Biochemical and Biophysical Research Communications **267,** 427–432 (2000)
doi:10.1006/bbrc.1999.1924, available online at http://www.idealibrary.com on IDEAL®

pCMB Treatment Reveals the Essential Role of Cysteinyl Residues in Conferring Functional Competence to the Regulatory Subunit of Protein Kinase CK2

Flavio Meggio,[1] Maria Ruzzene, Stefania Sarno, Mario A. Pagano, and Lorenzo A. Pinna
Dipartimento di Chimica Biologica and Centro di Studio delle Biomembrane del C.N.R., Università di Padova, Viale G. Colombo 3, 35121 Padua, Italy

Received November 14, 1999

To assess the functional role of the four conserved cysteinyl residues in the regulatory β-subunit of protein kinase CK2, the effect of pCMB and other reagents of sulfhydryl groups has been investigated. The pCMB-treated β-subunit has lost its ability to form either homodimers or regular $\alpha_2\beta_2$ heterotetramers with the catalytic subunit. It also fails to increase catalytic activity toward peptide substrates and to mediate the stimulatory effect of polylysine. The pCMB-treated β-subunit, however, is still able to prevent calmodulin phosphorylation and to physically interact with the α-subunit to form inactive complexes whose sedimentation coefficient is lower than that of CK2 holoenzyme. These inactive complexes upon treatment with reducing agents like DTT are converted into a fully active heterotetrameric holoenzyme. © 2000 Academic Press

Protein kinase CK2 (previously also termed "casein kinase-2") is one of the most pleiotropic protein kinases known so far: the continuously growing list of its substrates includes more than 160 proteins, many of which play relevant roles in signal transduction, gene expression and metabolic regulation (1). The striking variety of its targets in conjunction with a number of observations that correlate CK2 overexpression with neoplastic growth (2–6) and suggest the implication of host cell CK2 in viral infections (7) are rising increasing interest toward this ubiquitous and essential kinase [for review see 1, 7–9).

From a biochemical stand-point CK2 is intriguing in several respects: it uses GTP, besides ATP, as phosphate donor, it is extremely acidophilic in nature as it recognizes Ser/Thr and exceptionally also tyrosyl residues (10, 11) specified by numerous negatively charged side chains, and it displays high constitutive activity in the absence of second messengers and any other known effector. In most organisms and tissues CK2 is generally found as a stable heterotetramer composed by two catalytic (α and/or α′) and two non catalytic β-subunits (1, 12, 13), an observation which does not rule out the possibility that under some circumstancies the catalytic subunits may exist as isolated entities (14, 15).

The mode of regulation of CK2 is still enigmatic. A central question is in this respect the role of the β-subunit. At variance with other olygomeric protein kinases, such as PKA and CDKs, where the reversible equilibrium between the free catalytic subunits and the olygomeric holoenzyme plays a crucial role in turning on and off the catalytic activity, in the case of CK2 the holoenzyme is extremely stable and does not dissociate unless under denaturing conditions. Both the holoenzyme and the isolated catalytic subunits, moreover, are endowed with constitutive activity if tested with a variety of peptide and protein substrates, whereas in the case of PKA and CDKs only one of the two forms (the monomeric and the heterodimeric one, respectively) are active. Reconstitution experiments (16, 17) have shown that the β-subunit may exert opposite effects on the catalytic activity, depending on the phosphorylatable substrate. The negative regulation, which is especially evident with calmodulin and few other protein substrates whose phosphorylation is prevented by the β-subunit (17–19), is mediated by acidic residues clustered in the N terminal region of the molecule (17) which interact with basic residues of the catalytic subunit otherwise committed to substrate recognition (20). Positive regulation, which normally prevails over negative one especially with peptide substrates occurs through a mechanism which is still undecyphered and requires the integrity of the C-terminal part of the molecule (16). The C terminal domain is also required for tight interaction with the catalytic subunit (16, 21).

[1] To whom correspondence should be addressed at Dipartimento di Chimica Biologica, Viale G. Colombo 3, 35121 Padua, Italy. Fax: 39-049-8073310. E-mail: meggio@civ.bio.unipd.it.

0006-291X/00 $35.00

While the catalytic subunits alone exist as monomers, the β-subunits tend to dimerize, as judged from both the yeast two hybrid system approach (22–25) and chrystallographic analysis (26). In particular the solution of the crystal structure of a truncated form (1–182) of the β-subunit revealed the presence of a zinc finger mediating dimerization. The region involved is spanning between residues 105 to 146 with zinc ion tetrahedrically ligated to four highly conserved cysteinyl residues at positions 109, 114, 137, and 140. It is not clear from this picture, however, whether and to what extent β dimerization is a necessary prerequisite for functional association with α, all the more so because the C terminal segment responsible for high affinity interaction with the α-subunit was missing in the crystal structure of β (26).

To gain more information about this point and to assess the actual relevance of the four conserved cysteinyl residues in conferrring functional competence to the β-subunit we have examined the effect of reagents of sulfhydryl groups on the properties of CK2 β-subunit. The results of this study are reported here.

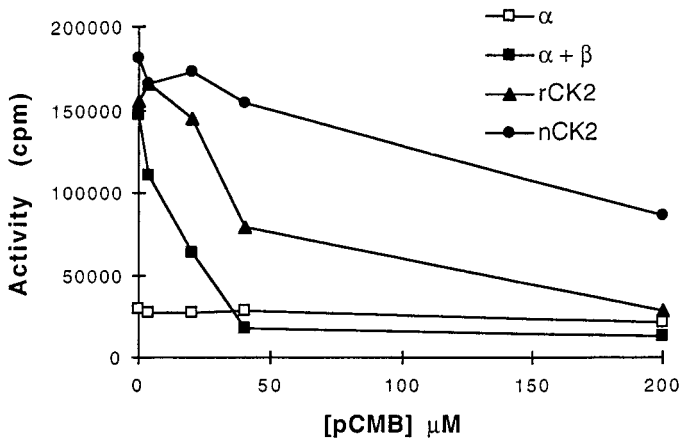

FIG. 1. Dose-dependent inhibition of CK2 activity by p-chloromercuribenzoic acid. The activity of rat liver CK2 holoenzyme (nCK2), recombinant CK2α and reconstituted CK2 holoenzyme treated with pCMB either during (α + β) or after reconstitution (rCK2) was assayed on the synthetic peptide substrate RRRAADSDDDDD (200 μM) in the presence of the indicated pCMB concentrations. Conditions are detailed under Materials and Methods.

MATERIALS AND METHODS

Materials. Recombinant calmodulin was kindly provided by Professor E. Carafoli (Zürich, Switzerland). Recombinant α and β subunits of human protein kinase CK2 were expressed in *E. coli* and purified as previously described (27). Native CK2 was isolated from rat liver cytosol and purified as described in (28). *p*-Chloromercuribenzoic acid (pCMB), *p*-chloromercuriphenylsulfonic acid (pCMPSA) and iodoacetamide (IAA) were from Sigma. *N*-Ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were from Boehringer.

Phosphorylation assay. Phosphorylation reactions were performed by incubating for 10 min at 37°C the phosphorylatable substrate at concentrations detailed in the figures in a medium containing 50 mM Tris/HCl pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl and 0.02 μM CK2 α subunit either alone or in the presence of equimolar amounts of CK2 β subunit. The reaction started with the addition of [γ-^{32}P]ATP (25 μM with a specific radioactivity of about 1000 cpm/pmol) and was stopped by putting the radiolabeled samples in ice. The phosphorylation of protein substrates was evaluated by subjecting samples to SDS–PAGE, staining and autoradiography or direct scanning on Instant Imager Apparatus (Canberra Packard). The radiolabeled peptide substrate was isolated and quantified by the phosphocellulose paper procedure (29).

Preparation of ^{32}P-labeled β subunit. 12.5 μg (0.5 nmol) of recombinant CK2 β subunit was phosphorylated by 60 min incubation under conditions described above with rat liver CK2 holoenzyme. The reaction was stopped by adding 0.5 ml of 30% acetic acid and [^{32}P]ATP was removed from the sample by ion exchange chromatography on Dowex-1 column before the lyophilization.

Treatment with pCMB of isolated CK2 α and β subunits. Samples of recombinant human CK2α and β subunits were incubated for 30 min in ice in the presence of 200 μM pCMB and then exhaustively dialyzed against 50 mM Tris-HCl, pH 7.5. Samples were also treated in parallel in the absence of pCMB as a control.

Sucrose gradient analysis. Linear gradients (4.2 ml) of sucrose 5–20% (w/v) were prepared in 50 mM Tris–HCl, pH 7.5, containing 500 mM NaCl and 0.01% NaN_3. Protein samples (100 μl) were loaded on the top of the gradient and centrifuged at 50,000 rpm in a Beckman SW60 rotor for 15 h. Twenty-three fractions were collected and monitored by either silver or Coomassie staining procedure and by autoradiography after PAGE/SDS. CK2 activity was checked on aliquots of the collected fractions by using synthetic peptide RRRAADSDDDDD (100 μM) as phosphorylatable substrate. Carbonic anhydrase (29 kDa) and bovine serum albumin (68 kDa) were run separately at the same conditions as molecular mass markers.

BIAcore analysis. A BIAcore X system was used to analyze molecular interactions by means of the surface plasmon resonance (SPR) phenomenon (30). CK2α was covalently linked to a Sensor Chip CM5 (BIAcore) (carboxymethylated dextrane surface) using amine coupling chemistry (31) as described in (32). A surface density of 5100 RU (resonance units) was generated. CK2β solutions (250 nM) were injected over the surface with a flow rate of 10 μl/min in running buffer HBS (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) at 25°C, in the absence or in the presence of 50 μM pCMB. At this concentration, pCMB alone does not evoke any SPR signal under these conditions. Surface was regenerated by injection of 10 mM NaOH (20 μl, flow rate of 30 μl/min). All samples were run simultaneously over a flow cell containing a blank surface (without any immobilized protein); each sensogram (time course of the SPR signal) was subtracted of the response observed in this control flow cell and normalized to a baseline of 0 RU.

RESULTS

To validate the functional relevance of the four conserved cysteinyl residues implicated in the formation of the zinc fingers which make the main intersubunit contact in the dimeric structure of CK2 β-subunit (26), the effect of a number of reagents of -SH groups on CK2 catalytic activity has been investigated, including among others *p*-chloromercuribenzoic acid (pCMB), *p*-chloromercuriphenylsulfonic acid (pCMPSA), *N*-ethylmaleimide (NEM), iodoacetamide (IAA), and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). It was found that treatment with pCMB (Fig. 1) and pCMPSA (not shown) promoted a dose-dependent inactivation of recombinant

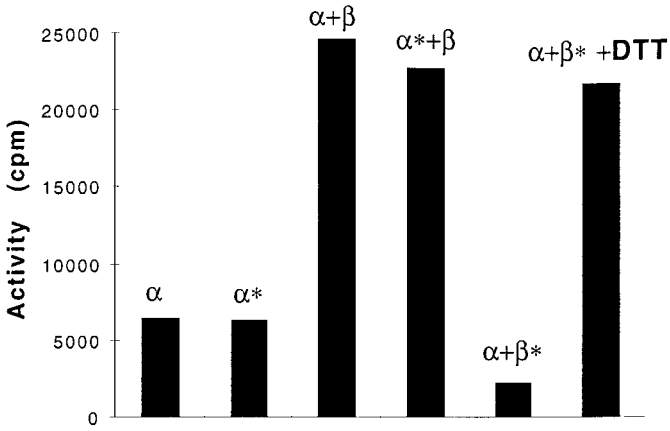

FIG. 2. The reversible effect of pCMB on CK2 activity is mediated by the β subunit. An asterisk denotes CK2 subunits which underwent pCMB treatment under conditions described under Materials and Methods. When present, DTT was 400 μM.

CK2 holoenzyme. The effect is especially dramatic if pCMB is added during the reconstitution of the holoenzyme, performed by mixing together equimolar amounts of the α and β subunits. Previously reconstituted holoenzyme is less susceptible to inactivation: 200 μM pCMB, however, causes a complete loss of activity under both conditions. In contrast native CK2 holoenzyme is much more refractory to pCMB treatment: its activity is only 50% lost after 200 μM pCMB treatment. As also shown in Fig. 1 the basal activity of the α subunit (which contains two conserved cysteinyl residues) is totally unaffected by pCMB, consistent with the concept that the target of pCMB effect is the β-subunit. This conclusion was strengthened by the experiment of Fig. 2 where CK2 α and β subunits were separately treated with pCMB prior to assemblance: it can be seen that the activity of the reconstituted holoenzyme is only lost if the β-subunit is pretreated with pCMB. Interestingly the residual activity is in this case even lower than that of the α subunit alone, either treated or untreated with pCMB, suggesting that pCMB treated β subunit exerts a negative effect on CK2α catalytic activity. Inactivation is fully reversed by subsequent DTT treatment which removes the pCMB adduct restoring cysteine in its reduced form.

The β-subunit of CK2 has a dual role (21, 33): while it increases catalytic activity toward peptide substrates and several protein substrates as well, it prevents the phosphorylation of certain protein substrates, with special reference to calmodulin. To check whether also this negative regulation by β-subunit was abolished by pCMB treatment the experiment of Fig. 3A was performed: clearly inhibition of calmodulin phosphorylation is quite evident with both untreated and pCMB treated β-subunit (compare lanes 2–3 and 5–6). This finding is in agreement with the above observation that pCMB treated β subunit also significantly reduces the phosphorylation of the peptide substrate (see Fig. 2). pCMB treated β-subunit, however, is severely defective in mediating the stimulatory effect of polylysine which promotes a striking phosphorylation of calmodulin by CK2 holoenzyme (17, 18) (compare lanes 4 and 7). Another known effect of polylysine is to prevent autophosphorylation of CK2 at its β-subunit, while inducing the autophosphorylation of the α-subunit (34). As shown in Fig. 3B pCMB treatment, which abolishes the stimulatory effect of β on the phosphorylation of peptide substrate (see Fig. 1) is only partially effective on the phosphorylation of the β-subunit itself (compare lanes 1–2 and 5–6), while it fully suppresses the polylysine induced autophosphorylation of α (compare lanes 3–4 and 7–8).

It has to be concluded from the data above that the modification of cysteinyl residues by pCMB suppresses the positive but not the negative regulatory potentials of the β-subunit and also reduces its ability to mediate the effects of polylysine. The finding that pCMB treated β-subunits still display their negative regulation and undergo appreciable autophosphorylation suggested that the modification of the cysteinyl residues might not entirely prevent association with the

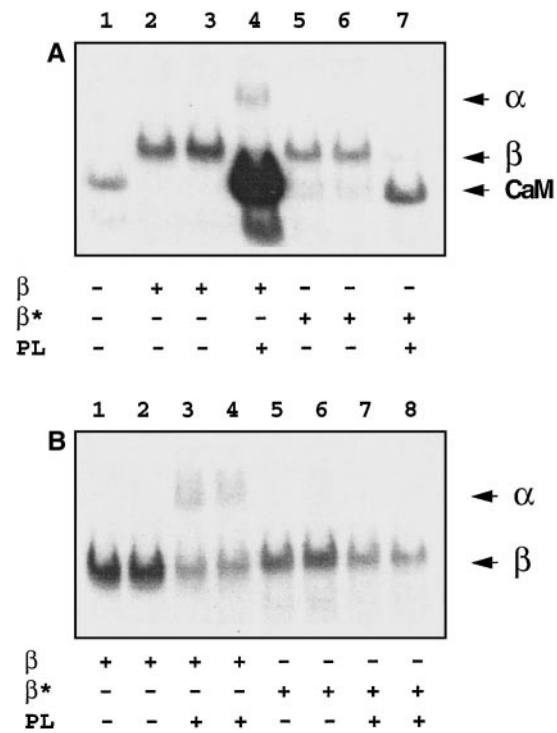

FIG. 3. Effect of pCMB treatment of the β subunit on calmodulin phosphorylation (A) and CK2 autophosphorylation (B). In A calmodulin (10 μM) was phosphorylated by CK2α subunit (2 pmol) either alone or in the presence of equimolar amounts of CK2β subunit under conditions described under Materials and Methods. The autoradiogram is shown. In B the autophosphorylation pattern of CK2 holoenzyme reconstituted *in vitro* by using either untreated or pCMB-treated β subunit (denoted by an asterisk) is shown. When indicated, polylysine was 420 nM.

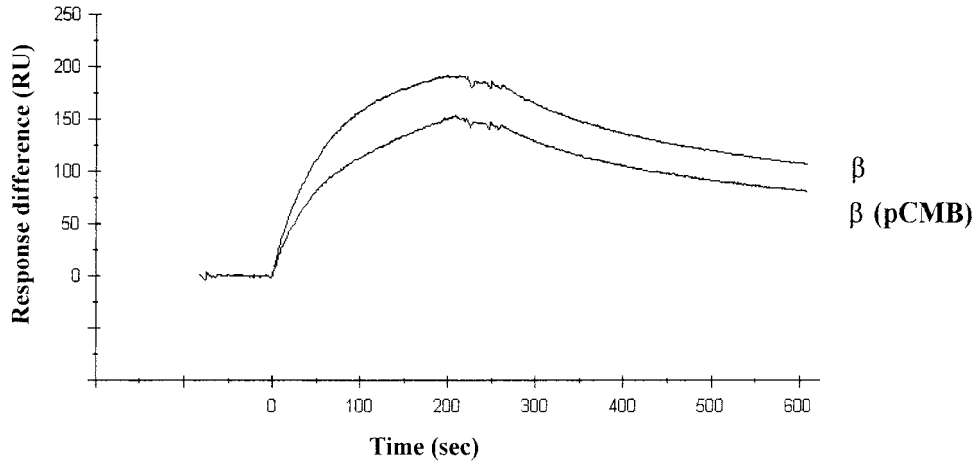

FIG. 4. Effect of pCMB treatment on CK2 α/β interactions detected by BIAcore analysis. Representative sensograms obtained by the injection of 35 μl of 250 nM CK2β in the absence (control) or in the presence (+pCMB) of 50 μM pCMB at a flow rate of 10 μl/min over a sensor surface containing 5100 RU of immobilized CK2α are shown. The response obtained with a control sensor surface (without CK2α) was subtracted to each sensogram. RU, resonance units.

catalytic subunit. This point of view was corroborated by plasmon resonance experiments in which the β-subunit, either unmodified or pCMB treated, was passed over the immobilized α-subunit. As shown in Fig. 4 in both cases the sensograms reveal a tight and high affinity interaction, although the pCMB treatment slightly reduces the SPR signal.

In order to get additional information about the residual ability of pCMB treated β-subunit to self-associate and to interact with the α-subunit a sucrose gradient ultracentrifugation analysis was performed (Fig. 5). As shown in Fig. 5A, unlike the regular homodimer β_2 formed by untreated β-subunit which peaks in fraction 17, pCMB treated β-subunit is recovered in fractions 14-15 denoting a higher sedimentation coefficient, consistent with formation of oligomers composed by more than two β-subunits. On the other hand the activity peak detected around fraction 12 upon ultracentrifugation of the regular $\alpha_2\beta_2$ heterotetramer (Fig. 5B) becomes negligible if the holoenzyme is reconstituted using pCMB treated β-subunit (Fig. 5C): in this case the low residual activity is spread in several fractions displaying lower sedimentation coefficient. Both the α and the β subunits are present in these fractions, with special reference to fraction 15, as judged from Western blots (not shown). If fraction 15 is treated with DTT the catalytic activity increases dramatically and upon resubmission to sucrose gradient ultracentrifugation it migrates to the same position as the regular holoenzyme, peaking in fraction 12 (see Fig. 5D). It has to be concluded therefore that the interaction between α and pCMB treated β-subunits gives rise to complexes smaller than the heterotetramer (presumably dimers $\alpha\beta$ and/or a mixture of $\alpha_2\beta/\alpha\beta_2$ trimers). These complexes, which are severely defective in catalytic activity can be readily converted to fully active regular heterotetramers upon removal of pCMB by DTT treatment.

DISCUSSION

The data presented here unambiguously show that many functional properties of CK2 β-subunit are either suppressed or deeply altered upon chemical modification of its cysteinyl residues by reagents of the sulfhydryl group, with special reference to pCMB, a compound widely used to assess the relevance of reduced cysteinyl residues in enzymes, included protein kinases (e.g., PKA, PKC and EGF-receptor kinase (35–37)). Upon pCMB treatment the β-subunit has lost its ability to generate the regular heterotetrameric holoenzyme $\alpha_2\beta_2$ and to exert its positive effect on the catalytic activity of the α-subunit. It is also unable to mediate the stimulatory effect of polylysine. These findings provide a biochemical validation to the observation, arising from the crystal structure of the β-subunit (26) that the four conserved cysteines play a crucial role in stabilizing a zinc finger which in turn is essential for β homodimerization. Consequently our data strongly support the view that regular homodimerization is a necessary prerequisite for functional competence of the β-subunit within CK2 holoenzyme.

pCMB-treated β-subunit is still able to aggregate, giving rise to oligomers larger than the β_2 dimer, and to associate with the α-subunit, generating complexes which conversely are smaller than the canonical holoenzyme. As judged from ultracentrifugation profiles these α-β complexes are heterogeneous and they may include substantial amounts of $\alpha\beta$ heterodimers. Once assembled into these anomalous complexes, pCMB-treated β subunit is still able to perform some of its

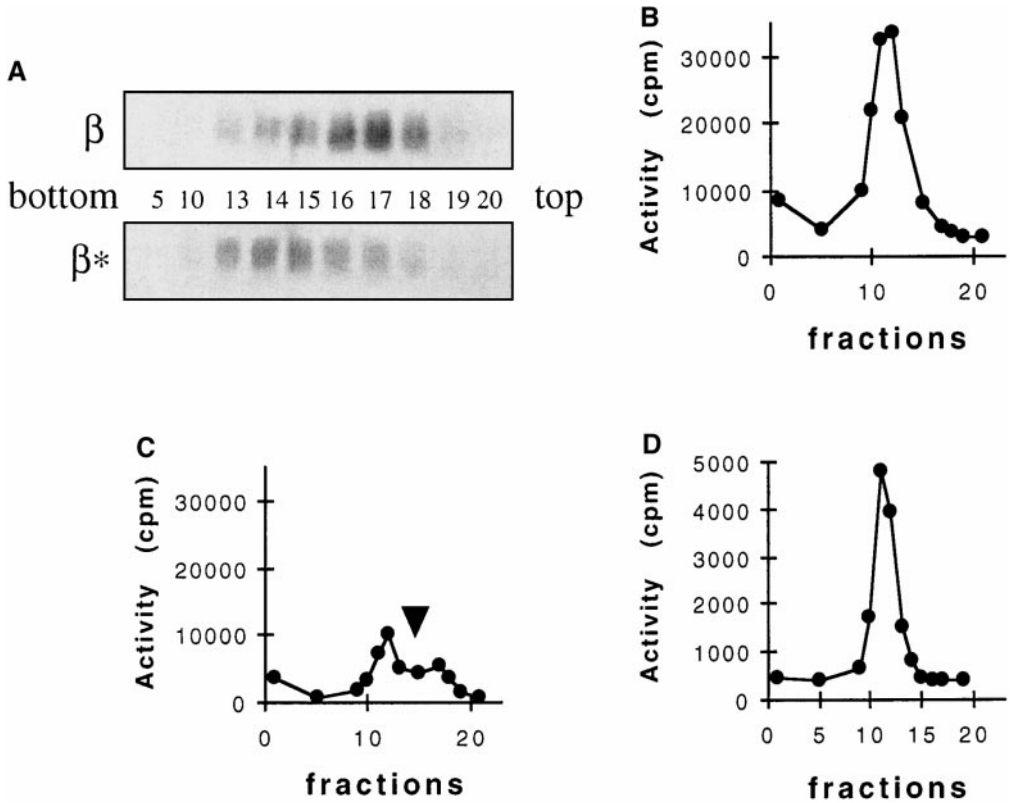

FIG. 5. Sucrose gradient ultracentrifugation of β subunit and reconstituted CK2 holoenzyme. Effect of pCMB. (A) 0.17 nmol of recombinant phosphoradiolabeled β subunit, prepared as described under Materials and Methods, was loaded on the top of a 5–20% sucrose gradient either before or after treatment with pCMB and subjected to ultracentrifugation under conditions described under Materials and Methods. Aliquots (50 μl) of the indicated fractions were analyzed by PAGE-SDS followed by autoradiography for the presence of ^{32}P-labeled β-subunit. (B and C) CK2 holoenzyme reconstituted *in vitro* by mixing 0.17 nmol of either untreated (B) or pCMB-treated β subunit (C) with equimolar amount of α was run under the same conditions as in A. CK2 activity was monitored in the collected fractions by using specific peptide substrate RRRAADSDDDDD. (D) 50 μl of fraction 15 from C (indicated by an arrow) was treated with 0.4 mM DTT and resubmitted to ultracentrifugation under the same conditions. CK2 activity was determined as in C.

functions, notably it prevents calmodulin phosphorylation as efficiently as untreated β subunit and it undergoes autophosphorylation, albeit to a lesser extent than untreated β.

The ability of pCMB treated β-subunit to interact both with itself and with the α-subunit did not come entirely as a surprise considering that a synthetic peptide (β[155–215]) reproducing the C-terminal region of the β-subunit displays a striking tendency to polymerize and to give multimeric complexes with the α-subunit (21) despite it spanning a region which is fully located downstream from the zinc finger (105–146). Those data and the present ones are also consistent with the indication, arising from the two-hybrid system analysis (24, 25) that tightly interacting elements and residues which attenuate this interaction, both located in the C-terminal moiety, cooperate to the formation of the canonical holoenzyme.

Interestingly the perturbing effects of pCMB on either structural or functional properties of CK2 β-subunit are readily reversed by reducing agents, like DTT and β-mercaptoethanol, which restore the cysteinyl residues in their -SH form. Especially striking is the finding that DTT converts an inactive low M_r complex formed by α and pCMB-treated β into a regular holoenzyme displaying both its normal basal activity and the expected sedimentation coefficient (see Fig. 5). These observations disclose the possibility that, as suggested for other protein kinases including PKA (35), PKC (36), and tyrosine protein kinases (37–39), also the activity of CK2 might be regulated through changes in the redox state of its sulfhydryl side chains. The first-choice candidate for performing this task would be redox enzymes of the protein disulfide isomerase family (PDI) exhibiting thiol dependent reductase activity (40). Our data suggest that in the case of CK2 the target for this kind of redox regulation would be the free β-subunit (whose presence in cells is well documented (12, 41)) rather than the β-subunit already incorporated into the holoenzyme. The recombinant holoenzyme in fact is less sensitive to pCMB treatment, at least *in vitro* (see Fig. 1). In this respect our data are in agreement with a previous study (42) showing the inhibition of recombinant CK2 holoenzyme by a number of thiol-sensitive chemicals. In the light of our data it is

likely that the cysteine residues affected in that study were not directly implicated in catalytic activity as suggested (42), but were essential for the functional competence of the regulatory β subunit. In this connection it may be worthy to note that native CK2 holoenzyme is much more refractory to pCMB than the recombinant holoenzyme generated by assembling the bacterially expressed α and β subunits. This provides a clearcut demonstration that recombinant CK2 holoenzyme is not indistinguishable from the native one, suggesting that the latter undergoes some kind of posttranslational processing which cannot take place in bacteria. Decyphering the structural features underlying these functional differences will also help to get e deeper insight into the enigmatic mode of control of CK2.

ACKNOWLEDGMENTS

This work was supported by grants (to L.A.P.) from CNR (97.03614.PS14 and T.P on Biotechnology), from MURST (PRIN 1997 to L.A.P. and F.M.), from AIRC, Italian Ministry of Health (Progetto AIDS, Istituto Superiore di Sanità), the European Commission (Biomed-2 BMH4-CT96-0047), and from the Armenise–Harvard Foundation.

REFERENCES

1. Pinna, L. A., and Meggio, F. (1997) *Prog. Cell Cycle Res.* **3,** 77–79.
2. Issinger, O.-G. (1993) *Pharmac. Ther.* **59,** 1–30.
3. Seldin, D. C., and Leder, P. (1995) *Science* **267,** 894–897.
4. Kelliher, M. A., Seldin, D. C., and Leder, P. (1996) *EMBO J.* **15,** 5160–5166.
5. Landesman-Bollag, E., Channavajahala, P. L., Cardiff, R. D., and Seldin, D. C. (1998) *Oncogene* **16,** 2965–2974.
6. Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A., and Oliviero, S. (1998) *J. Biol. Chem.* **273,** 21291–21297.
7. Guerra, B., and Issinger, O.-G. (1999) *Electrophoresis* **20,** 391–408.
8. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054,** 267–284.
9. Allende, C. C., and Allende, J. E. (1998) *J. Cell. Biochem. Suppl.* **30/31,** 129–136.
10. Wilson, L. K., Dhillon, N., Thorner, J., and Martin, G. S. (1997) *J. Biol. Chem.* **272,** 12961–12967.
11. Marin, O., Meggio, F., Sarno, S., Cesaro, L., Pagano, M. A., and Pinna, L. A. (1999) *J. Biol. Chem.* **274,** 29260–29265.
12. Bosc, D. G., Lùscher, B., and Litchfield, D. W. (1999) *Mol. Cell. Biochem.* **191,** 213–222.
13. Vilk, G., Saulnier, R. B., St. Pierre, R., and Litchfield, D. W. (1999) *J. Biol. Chem.* **274,** 14406–14414.
14. Heriché, J.-K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E. M., and Goldberg, Y. (1997) *Science* **276,** 952–955.
15. Stigare, J., Buddelmeijer, N., Pigon, A., and Egyhazi, E. (1993) *Mol. Cell. Biochem.* **129,** 77–85.
16. Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O.-G. (1993) *Biochemistry* **32,** 12672–12677.
17. Meggio, F., Boldyreff, B., Issinger, O.-G., and Pinna, L. A. (1994) *Biochemistry* **33,** 4336–4342.
18. Meggio, F., Boldyreff, B., Marin, O., Pinna, L. A., and Issinger, O.-G. (1992) *Eur. J. Biochem.* **204,** 293–297.
19. Bodenbach, L., Fauss, J., Robitzki, A., Krehan, A., Lorenz, P., Lozeman, F. J., and Pyerin, W. (1994) *Eur. J. Biochem.* **220,** 263–273.
20. Sarno, S., Vaglio, P., Marin, O., Meggio, F., Issinger, O.-G., and Pinna, L. A. (1997) *Eur. J. Biochem.* **248,** 290–295.
21. Marin, O., Meggio, F., Sarno, S., and Pinna, L. A. (1997) *Biochemistry* **36,** 7192–7198.
22. Kusk, M., Bendixen, C., Duno, M., Westergaard, O., and Thomsen, B. (1995) *J. Mol. Biol.* **253,** 703–711.
23. Gietz, R. D., Graham, K. C., and Litchfield, D. W. (1995) *J. Biol. Chem.* **270,** 13017–13021.
24. Boldyreff, B., Mietens, U., and Issinger, O.-G. (1996) *FEBS Lett.* **379,** 153–156.
25. Kusk, M., Thomsen, B., Bendixen, C., Issinger, O.-G., and Boldyreff, B. (1999) *Mol. Cell. Biochem.* **191,** 51–58.
26. Chantalat, L., Leroy, D., Filhol, O., Nueda, A., Benitez, M. J., Chambaz, E. M., Cochet, C., and Dideberg, O. (1999) *EMBO J.* **18,** 2930–2940.
27. Grankowski, N., Boldyreff, B., and Issinger, O.-G. (1991) *Eur. J. Biochem.* **198,** 25–30.
28. Meggio, F., Donella Deana, A., and Pinna, L. A. (1981) *J. Biol. Chem.* **256,** 11958–11961.
29. Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) *Anal. Biochem.* **87,** 566–575.
30. Malmqvist, M. (1999) *Biochem. Soc. Trans.* **27,** 335–340.
31. Johnsson, B., Lofas, S., and Lindquist, G. (1991) *Anal. Biochem.* **198,** 268–277.
32. Ruzzene, M., Brunati, A. M., Sarno, S., Donella-Deana, A., and Pinna, L. A. (1999) *FEBS Lett.*, in press.
33. Marin, O., Meggio, F., Boldyreff, B., Issinger, O.-G., and Pinna, L. A. (1995) *FEBS Lett.* **363,** 111–114.
34. Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O.-G. (1994) *J. Biol. Chem.* **269,** 4827–4831.
35. Saijoh, K., Inoue, Y., Katsuyama, H., and Sumino, K. (1991) *Pharmacol. Toxicol.* **68,** 96–99.
36. Inoue, Y, Saijoh, K., and Samino, K. (1988) *Pharmacol. Toxicol.* **62,** 278–281.
37. Woltjer, R. L., and Staros, J. V. (1997) *Biochemistry* **36,** 9911–9916.
38. Nakamura, K., Hori, T., Sato, N., Sugie, K., Kawakami, T., and Yodoi, J. (1993) *Oncogene* **8,** 3133–3139.
39. Monteiro, H. P., and Stern, A. (1996) *Free Radical Biol. Med.* **21,** 323–333.
40. Ferrari, D. M., and Söling, H.-D. (1999) *Biochem. J.* **339,** 1–10.
41. Lüscher, B., and Litchfield, D. W. (1994) *Eur. J. Biochem.* **220,** 521–526.
42. Zhang, P., Davis, A. T., and Ahmed, K. (1998) *J. Cell. Biochem.* **69,** 211–220.