

Regulation of casein kinase 2 by phosphorylation/dephosphorylation

Patrizia AGOSTINIS,* Jozef GORIS,* ‡ Lorenzo A. PINNA† and Wilfried MERLEVEDE*

*Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, B-3000 Leuven, Belgium, and †Istituto di Chimica Biologica, Università di Padova, Padova, Italy

The effects of various polycation-stimulated (PCS) phosphatases and of the active catalytic subunit of the ATPMg-dependent (AMD_C) protein phosphatase on the activity of casein kinase 2 (CK-2) were investigated by using the synthetic peptide substrate Ser-Glu-Glu-Glu-Glu-Glu, whose phosphorylated derivative is entirely insensitive to these protein phosphatases. Previous dephosphorylation of native CK-2 enhances its specific activity 2–3-fold. Such an effect, accounted for by an increase in V_{max} , is more readily promoted by the PCS phosphatases than by the AMD_C phosphatase. The phosphate incorporated by auto-phosphorylation could not be removed by the protein phosphatases, suggesting the involvement of phosphorylation site(s) other than the one(s) affected by intramolecular autophosphorylation. The activation of CK-2 by the phosphatase pretreatment is neutralized during the kinase assay; the mechanism of this phenomenon, which is highly dependent on the kinase concentration, is discussed.

INTRODUCTION

Casein kinase 2 (CK-2) is a cyclic nucleotide-independent Ca^{2+} /calmodulin-insensitive protein kinase with a pronounced preference for acidic proteins, such as casein or phosvitin, over histones as model substrates. Its ubiquity among eukaryotic organisms and the recognition of several endogenous substrates, including among others the modulator subunit of the ATPMg-dependent protein phosphatase (DePaoli-Roach, 1984; Agostinis *et al.*, 1986; Holmes *et al.*, 1986), initiation factors (Hathaway & Traugh, 1979), RNA polymerase (Dahmus, 1981), troponin T (Pinna *et al.*, 1981), glycogen synthase (DePaoli-Roach *et al.*, 1981; Cohen *et al.*, 1982), the type 2 regulatory subunit of cyclic AMP-dependent protein kinase (Carmichael *et al.*, 1982; Hemmings *et al.*, 1982) and ornithine decarboxylase (Meggio *et al.*, 1984a), suggest the involvement of CK-2 in the integration of cellular metabolism. The enzyme isolated from most sources has an oligomeric structure consisting of α (or α') and β subunits combined in $\alpha_2\beta_2$ (or $\alpha\alpha'\beta_2$) tetramers; the 35–44 kDa α subunit displays the catalytic activity (Hathaway & Traugh, 1982). The smaller 24–28 kDa β subunit, which is rapidly auto-phosphorylated *in vitro*, presumably plays a regulatory role (Meggio & Pinna, 1984). CK-2 specifically recognizes serine and threonine residues *N*-terminal to clusters of acidic amino acids (Pinna *et al.*, 1986). Polyanions, such as heparin and polyglutamate (Hathaway *et al.*, 1980; Meggio *et al.*, 1983), strongly inhibit the enzyme activity *in vitro*, whereas polycations, such as protamine (Yamamoto *et al.*, 1979; Meggio & Pinna, 1984), spermine and spermidine (Cochet *et al.*, 1980; Hara & Hendo, 1982; Hathaway & Traugh, 1984), are stimulatory.

The mechanism controlling the activity of CK-2

in vivo, including the significance of the auto-phosphorylation of the β subunit, is still unknown. Autophosphorylation appears to be a common feature of most, if not all, protein kinases, and in some cases, e.g. phosphorylase kinase (King *et al.*, 1983; Ramachandran *et al.*, 1987), the type 2 cyclic AMP-dependent protein kinase (Nesterova *et al.*, 1981), the insulin-receptor tyrosine protein kinase (Rosen *et al.*, 1983) and Ca^{2+} -dependent protein kinase (Lou *et al.*, 1986), a relationship between autophosphorylation and activation has been demonstrated. It is therefore possible that auto-phosphorylation may control the activity of CK-2, too. Qi *et al.* (1986) have shown that the subunits of protein kinase NII, the nuclear counterpart of CK-2, are phosphorylated to various degrees, as disclosed by changes in isoelectrofocusing mobility upon treatment with alkaline phosphatase.

We have investigated the effect of different protein phosphatases (Merlevede, 1985), namely the polycation-stimulated (PCS_{HI} , PCS_M , PCS_L , PCS_C) phosphatases and the active catalytic subunit of the ATPMg-dependent (AMD_C) phosphatase, on native CK-2 as well as on autophosphorylation of the enzyme. CK-2 activity could be measured under dephosphorylating conditions with the synthetic peptide substrate Ser-Glu-Glu-Glu-Glu-Glu. This acidic peptide, reminiscent of the CK-2 phosphorylation site in glycogen synthase, is a very good substrate for CK-2 (Meggio *et al.*, 1984b; Marin *et al.*, 1986) and is not dephosphorylated by the PCS phosphatases nor by the AMD_C phosphatase (the present paper).

MATERIALS AND METHODS

Phosphorylase *b* (Fisher & Krebs, 1958), PCS_{HI} , PCS_M , PCS_L (Waelkens *et al.*, 1987) and PCS_C

Abbreviations used: PCS phosphatases, polycation-stimulated (PCS_{HI} , PCS_M or PCS_L : high-, medium-, low- M_r) phosphatases; PCS_C , catalytic subunit of the polycation-stimulated protein phosphatases; AMD_C , active catalytic subunit of the ATPMg-dependent protein phosphatase; CK-2, casein kinase 2.

‡ To whom correspondence should be addressed.

phosphatase (Ramachandran *et al.*, 1987) were prepared from rabbit skeletal muscle; the AMD_c phosphatase was prepared from dog liver (Goris *et al.*, 1984). *Escherichia coli* alkaline phosphatase type III-R (specific activity 44000 units/mg; 1 unit of enzyme hydrolyses 1 nmol of *p*-nitrophenyl phosphate/min at pH 10.4 and 37 °C) was obtained from Sigma. CK-2 [specific activity 400 units/mg; 1 unit incorporates 1 nmol of phosphate/min into casein (2 mg/ml)] at 37 °C, was isolated from rat liver cytosol (Meggio *et al.*, 1981). Whole casein was prepared as described by Mercier *et al.* (1968). The peptide Ser-Glu-Glu-Glu-Glu-Glu was generously given by Dr. F. Marchiori (Padova, Italy). [γ -³²P]ATP was obtained from Amersham International, Dowex 1-X8 ion-exchange resin from Bio-Rad, Sephadex G-50 from Pharmacia, and the standard molecular-mass markers used in SDS/polyacrylamide-gel electrophoresis were from Bethesda Research Laboratories.

Autophosphorylation of CK-2 was evaluated by incubating the enzyme (20–30 μ g/ml final concn.) at 30 °C for 20 min in 20 μ l of a medium containing 100 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, and 5 μ M- or 10 μ M-[γ -³²P]ATP (approx. 2000 c.p.m./pmol). The dephosphorylation of the autophosphorylated CK-2 was assayed directly or after blocking the ³²P-labelling with 1 μ g of heparin/ml, by a 100-fold dilution with unlabelled ATP or after thermal denaturation (5 min at 100 °C), incubating the radiolabelled substrate with 5 units (phosphorylase phosphatase units as defined by Yang *et al.*, 1980) of the different protein phosphatases/ml for 10 min at 30 °C in a final volume of 20 μ l. The incubation was stopped by addition of the electrophoresis sample buffer [final concns. 2% (v/v) SDS, 10% (v/v) glycerol, 1% 2-mercaptoethanol and 0.5 M-Tris/HCl, pH 8.3]. The samples were boiled for 3 min, followed by gel electrophoresis on vertical plates of 10%-polyacrylamide containing SDS, by the Laemmli (1970) method. The slabs were stained with 0.25% Coomassie Brilliant Blue, dried and autoradiographed for evaluation of the ³²P label incorporated into the CK-2. The autoradiograms were prepared with Agfa-Gevaert Curix RP1 X-ray film and intensifying screens. The phosphate incorporated was measured by cutting out the ³²P-labelled polypeptide and counting the radioactivity in scintillation fluid, or by densitometric analysis of the autoradiograms with an LKB Density Laser Ultrosan. CK-2 phosphatase activity was also measured after re-isolation of maximally autophosphorylated (in the presence of 100 mM-NaCl) CK-2 by Sephadex G-50 gel filtration, followed by dialysis against poly(ethylene glycol) 10000. The latter procedure was done to avoid any interference by the autophosphorylation medium in the dephosphorylation reaction or the re-incorporation of phosphate.

Casein kinase activity was routinely assayed at pH 7.5 with whole casein (2 mg/ml) as previously described (Meggio *et al.*, 1981). The phosphorylation of Ser-Glu-Glu-Glu-Glu-Glu was carried out at 30 °C in 50 μ l containing 100 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 100 mM-NaCl, 30 μ M-[γ -³²P]ATP (5000–20000 c.p.m./pmol), 0.5 mg of substrate/ml and the CK-2 concentrations as specified. ³²P label incorporated into the trichloroacetic acid-soluble peptide was determined by the procedure described by Kemp *et al.* (1975). The reaction was stopped by adding 30% (v/v) acetic acid (final concn.), and the radioactive peptide was centrifuged

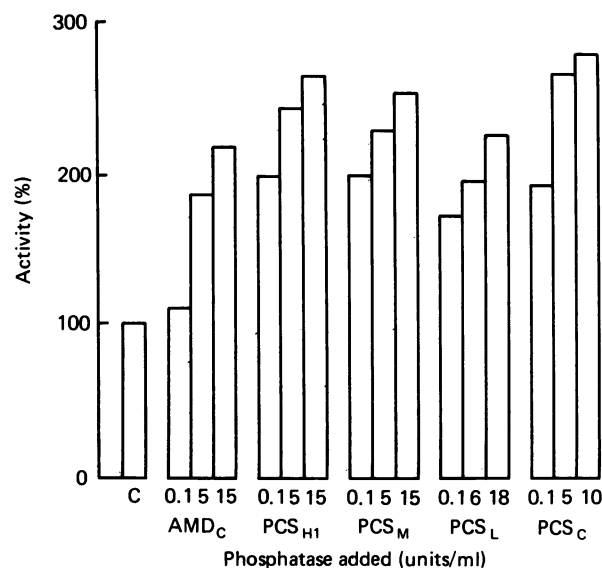


Fig. 1. CK-2 activation by protein phosphatases

CK-2 (20 μ g/ml) was preincubated for 10 min at 30 °C in the presence of the indicated concentrations of phosphatases in 10 μ l containing 20 mM-Tris/HCl, pH 7.4, 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml. Subsequently the CK-2 was diluted with 5 mM-Tris/HCl, pH 7.4, to give a concentration of 1.75 μ g/ml. The CK-2 activity was measured with the synthetic peptide as indicated in the Materials and methods section, for 10 min at 30 °C. Results are expressed as percentages of the control (C) activity.

(500–1000 *g*-min) through 1 ml of Dowex 1-X8 anion-exchanger equilibrated in 30% acetic acid. Thus the free ATP was tightly bound to the column, while the phosphopeptide is protonated and more than 90% could be recovered in the first wash (0.5 ml of 30% acetic acid) and the label counted.

When the phosphorylated peptide was used as a substrate in the phosphatase reactions, the acetic acid was evaporated after washing the column, and the sample was dissolved in the appropriate amount of 20 mM-Tris/HCl (pH 7.4)/0.5 mM-dithiothreitol, giving a ³²P-labelled peptide concentration of 1 mM. For the latter evaluation it was assumed that the specific radioactivity of the ³²P incorporated into the substrate was equal to that of the [³²P]ATP employed. The phosphatase activity toward the phosphorylated Ser-Glu-Glu-Glu-Glu-Glu peptide was checked by incubating 10 μ l portions of the phosphorylated substrate, at pH 7.4 and 30 °C for PCS and AMD_c protein phosphatases and at pH 8.4 and 37 °C for the alkaline phosphatase, in a total volume of 20 μ l for 10 min. After stopping the reaction by the addition of 1.2 ml of isobutanol/toluene (1:1, v/v) and 0.8 ml of a solution containing 5 mM-silicotungstate and 1 mM-H₂SO₄, the ³²P liberated was measured as described by Shacter (1984).

RESULTS AND DISCUSSION

In order to check whether the treatment with protein phosphatases could affect the activity of CK-2, the peptide Ser-Glu-Glu-Glu-Glu-Glu was employed as

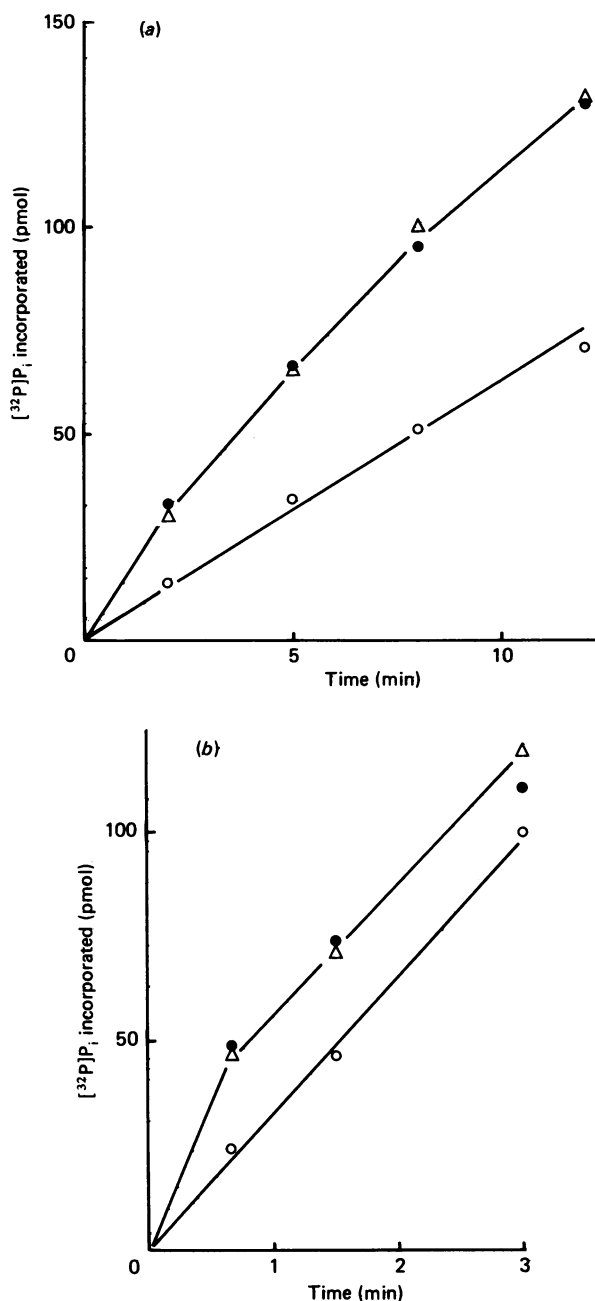


Fig. 2. Time course of the synthetic-peptide phosphorylation by protein phosphatase-activated CK-2 compared with untreated CK-2

CK-2 (*a*, 25 µg/ml; *b*, 50 µg/ml) was preincubated in the absence (○) or in the presence of 5 units of PCS_M/ml (△) or 5 units of AMD_C/ml (●) protein phosphatases for 10 min at 30 °C as in Fig. 1. After this preincubation, the CK-2 was diluted and phosphorylation medium was added to give a final enzyme concentration of 1.25 µg/ml (*a*) or 10 µg/ml (*b*). At the indicated time intervals, CK-2 activity was measured in 10 µl samples described in the Materials and methods section.

phosphorylatable substrate. This peptide was the substrate of choice for this kind of study as it is very readily phosphorylated by CK-2 but is totally unaffected by as much as 10 units of either PCS or AMD_C phosphatase/ml (results not shown). Consequently, with this peptide

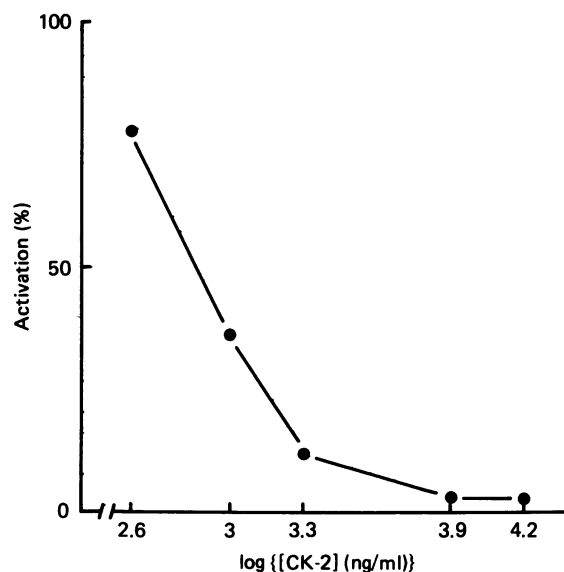


Fig. 3. Effect of the concentration of CK-2 on the activation by PCS_C protein phosphatase

Different concentrations of CK-2 (ranging from 67 to 2 µg/ml) were preincubated with 5 units of PCS_C phosphatase/ml for 10 min at 30 °C as specified in Fig. 1. After the pretreatment, the CK-2 was diluted 2-fold and the peptide phosphorylation medium was added to give the final indicated concentrations of the protein kinase. The phosphorylation reaction was carried out for 10 min at 30 °C and measured as described in the Materials and methods section.

the activity of CK-2 can be assayed even in the presence of high concentrations of protein phosphatases used to dephosphorylate CK-2.

From Fig. 1, showing the activity changes of CK-2 after preincubation with increasing concentrations of different protein phosphatases, it appears that all the protein phosphatases tested induce a 2–3-fold increase in protein kinase activity. Taking phosphorylase *a* as the substrate of reference, no clear specificity of the different protein phosphatases was observed. This observation is in contrast with the dephosphorylation of synthetic peptides and phosphatase inhibitor-1 (Agostinis *et al.*, 1987), deinhibitor protein (Goris *et al.*, 1986), modulator protein (Agostinis *et al.*, 1986) or autophosphorylated protein kinase C (Parker *et al.*, 1986), where large differences in specificity between the AMD_C and the PCS protein phosphatases, or even among the PCS phosphatases, were observed. Nevertheless, normalizing the activity of all phosphatases toward phosphorylase *a*, 0.1 unit of AMD_C phosphatase/ml is still ineffective, whereas the same amount of PCS phosphatases induces a significant activation. Addition of 50 mM-NaF, which inhibits the protein phosphatase without affecting the CK-2 activity, entirely prevents the activation of CK-2 (result not shown). An activation comparable with that induced by the protein phosphatases could also be obtained with alkaline phosphatase.

Time-course studies of synthetic-peptide phosphorylation by untreated CK-2 or by CK-2 preincubated with either AMD_C or PCS_M phosphatase are shown in Fig. 2. With low CK-2 concentrations (1.25 µg/ml), phos-

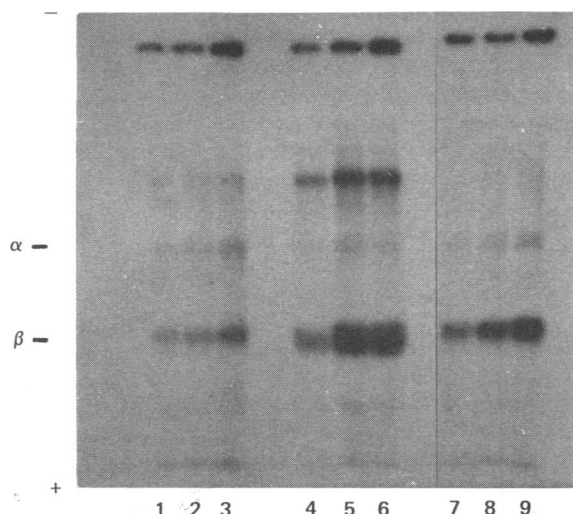


Fig. 4. Autoradiogram of the time course of CK-2 autophosphorylation without and with phosphatase pretreatment

CK-2 (44 $\mu\text{g}/\text{ml}$) was preincubated without (lanes 1–3) or with 1 unit of PCS_M phosphatase/ml (lanes 4–6), 5 units of AMD_C phosphatase/ml (lanes 7–9), in 30 μl of the preincubation medium for 10 min at 30 °C. Then 15 μl of this mixture was taken and added to the autophosphorylation medium (see the Materials and methods section) to give a final volume of 32 μl . After 20 min (lanes 1, 4, 7), 40 min (lanes 2, 5, 8) and 80 min (lanes 3, 6, 9) at 30 °C, 8 μl samples of the autophosphorylation mixture were taken and the reaction was stopped as described in the Materials and methods section. The phosphate incorporation into the β subunit of CK-2, estimated by densitometric analysis and expressed as arbitrary units, was as follows: control, 2.5, 5.4, 9.8; pretreated with PCS_M phosphatase, 8.7, 23, 21.9; pretreated with AMD_C phosphatase, 9.3, 12.3, 20.1.

phorylation is linear for at least 10 min for both the control and the phosphatase-treated enzyme (Fig. 2a). Under these conditions, activation by protein phosphatase was accounted for by an increment in V_{max} (from 297 to 512 nmol/min per mg) rather than by a decrease in K_m , which was unchanged (240 μM). With higher concentrations of CK-2 (10 $\mu\text{g}/\text{ml}$), however, stimulation by protein phosphatase was considerable only at short times of incubation (Fig. 2b): after 1 min the phosphorylation efficiency of phosphatase-treated CK-2 declines and tends to equal that of the control proceeding linearly (Fig. 2b).

Consequently, the extent of activation by the protein phosphatases, measured after a 10 min preincubation, is inversely proportional to the concentration of CK-2 (Fig. 3). This finding, together with the time-dependent decline in the effect of activation (Fig. 2b), are suggestive of some kind of interaction between CK-2 molecules, which could antagonize the activation induced by protein phosphatases. In particular, it was conceivable that re-incorporation of the phosphate removed from the β subunit by the protein phosphatases through (intra-molecular) CK-2 autophosphorylation (Meggio & Pinna, 1984) could account for the CK-2 deactivation after the phosphatase-induced activation. However, this is not the case, since the previously autophosphorylated β subunit of CK-2 is entirely resistant to the same

Table 1. Effect of protein phosphatase pretreatment on CK-2 autophosphorylation

CK-2 (48 $\mu\text{g}/\text{ml}$) was preincubated with the different protein phosphatases at the concentrations specified, for 10 min at 30 °C in a 20 μl volume as in Fig. 1. After the preincubation, 10 μl of the mixture was added to the autophosphorylation medium and the kinase activity was evaluated as described in the Materials and methods section. The ^{32}P content of the β subunit was estimated either by densitometric analysis of the autoradiogram, the control value being termed 100%, or by cutting out the radiolabelled polypeptide as described in the Materials and methods section.

Pretreatment	Phosphate incorporation into the β subunit of CK-2	
	Densitometric analysis (%)	mol of P/mol of CK-2
None	100	0.4
PCS_{H1} (6 units/ml)	160	0.64
PCS_M (2.5 units/ml)	340	1.52
PCS_L (7.5 units/ml)	200	0.96
PCS_C (6 units/ml)	300	1.44
AMD_C (6 units/ml)	320	1.6

phosphatases inducing activation (results not shown), thus ruling out the involvement of this phosphate incorporation in the deactivation mechanism. This crucial negative result was obtained repeatedly under various conditions by adding up to 5 units of the protein phosphatases/ml, before and after further radiolabelling of the β subunit of CK-2 had been blocked by different procedures including addition of 1 μg of heparin/ml, a 100-fold isotopic dilution with unlabelled ATP or thermal denaturation (5 min at 100 °C). There was no significant release of ^{32}P label by the phosphatases from the autophosphorylated CK-2 separated from ATPMg by gel filtration. Dephosphorylation of autophosphorylated CK-2 could be obtained by alkaline phosphatase in the presence of heparin (1 $\mu\text{g}/\text{ml}$), but the high phosphatase concentrations required for this observation could not be used in the presence of the synthetic phosphopeptide because of its sensitivity to alkaline phosphatase (result not shown). On the other hand, it should be noted that preincubation of CK-2 with protein phosphatases enables the kinase to undergo a much more pronounced autophosphorylation than the untreated enzyme (Fig. 4 and Table 1). This would indicate the presence in CK-2 of additional phosphorylation sites whose availability depends on previous treatment with phosphatases.

It is also possible that such a phosphatase-promoted phosphorylation occurs concomitantly to a polymerization of CK-2, resulting in inactive filamentous forms, in accord with the model proposed by Glover (1986). This would be consistent with the time- and concentration-dependence of the spontaneous CK-2 deactivation in the presence of protein phosphatases (Fig. 2), assuming that within the forming polymer the phosphorylated residues are no longer readily accessible to the phosphatases.

The data presented show unambiguously that the

activity of rat liver CK-2 can be substantially increased by incubation with PCS protein phosphatases and, albeit to a lesser extent, with ATPMg-dependent protein phosphatase, only under conditions allowing for dephosphorylation. Such an effect has been reproducibly obtained with five different preparations of CK-2. Some observed variability in the extent of stimulation might be accounted for by a different degree of phosphorylation of the native CK-2. Accordingly, the most responsive preparations appeared to be those with lowest intrinsic specific activity. Seemingly, the stimulatory effect of protein phosphatases is not due to the removal of the same phosphate(s) which can be incorporated *in vitro* into the β subunit by intramolecular autophosphorylation of CK-2, since the latter phosphate is fully resistant to the protein phosphatase treatments that are able to promote activation. It is therefore conceivable that other phosphate(s) incorporated *in vivo* and more readily susceptible to protein phosphatases are responsible for a down-regulation of CK-2.

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