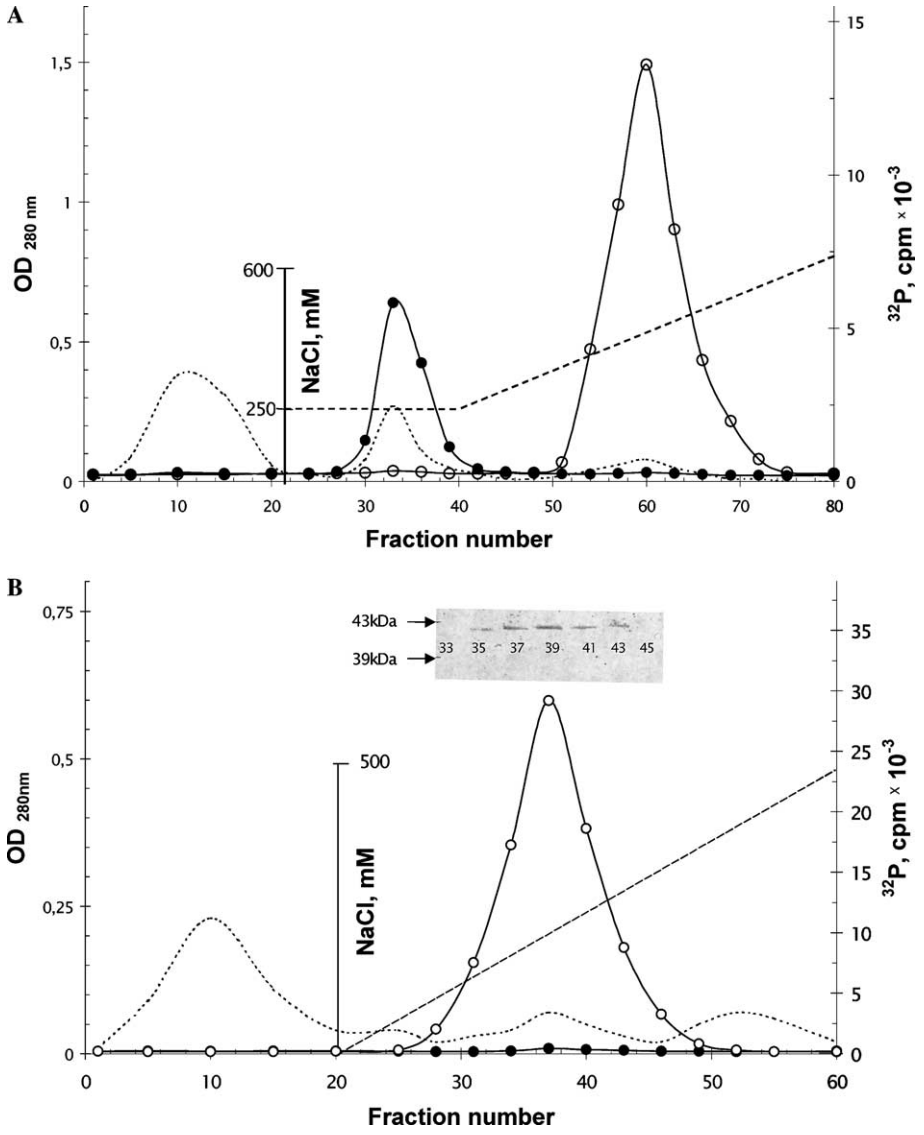

Fig. 1. Chromatography of the CK2α′ on the α-casein–agarose (A) and the SOD1-A-peptide–Sepharose 4B (B). (A) About 11.8 mg dialysed P-protein-specific kinase activity after ion-exchange chromatography on P-cellulose (Materials and methods) was applied on α-casein–agarose (0.9 × 7.5 cm). After elution of CK1 kinase with 250 mM NaCl in buffer A, the protein kinase CK2α′ was recovered in a 40-ml linear gradient of NaCl over 250–600 mM in the same buffer (---) Activities of protein kinases were detected under standard protein kinase assay, using 2 μg of ribosomal P2B protein (○) or 15 μg of whole casein (●) and 10 μl aliquots form the column fractions. A discontinuous lane indicates the optic density of the eluting solution measured at 280 nm. (B) Dialysed CK2α′ activity after α-casein–agarose chromatography (1.4 mg) was applied on SOD1-A-peptide–agarose (0.9 × 4 cm) equilibrated with buffer A. The CK2α′ was eluted from the column in a 25-ml linear gradient of NaCl over 0–500 mM (---). The activity of protein kinase was detected as described in (A). Inset photo shows Coomassie brilliant blue-stained protein kinase fractions on 12.5% SDS–PAGE. Position of 43-kDa protein standard (ovalbumin) is shown by an arrow.

A–Sepharose column equilibrated with the same buffer. Protein kinase active material was eluted from the column with 25 ml linear gradient of NaCl over 250–600 mM in buffer A (Fig. 1A). Kinase fractions active with ribosomal protein P2B were concentrated, dialysed in buffer A with 20% glycerol, and kept at −30 °C for several weeks without a substantial decrease in activity.

A standard reaction mixture (50 μl of final volume) for the determination of protein kinase activity (CK1, CK2, and CK2α′) contained 20 mM Tris–HCl buffer, pH 7.5, 10 mM Mg^{2+}, and 20 μM [γ-^{32}P]ATP (specific radioactivity 500–1000 cpm/pmol) sample of appropriate enzyme and 0.1–1.5 mg/ml of protein substrate. Incubation was performed at 30 °C for 20 min.

One unit kinase activity is defined as the amount of the enzyme required for the incorporation of 1 pmol phosphate from [γ-^{32}P]ATP into a protein substrate per minute under conditions described above.

For analysis of protein kinases activity in mutant yeast strains Y01428, Y01837, and Y01963 the flow-through column fractions after DEAE-cellulose were used. In each case this activity was compared with the activity of appropriate enzyme from wild strain.

Renaturation assay for protein kinases. Detection of protein kinase after SDS–PAGE was performed using the modified methods of Wang and Erikson [53] and Kameshita and Fujisawa [54]. Protein fractions were resolved on 12.5% SDS–PAGE containing yeast recombinant P2B protein in a concentration of 0.15 mg/ml in a running part of the

gel. A sample of P-proteins-specific protein kinase was mixed with an equal volume of sample buffer (2.5% SDS, 25% sucrose, 25 mM Tris–HCl, pH 8.0, 2.5 mM EDTA, and 15 mM 2-mercaptoethanol) and then heated for 3 min in boiling water prior to loading on the gel. Afterwards, the electrophoresis gel was carefully placed in a clean glass dish with a tight lid for the remaining steps. All the incubations took place on an orbital shaker in room temperature. The gel was washed twice with 50 mM Tris–HCl, pH 8.8 (buffer A) containing 20% isopropanol and twice with 100 ml of buffer A containing 5 mM 2-mercaptoethanol, 30 min each time, to remove the SDS. Afterwards, the gel was washed for 30 min with 75 ml of 6 M guanidine in buffer A to denature proteins, which were later renaturated by soaking the gel in 100 ml of buffer A containing 0.4% Nonident NP-40 during 10 min at room temperature and then overnight at 4 °C. The gel was washed thereafter in 100 ml of the same fresh solution for 30 min and then during the 30 min it was equilibrated in 90 ml of buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM glycerophosphate, and 0.3 mM orthovanadate (phosphorylation buffer). The protein kinase activity test was done in sealed plastic bag containing gel soaked in 2.5 ml of phosphorylation buffer containing 50 μM [γ-^{32}P]ATP (100 μCi). After 2-h reaction at 30 °C the gel was washed 10 times with 100 ml of 0.1% sodium pyrophosphate in 5% TCA. Finally, the gel was dried and exposed to Kodak X-Omat film to detect labelled bands.

Purification of SOD. The superoxide dismutase from the yeast postribosomal extract was purified almost to homogeneity as previously described [48]. The pure SOD1 protein was stored at −30 °C in 20 mM Tris–HCl buffer (pH 7.5) containing 20% glycerol. Superoxide dismutase activity was tested using Supeoxide Dismutase Assay Kit (Calbiochem).

Determination of the size of the CK2α′–SOD1 complex by centrifugation. Briefly, 0.2 mg of pure protein kinase CK2α′ was mixed with 0.5 mg of SOD1 protein (both in 0.25 ml of buffer A containing 50 mM NaCl), incubated for 15 min in ice, and layered onto 15–40% glycerol linear density gradient (10 ml) in the same buffer. Samples were centrifuged for 40 h at 40,000 rpm (∼200,000*g*) in a Sorval TH641 rotor. Fractions (0.4 ml) were collected by upward displacement and monitored at 280 nm. In parallel tubes a gradient containing 0.2 mg of pure protein kinase CK2α and a gradient with protein standards: soybean trypsin inhibitor (20.1 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa), and phosphorylase a (97 kDa) were run at the same time. Fractions were assayed for acidic protein kinase and SOD1 activity and analysed by SDS–PAGE to localise both kinase and SOD1, and protein standards.

Synthetic peptides. Three synthetic peptides, two with amino acid sequences derived from SOD1 protein (peptide A—RRREQASESEPTT and peptide B—RRRGKGDTEESLKT), and third, with amino acid sequence typical for CK2 (peptide C—KKKEEESEEE), were prepared. Each peptide had a triplet basic amino acid residue additionally attached to the N-terminus to serve as a "sticky end," with which it attached itself to phosphocellulose paper. Synthetic peptides were prepared on a peptide synthesiser Labortec AG model SP650 using the Fmoc-polyamide method on 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)-phenoxy resin. Side chain protections were as follows: 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl (Pmc) for arginine, t-butyloxycarbonyl (Boc) for lysine, and t-butyl (t-But) for aspartic acid, glutamic acid, serine, and threonine. After synthesis peptides were cleaved from the resin with trifluoroacetic acid (TFA)/phenol/triisopropylsilane/water (88:5:2:5, by vol.). The crude peptides were purified on a preparative RP-HPLC Vydac C-18 column eluted with a 5–30% linear gradient of acetonitrile. The purity of 95% was evaluated by amino acid analysis on analytical HPLC System Gold Beckman using a Spherisorb S 5X C-18 column.

Electrophoretical techniques. Electrophoresis of proteins was performed in SDS 10–15% polyacrylamide (w/v) slab gels according to Laemmli [55]. The relative molecular mass of proteins was calculated, using the following marker proteins: phosphorylase a (97 kDa), bovine serum albumin (67 kDa), albumin from egg (43 kDa), soybean trypsin inhibitor (20.1 kDa), and yeast P2B ribosomal protein (11 kDa).

Isoelectric focusing (IEF) of ribosomes and ribosomal proteins was performed on 5% polyacrylamide gel slabs (225 × 100 × 0.8 mm) containing 2% Pharmalyte, pH range 2.5–5.0 (Sigma), as previously described [32].

Mass spectrometric analyses. The homogeneous protein band was excised from a Coomassie blue-stained SDS–PAGE gel (run separately with higher load for these analyses) and digested in situ with porcine trypsin as described [56]. After digestion and extraction, a small aliquot of the peptide mixture was analysed by PMF, and the peptide mass list was used to search the protein's identity via the search engine ProFound.

Other procedures. Protein concentration was determined by the Bradford procedure [57] with bovine serum albumin as a standard.

Synthetic peptide A, with amino acid sequence corresponding to CK2 substrate recognition site and reproducing the sequence 21–30 of SOD1 protein was coupled with CNBr-activated Sepharose 4B according to the manufacturer's instruction.

Results

Yeast kinase specific for ribosomal acidic proteins was purified from *S. cerevisiae* strain W303 using existing procedures [31,42] with modifications which include two affinity chromatography steps on α-casein–agarose and peptide-A–Sepharose 4B (Figs. 1A and B, respectively). The early fractions from α-casein–agarose contained a trace amount of casein kinase type 1 as a contamination from activity eluted from the column at 250 mM NaCl concentration. This CK1 has been identified as a yeast casein kinase I homologue 1 (Gene: YCK1 or CKI2 or YHR135C). The P-protein-specific kinase was eluted from the second affinity column at about 240 mM NaCl as a symmetrical peak of activity. The polypeptide composition of the P-protein kinase fraction obtained during purification was analysed by SDS–polyacrylamide gel electrophoresis, as shown in Fig. 1B (inset) and Fig. 2 (lane B). As shown the enzyme exists as a single, electrophoretically pure protein band with a molecular weight of around 38 kDa. Interestingly, analysis of the protein kinase under protein renaturation conditions coincides with the position of the silver stained band in the SDS–PAGE (Fig. 2).

After in-gel digestion of the 38-kDa protein and analysis by PMF the protein kinase known as PK60S [31] or protein kinase 1B [40] was identified as the catalytic α′ subunit of casein kinase type 2 (CKA2). The "*z* score" (ProFound specific measure of significance) was 1.97 with nine peptides covering 29% of the sequence of the kinase.

In our previous studies, we have shown that by DEAE-cellulose anion exchange chromatography resolves two major P-protein-specific kinase activities: CK2α′ (former PK60S) localised in flow-through protein fraction and holoenzyme of CK2 eluted at 350 mM NaCl [52]. In addition, the DEAE-cellulose flow-through protein fraction contains the casein kinase type 1, SOD1 protein [48], and a protein phosphatase type

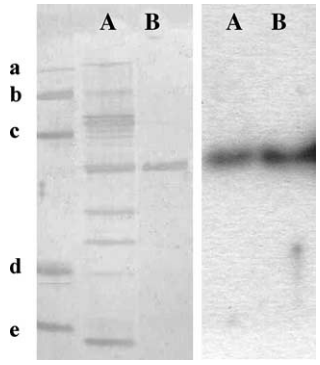

Fig. 2. Identification of the CK2α′ activity in protein fractions isolated from yeast cytoplasm. (Left panel) Silver-stained CK2α′ protein fraction purified from *S. cerevisiae* obtained after the α-casein–agarose (A) and the SOD1-A-peptide–Sepharose (B) chromatography resolved on 12.5% SDS–PAGE. As protein markers were used: a—phosphorylase a (97 kDa); b—bovine serum albumin (67 kDa); c—albumin from egg (43 kDa); d—soybean trypsin inhibitor (20.1 kDa); and e—purified rYP2B protein (11 kDa). (Right panel) On the 12.5% SDS–PAGE containing 0.15 mg/ml of the recombinant P2B protein: ~15 μg of the CK2α′ kinase fraction after α-casein–agarose (A) and ~1.2 μg of the enzyme after the SOD1-A-peptide–Sepharose (B) chromatography were separated. Separated proteins were treated with guanidine and alloyed to renature overnight. The renatured proteins were incubated with 2.5 ml of reaction buffer containing [γ-^{32}P]ATP as described in Materials and methods. Dried gel was autoradiographed.

2A (PP2A) active with P-proteins [53]. To confirm our result, the DEAE-cellulose flow-through protein fractions obtained from wild-type yeast (W303) and single deletion yeast mutants $CKA1_{\Delta}$ (strain Y01428), $CKA2_{\Delta}$ (strain Y01837), and $CKI1_{\Delta}$ (strain Y01963) were analysed for the presence of protein kinase activities with two protein substrates: casein and ribosomal recombinant P2B proteins. Results shown in Fig. 3 (lane 3) confirm our MALDI-TOF analysis. This result gives

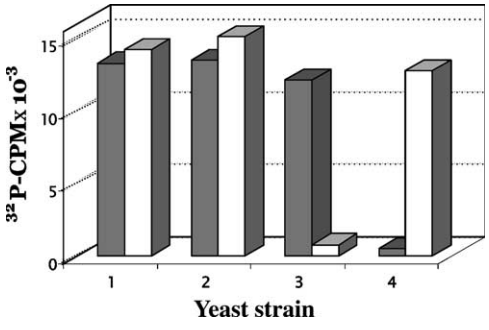

Fig. 3. Detection of casein and P-protein-specific protein kinases in the cytoplasm fraction of different yeast strains: (1) wild-type, (2) CKA1 deletion mutant, (3) CKA2 deletion mutant, and (4) CKI1 deletion mutant. Protein kinase activity from the flow-through column fractions after DEAE-cellulose was used (Materials and methods). An obtained protein fraction was monitored with 15 μg casein (grey blocks) and with 4 μg of pure recombinant P2B protein (white blocks) in the reaction mixtures containing 20 μM [γ-^{32}P]ATP in 20 mM Tris–HCl buffer, pH 7.5, as described in Materials and methods.

additional information that the protein kinase CK2α′ phosphorylates only P2B protein and exogenous casein fraction is phosphorylated by casein kinase CKI1 but not by CKA2 (Fig. 3, lanes 3 and 4).

As noted earlier [35–38,58,59], CK2 has been considered to be a tetrameric $\alpha\alpha'\beta_2$ or in yeast $\alpha\beta\alpha'\beta'$ enzyme with the β(β′) regulatory subunit controlling of the catalytic activity of CK2 at a number of possible levels. In our report, we present existing free and active CK2α′ subunit. The substrate specificity and influence of different factors on the CKA2 were tested and compared with purified yeast CK2 tetrameric enzyme. The results are shown in Table 1. As can be seen CK2α′ has properties typical for casein kinase type 2 with some exceptions, which are probably associated with lacking of regulatory β subunit. These exceptions are, lack of inhibition by heparin and stimulation by polyamines, and probably difference in substrate specificity [37]. The CK2α′ phosphorylates ribosomal acidic P-proteins and calmodulin but casein—typical substrate for this type of protein kinases is phosphorylated very poorly. The potential recognition site for both CK2α′ and CK2 holoenzymes seems to be similar to what is shown in Table 2. Both protein kinases recognise the sequences representing a requirement determined for casein kinase type 2 [60,61].

Table 1
Comparison of two forms of CK2 from *S. cerevisiae*

Quantity	Form of CK2[a]	
	CK2α′	CK2 holoenzyme
ATP, K_m (μM)	11.5	7.5
GTP, K_m (μM)	60	55
Optima		
PH	7.5	7.5
Mg^{2+} (mM)	10	8
Na^{+} (mM)	0	200
Protein substrates[b]		
80S ribosomes	110	231
rYP2A (rYP2B)	592 (324)	673 (548)
Total casein	4.2	396
Calmodulin (bovine)	867	49
Effectors[c]		
Heparin, K_i (μg/ml)	>20	0.1
DRB, K_i (μM)	>200	32
TBBt, K_i (μM)	0.1	0.7
SOD1, K_i (μM)	2.8	>50
Polyamines	No effect	Stimulation

[a] Protein kinase activity measurements were performed for 20 min at 30 °C in a medium (50 μl of final volume) containing 50 mM Tris–HCl buffer, pH 7.5, 10 mM Mg^{2+}, 20 μM [γ-^{32}P]ATP (specific radioactivity 500–1000 cpm/pmol), 4 U of appropriate enzyme, and 0.1–1.5 mg/ml of protein substrate.

[b] Incorporation of ^{32}P (picomolar per minute per milligram of protein substrate).

[c] Effectors influence on protein kinases was tested with 6 μg of rYP2A protein used as protein substrate in the reaction mixture.

Table 2
Phosphorylation of synthetic peptides by two forms of yeast protein kinase CK2

Peptide	Phosphorylation (pmol/sample)	
	CK2α′	CK2 (holoenzyme)
(A) RRREQASESEPTT	12.80	7.17
(B) RRRGKGDTEESLKT	0.94	0.49
(C) KKKEEESEEE	114.06	92.15

Phosphorylation of 5 nmol of synthetic peptides was carried out for 15 min under standard conditions. Phosphorylation was quantitated by the phosphocellulose paper procedure [51]. The triple basic residues at the N-terminus of each peptide are required for the phosphocellulose paper assay as described in Materials and methods.

As previously noted halogenated benzimidazoles and benzotriazoles, which are inhibitors competitive with respect to ATP, were found to be effective for CK2 but not CK1 [37,44,45]. As can be seen in Table 1 DRB has low effect on CK2α′ but TBBt shows strong potency in inhibition of both forms of the protein kinase.

Attention was then directed to the effect of the SOD protein shown in our previous work as an inhibitor of PK60S [48] on activity of both forms of yeast CK2 phosphorylating acidic protein substrates (casein, 80S ribosomes, and ribosomal P proteins). Therefore, we have examined whether yeast cytosolic dismutase inhibits phosphorylation of all five P1/P2 polypeptides and whether SOD inhibits both enzymes. The 80S ribosomes phosphorylated by CK2α′ or CK2 in the absence or presence of the SOD protein (Fig. 4, lanes B and C, respectively) were resolved by isoelectric focusing (Fig. 4A), followed by autoradiography (Figs. 3B and C). As shown, inhibition of phosphorylation by SOD applies only to free catalytic α′ subunit and is similar among all P1/P2 ribosomal proteins.

As previously shown, differences in activity of yeast CK2α′, depending on the growth phase of the cells were observed [48]. This observation was in close correlation between the state of phosphorylation of ribosomal P proteins isolated from logarithmic and diauxic shift phase cells and presence of the SOD1 protein.

Based on these observations, we presume that SOD1 protein may form some kind of regulatory complex with CK2α′. To confirm this presumption pure as well as combined α′ subunit of CK2 and dismutase (SOD1) were centrifugated in the 10–40% glycerol gradient (Fig. 5). Gradients analysis shows that CK2α′ interacts with SOD1 and forms a complex of molecular weight of ~73 kDa, which suggests its structure as a CK2α·$(SOD1)_2$ (Fig. 5A). Interestingly, the activity of the CK2α kinase in the complex is inhibited and this interaction can be inverted by addition of protein substrate, which suggests that both (P2B protein and SOD) interact with CK2α′ at the substrate interaction site. In the same gradient α′ subunit of CK2 migrates as a single protein of molecular weight of ~39 kDa (Fig. 5B) and dismutase migrates as a ~35-kDa dimer (Fig. 5A—position shown by arrow).

Discussion

In this study, we have obtained evidence for the existence of a cytoplasmic protein kinase specific for acidic ribosomal P-proteins from yeast (*S. cerevisiae*), which can be assigned as a free α′ subunit of CK2 on the basis of the following criteria. (a) The amino acid sequence of purified protein kinase shows identity with CK2α′ sequence from yeast. (b) The purified enzyme shares biochemical properties of typical protein kinase type 2 (CK2) with some exceptions, e.g., lack of casein phos-

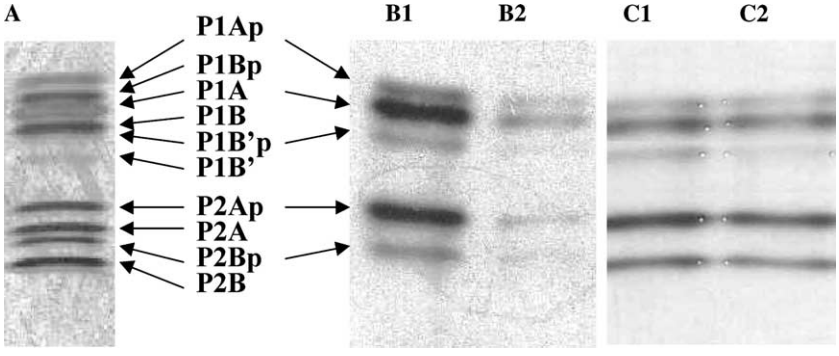

Fig. 4. Separation of yeast P-proteins on a polyacrylamide gel by isoelectric focusing. (A) Silver-stained yeast P proteins from the ribosome isolated from log cells. (B,C) Samples of 800 μg ribosomes isolated from logarithmic cells were phosphorylated by CK2α′ (B1 and B2) or CK2 holoenzyme (C1 and C2) in the absence (lanes 1) and in the presence (lanes 2) of 2 μg of the pure SOD protein as described in Materials and methods. Four units every CK2 form was used in each phosphorylation sample. Reaction was stopped after 20 min by addition of IEF-buffer containing 8 M urea. RNA was removed from ribosomal particles by addition of 2.5 μg of pancreatic RNAse for 10 min at room temperature and the total ribosomal proteins were directly subjected to isoelectrofocusing on the polyacrylamide gel (pH 2.5–5.0) The IEF-separated acidic proteins were silver-stained (A) and autoradiographed (B,C).

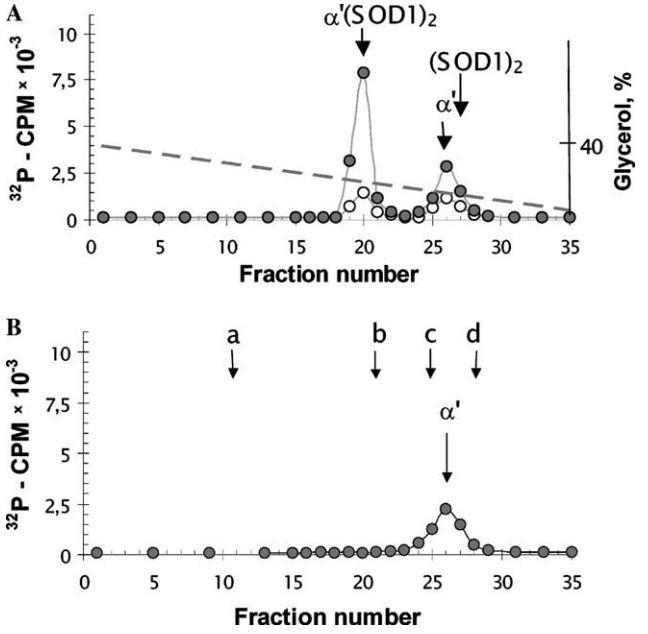

Fig. 5. Glycerol gradient centrifugation of the CK2α′ kinase (B) and the CK2α′ in the presence of the SOD1 protein (A). (A) Pure CK2α′ (0.12 mg) was mixed with 0.2 mg of SOD1 protein, incubated for 15 min in ice loaded on 10–40% glycerol gradient and ultracentrifugated 44 h as described in Materials and methods. Activity of kinase was measured at standard conditions using as a protein substrate 3 μg (○) and 9 μg (●) of recombinant ribosomal P2B protein in phosphorylation assay. The arrows denote positions of free SOD1 dimer, CK2α′, and complex formed by those two proteins. (B) Pure CK2α′ (0.12 mg) was incubated for 15 min in ice loaded on 10–40% glycerol gradient and ultracentrifugated. The arrows denote the positions of migration by a—dimer of phosphorylase b (97 kDa), b—bovine serum albumin (67 kDa), c—ovalbumin (43 kDa), and d—soybean trypsin inhibitor (20.1 kDa).

phorylation. (c) Activity of the ribosomal P-protein-specific kinase is not observed in the yeast strain lacking *CKA2*. (d) In addition, a catalytic activity of CK2α′ protein kinase is strongly inhibited by Cu,Zn-superoxide dismutase and together with this protein can form an inactive complex with possible structure CK2α′·(SOD1)$_2$.

Protein kinase CK2 is ubiquitously distributed in eukaryotic organisms where it appears to exist as a tetrameric complex consisting of two catalytic subunits (α_2 or αα′) and two regulatory subunits (β_2 or ββ′). Distinct isozymic forms of the catalytic subunit of CK2 have been identified in many organisms [36–38,47,58,59,62]. Only a single regulatory β subunit has been identified in humans, but multiple β and β′ forms have been identified in lower eukaryotes such as yeast [36]. This subunit was reported to play an important role in the assembly of tetrameric CK2 complexes, in enhancing the catalytic stability and activity of the whole enzyme, and in modulation of the substrate selectivity of CK2 [37,63,64]. In our report, we describe for the first time that CK2α′ can exist as a single and active protein. Although previous studies demonstrate that tetrameric CK2 complexes cannot be dissociated in vitro without denaturing agents [65]. Recent evidence from dynamic localisation studies of the individual subunits of CK2 provides indication of independent movements of CK2α and CK2β units within cells [66].

CK2 has been classified as a kinase independent of second messengers [43]. A number of compounds have been proposed to regulate CK2. Potential activators are basic oligoamines such as spermidine. In contrast to activators the inhibitors have acidic charged groups. One of them is heparin with $K_i = 1.4$ nM [43]. Several experimental evidences are consistent with a conclusion that both polyanionic inhibitors and polycationic activators regulate the activity of CK2 through CK2β subunit [63,67]. For example polyamines, known as in vitro activators of CK2, bind to the acidic cluster DLEPDEELED of β subunit (CK2β$^{55–64}$) [63]. This explains that lack of interaction of the CK2α′ with polyamines and heparin is due to lack of regulatory CK2β subunit. Enzyme shows only sensitivity to the modulators interacting at ATP binding site, e.g., TBBt.

For forms of CK2–CK2 holoenzyme and CK2a′, we propose alternative ways of regulation. In our previous paper, we have found correlation between phosphorylation of acidic ribosomal P-proteins and induction of copper–zinc superoxide dismutase in stress condition [48]. Until now SOD1 was known as one of the major constituents in protecting cellular components against reactive oxygen species. *S. cerevisiae* contains at least two distinct SOD enzymes: Mn-SOD, residing in the mitochondrial matrix, and Cu,Zn-SOD, localised mainly in the cytoplasm, nucleus, and in the mitochondrial intermembrane space. SOD1 has been shown to be required for maintaining the redox state of the cell [68], resistance to dioxygen [69], freeze–thaw tolerance [70], and stationary-phase survival [71], and protects iron–sulfur clusters of enzymes against inactivation due to superoxide-induced iron release [72]. In *S. cerevisiae*, level of SOD changes in the cell, depending on different stimuli, e.g., exposure to the redox active molecules or oxygen concentration in the medium [68].

In this report, we show that SOD protein may have a direct influence on activity of the protein kinase CK2α′ regulating translational activity of the ribosome. We have shown (Fig. 5) that in oxygen stress condition activity of the CK2α′ can be abolished by dimer of SOD1 protein and formation of inactive CK2α′·(SOD1)$_2$ complex of molecular weight ~73 kDa. One of the possible mechanisms of regulation of kinase activity may be similar to the way of regulation of CK2α/α′ catalytic subunit by regulatory CK2β subunit. SOD1 protein contains an amino acid cluster similar to those present in P1/P2 proteins and recognised by the CK2α′ [37,38]. Such "pseudosubstrate" sequences have been reported as autoinhibitory domains of protein kinases

[73] and are present in CK2β [63]. Maybe there are some other than SOD1 proteins present in different cell compartments regulating free CK2α and α′ catalytic subunits in the way similar to regulatory B′/B″ subunits of protein phosphatases type 2A [74].

From this perspective, it will be highly interesting to study in detail the structure and mechanism of regulation of CK2α′ structure with SOD1 protein and its influence on ribosomal stalk and protein synthesis machinery.

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