Reconstitution of Normal and Hyperactivated Forms of Casein Kinase-2 by Variably Mutated β-Subunits†

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ABSTRACT: Twenty-one mutants of the noncatalytic β-subunit of human casein kinase-2 have been created, expressed in *Escherichia coli*, and purified to homogeneity. They are either modified at the autophosphorylation site (mutants βΔ1–4 and $\beta A^{5,6}$) or bear variable deletions in their C-terminal part (mutants βΔ209–215, βΔ194–215, βΔ181–215, βΔ171–215, βΔ150–215) or have undergone Ala substitutions for the acidic and basic residues which are concentrated in the sequences 55–70 and 171–180, respectively. All these mutants have been examined for their ability to functionally replace the wild type β-subunit. All substitutions and the deletions Δ1–4, Δ194–215, and Δ209–215 are compatible with effective binding of the catalytic α-subunit, as judged by sucrose density gradient analysis, stimulation of catalytic activity, and protection against thermal denaturation. Deletions Δ171–215 and Δ150–215, however, give rise to truncated molecules which are unable to associate with the α-subunit. The intermediate deletion Δ181–215 is still compatible with association, albeit the reconstituted holoenzyme exhibits an altered sedimentation coefficient. The holoenzymes reconstituted with substituted mutants $\beta A^{55,57}$, βA^{55-57}, and, to a lesser extent, βA^{59-61}, $\beta A^{63,64}$, and $\beta A^{5,6}$ display a basal activity which is higher (up to 4-fold) than that of the wild type holoenzyme. These data map two functional domains of the β-subunit: (1) a C-terminal segment which is essential for the association with the α-subunit, whose crucial element appears to be the overall conformation of the 171–180 sequence and (ii) an acidic N-terminal region including a number of carboxylic residues (notably Asp-55 and Glu-57) responsible for an intrinsic negative regulation of CK2 basal activity and possibly implicated in the responsiveness to various effectors.

Casein kinase-2 (CK2) is an ubiquitous and pleiotropic Ser/Thr specific protein kinase supposed to play a central role in cell regulation (reviewed by Pinna, 1990 and Tuazon & Traugh, 1991). While a multitude of protein targets for CK2 are known, many of which are involved in gene expression and cell proliferation, the mechanism by which this protein kinase is regulated remains undeciphered. In this connection the quaternary structure of CK2, composed by two catalytic (α and/or α′) subunits and two noncatalytic β-subunits, has attracted special attention. Unlike PKA, however, which is the only other known protein kinase sharing the same heterotetrameric structure, CK2 does not appear to undergo dissociation/activation inducible by physiological effectors; rather, dissociation under drastic conditions (Cochet & Chambaz, 1983) and reconstitution experiments (Grankowski et al., 1991; Filhol et al., 1991; Birnbaum et al., 1992) support the concept that the noncatalytic β-subunits are required for full activity toward most of the substrates tested with the intriguing exception of calmodulin whose phosphorylation by the recombinant α-subunit in the absence of polycationic effectors is indeed suppressed by the reconstitution of the $\alpha_2\beta_2$ tetramers (Meggio et al., 1992). This finding as well as the different activity spectra of α-subunit *vs* reconstituted holoenzymes toward a variety of peptide substrates supported the view that the β-subunit, besides conferring to the catalytic subunit a more ordered structure (Issinger et al., 1992) and an increased resistence to denaturing agents (Meggio et al., 1992a), can also alter its specificity, thus providing CK2 with a subtle regulatory device. In order to shed light on the mechanism by which the β-subunit affects the catalytic properties of CK2 and to dissect the molecular interactions between the α- and β-subunits a number of mutants of the β-subunit were created (Boldyreff et al., 1992). These mutants as well as additional ones have now been tested for their ability to display a number of known properties of the wild type β-subunit. This paper reports on these experiments, disclosing the existence in the β-subunit of distinct domains affecting in opposite manners the catalytic activity of the α-subunit.

MATERIALS AND METHODS

Materials. Heparin was from Serva and phosphocellulose paper (P81) from Whatman. [γ-^{32}P]ATP (2 mCi/mL) was purchased from Amersham. All other reagents were of the highest purity available.

Cloning and Expression of Mutants. Mutants with deletions at the N- or C-terminus were created by a PCR based method as described by Boldyreff et al. (1992) using the expression plasmid pBB4 (Grankowski et al., 1991) as a template. For mutants with amino acid exchanges between amino acids 55–70 and 171–178 the 0.58 kb *Eco*RI/*Hind*III fragment of expression plasmid pBB4 containing the coding region of human CK2 β cDNA from nucleotide 58 till the end was excised and cloned into vector M13. This construct was used for the oligonucleotide directed mutagenesis as described by Boldyreff et al. (1992). For mutant $\beta A^{5,6}$ a mutagenic primer representing the first 30 nucleotides of the coding region of the CK2 β subunit was used. This primer contained instead

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of the codons GAG coding for glutamic acids at positions 5 and 6 the codons GCG coding for alanine. The mutant $\beta A^{5,6}$ was created by PCR using a second primer complementary to the last 20 amino acids of the coding region of the β-subunit following the described protocol (Boldyreff et al., 1992). All mutated constructs were confirmed by dideoxy chain termination DNA sequencing of the entire coding region of the β-subunit (Sanger et al., 1977). Expression of the mutated CK2 β-subunits was done as described earlier (Grankowski et al., 1991). All mutants were purified to >95% homogeneity as judged by Coomassie Blue staining as described earlier (Boldyreff et al., 1992).

Phosphorylation of Synthetic Peptides. Peptides to be used for in vitro phosphorylation assays (RRRDDDSDDD, RRRAADSDDDDD, and RKMKDTDSEEEIR) were prepared by continuous flow solid-phase synthesis using an automatic synthesizer (Model 431A, Applied Biosystems). Kinase reactions were carried out in a total volume of 50 μL containing 50 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl (unless otherwise indicated), 0.3 mM peptide substrate, 20 μM [γ-^{32}P]ATP (specific activity 500–1000 cpm/pmol), and recombinant human CK2 α-subunit prepared as described by Grankowski et al. (1991) either isolated or previously mixed with β-subunit to give rise to variably reconstituted holoenzymes. The reaction was initiated by addition of ATP, incubated at 37 °C for 10 min, and stopped by cooling in ice. The P_i incorporated was evaluated following the phosphocellulose paper procedure (Glass et al., 1978).

Determination of Kinetic Constants. Kinetic constants of the α-subunit and various holoenzymes for the synthetic peptides RRRAADSDDDDD and RKMKDTDSEEEIR were determined from Lineweaver–Burk plots by linear regression analysis. Data were calculated on the average of three assays run independently.

Sucrose Density Gradient Analysis. A linear gradient (3.5 mL) of sucrose 5–25% w/w was prepared in 25 mM Tris-HCl, pH 8.5, containing 300 mM NaCl. Protein samples containing 30 μg of recombinant human CK2 α-subunit, prepared as described by Grankowski et al. (1991), and equimolar amounts of wild type or mutated CK2 β-subunit were loaded on top of the gradient and centrifuged at 48 000 rpm in a Kontron TST 60 rotor for 14 h at 4 °C. Twenty fractions were collected and monitored by Coomassie staining and enzyme activity as described by Grankowski et al. (1991).

Thermal Stability. Isolated α-subunit and variably reconstituted holoenzymes were preincubated at 45 °C for 0–10 min in 50 mM Tris-HCl pH 7.5 buffered solution. The samples were immediately ice-cooled, and the residual activity was determined as described above.

RESULTS

β-Subunit Mutants. The mutants of the β-subunit used in this study are shown in Table I. The mutants can be roughly divided into two categories: (1) Deleted mutants lacking either the two short stretches including the autophosphorylation site (Ser-2) and the site phosphorylated by $p34^{cdc2}$ (Ser-209), respectively, or larger segments from the C-terminal region, which has been shown by preliminary experiments (Boldyreff et al., 1992) to be essential for the reconstitution of the holoenzyme. (2) Substituted mutants in which alanyl residue(s) have been variably replaced either for glutamic and/or aspartic acids belonging to a highly conserved acidic sequence spanning between residues Asp-55 and Asp-70 or for charged residues located in the 171–178 segment. The interest for the 55–70 acidic stretch of the β-subunit was elicited by the observation that this segment is entirely lacking in the product of the stellate gene, a protein otherwise displaying a high degree of homology with the CK2 β-subunit (Livak, 1990) and by the finding that a synthetic peptide reproducing this sequence could either mimick or counteract some of the properties of the β-subunit (Meggio et al., 1992a). In addition a substituted mutant has been also constructed in which the acidic residues Glu-5 and Glu-6 supposed to act as specificity determinants for the autophosphorylation occurring at Ser-2/Ser-3 have been replaced by alanines.

Table I: Mutants of Human β-Subunit

1.	Δ1–4	deletion of residues 1–3 and Met replaced for Ser4
2.	Δ209–215	deletion of residues 209–215
3.	Δ194–215	deletion of residues 194–215
4.	Δ181–215	deletion of residues 181–125
5.	Δ171–215	deletion of residues 171–215
6.	Δ150–215	deletion of residues 150–215
7.	$A^{5,6}$	Ala replaced for Glu5 and Glu5
8.	A^{55-57}	Ala replaced for Asp^{55}, Leu^{56}, and Glu^{57}
9.	$A^{55,57}$	Ala replaced for Asp^{55} and Glu^{57}
10.	A^{55}	Ala replaced for Asp^{55}
11.	$A^{5}17$	Ala replaced for Glu^{57}
12.	A^{59-61}	Ala replaced for Asp^{59}, Glu^{60}, and Glu^{61}
13.	$A^{59-61,63,64}$	Ala replaced for Asp^{59}, Glu^{60}, Glu^{61}, Glu^{63}, and Asp^{64}
14.	$A^{63,64}$	Ala replaced for Glu^{63} and Asp^{64}
15.	A^{63}	Ala replaced for Glu^{63}
16.	A^{64}	Ala replaced for Asp^{64}
17.	A^{70}	Ala replaced for Asp^{70}
18.	A^{171}	Ala replaced for His^{171}
19.	A^{173}	Ala replaced for Glu^{173}
20.	A175	Ala replaced for Arg^{175}
21.	$A^{177,178}$	Ala replaced for Lys^{177} and Arg^{178}

Reconstitution of CK2 Holoenzyme from Mutants of the β-Subunit. In order to check whether and to what extent the mutants of the β-subunit were able to assemble with the α-subunit to give rise to canonical heterotetramers of the expected molecular mass, ultracentrifugation experiments were performed with equimolar mixtures of α-subunit plus variably mutated β-subunits. As outlined in Figure 1 under our conditions α-subunit alone is peaking, both in terms of activity and Coomassie staining (right side of Figure 1) at fraction 12 (sedimentation coefficient 4.5 S), while the activity peak is shifted to fraction 6 (sedimentation coefficient 7.0 S) upon addition of equimolar amounts of the β^{wt}-subunit. The PAGE/SDS patterns of Figure 1 provide the evidence that the higher sedimentation coefficient of CK2 activity is due to the reconstitution of the canonical $\alpha_2\beta_2$ tetramer since staining reveals the cosedimentation of the α- and β-subunits with the activity peak. It should be noted that this pattern is indistinguishable from that of native CK2 (indicated by arrow). By the same approach it could be shown that normal reconstitution of the holoenzyme was also observed with the shortly deleted mutants $\beta\Delta$1–4 and $\beta\Delta$209–215 lacking the autophosphorylation and the $p34^{cdc2}$ phosphorylation sites, respectively, and with the mutants $\beta A^{5,6}$, βA^{59-61}, $\beta A^{63,64}$, βA^{63}, βA^{64}, βA^{70}, βA^{171}, βA^{173}, βA^{175}, $\beta A^{177,178}$, and $\beta\Delta$194–215 whose patterns were superimposable to that of β^{wt} (Figure 1C). The previous finding that the activity of the holoenzyme reconstituted with $\beta A^{63,64}$ did not peak only at the position of the $\alpha_2\beta_2$ tetramer but was distributed along the gradient (Boldyreff et al., 1992) was arising from overloading of the tube. On the contrary the largely C terminal deleted mutants $\beta\Delta$150–215 and $\beta\Delta$171–215 proved totally uncapable to assemble with the α-subunit, even if added in an excess molar ratio (10:1) as judged by their failure to shift the α-subunit catalytic activity along the ultracentrifugation gradient (Figure 1D). A somewhat intermediate behavior was displayed by the $\beta\Delta$181–215 mutant, whose addition to the α-subunit at

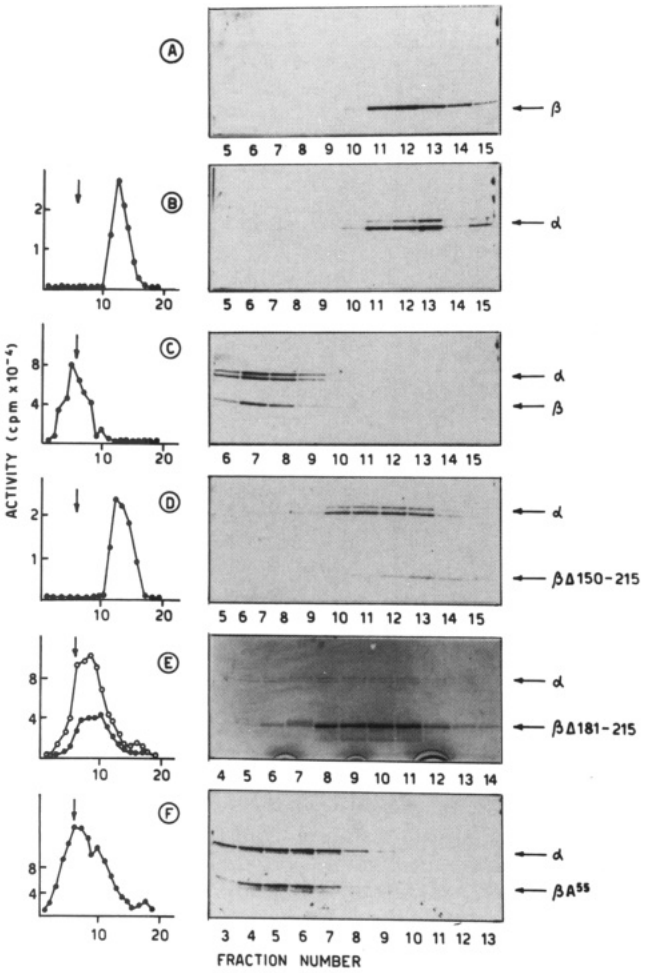

FIGURE 1: Sucrose density gradient analyses of CK2 holoenzymes reconstituted by combining α-subunit with wild type and mutated β-subunits. Ultracentrifugation conditions are described under Materials and Methods. (A) isolated wild type β-subunit; (B–F) α-subunit either alone (B) or combined at a 1:1 ratio with wild type β-subunit (C), mutant $\beta\Delta$150–215 (D), mutant $\beta\Delta$181–215 at 1:1 (●) and 1:10 (○) molar ratio (E), and mutant βA55 (F). Left panels: the kinase activity profiles with the synthetic peptide RRRDDDSDDD as a substrate are shown. The arrows indicate the position of native CK2 holoenzyme. Right panels: Coomassie staining of PAGE/SDS analysis. In (E) only the pattern corresponding to a 1:10 ratio is shown. The sedimentation coefficient of the activity peaks, using marker proteins of known molecular mass, was found to be 4.5 S for recombinant α-subunit and for its association with either $\beta\Delta$171–215 (not shown) or $\beta\Delta$150–215. A value of 7.0 S was determined for all other reconstituted CK2 holoenzymes with the only exception of mutant $\beta\Delta$181–215 whose association with α-subunit shows an activity peak at 5.8 S.

a 1:1 ratio and, even more effectively, 10:1 molar ratio does clearly promote the formation of catalytic entities with sedimentation coefficients higher than that of the α-subunit alone and composed by both α and $\beta\Delta$181–215 (see Figure 1E). In this case however the reconstituted holoenzyme still displays a sedimentation coefficient (5.8 S) lower than that of the canonical holoenzyme (7.0 S). Such a value is too low for being entirely accounted for by the lower molecular mass of the truncated β-subunit (21 kDa instead of 25 kDa) and indeed the maize α-subunit which is 50 amino acids shorter than the human α-subunit combines with the human β-subunit to give a complex with the same sedimentation coefficient (Boldyreff et al., 1993). It is more reasonable to assume therefore that $\beta\Delta$181–215 gives rise to dimeric rather than tetrameric forms of CK2. The only substitutions, among the ones tested, that seem to significantly alter the reconstitution of CK2 holoenzyme are those affecting the acidic residues in the 55–64 sequence since the mutants $\beta A^{55,57}$, βA^{55}, βA^{57},

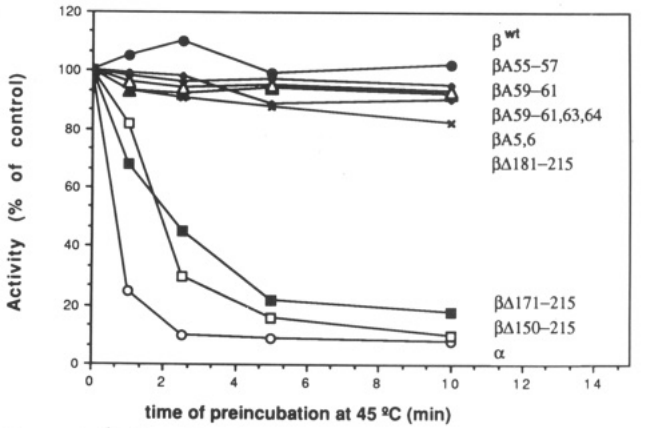

FIGURE 2: Effect of wild type and mutated β-subunits on thermal inactivation of the catalytic α-subunit. Assay conditions are described under Materials and Methods. The residual activity was normalized assuming 100% as the activity of the untreated sample. The full protection shown by wild type β-subunit was also observed with $\beta\Delta$1–4, $\beta A^{55,57}$, βA^{55}, βA^{57}, $\beta A^{63,64}$, βA^{63}, βA^{64}, βA^{70}, βA^{171}, βA^{173}, βA^{175}, $\beta A^{177,178}$, $\beta\Delta$209–215, and $\beta\Delta$194–215.

and $\beta A^{59-61,63,64}$ give rise to holoenzymes whose ultracentrifugation pattern, albeit reminiscent of those of canonical holoenzyme, displays a shoulder of activity at a position intermediate between those of CK2 heterotetramer and the free α-subunit (shown in Figure 1F for βA^{55}).

Thermostability. Increased thermostability is among the features of CK2 holoenzyme *vs* isolated α-subunit (Meggio et al., 1991a). As shown in Figure 2 the prompt heat inactivation of the α-subunit can be markedly counteracted by addition of equimolar amounts not only of β^{wt}-subunit but also of a number of β mutants with either Ala for Asp/Glu substitutions or short deletions which proved more or less effective in the reconstitution experiments as well (see Figure 1). The behavior of β mutants with large C-terminal deletions is also in good agreement with the reconstitution experiments since neither $\beta\Delta$150–215 nor $\beta\Delta$171–215, both unable to assemble with the α-subunit (see Figure 1D), efficiently protect the α-subunit against heat inactivation (Figure 2), while an almost full protection is afforded by $\beta\Delta$181–215 which also displayed reconstitution capability. These data corroborate the concept that unlike the 171–215 deletion, the ten residues shorter deletion 181–215 consents the assembly with the α-subunit, although not to a canonical tetramer as compared to the full length β-subunit. Point mutations in the 171–181 sequence, however, did not impair the ability of the β-subunit to protect against thermal inactivation (not shown).

Variable Activation by Mutants of the β-Subunit. Both dissociation (Cochet & Chambaz, 1983) and reconstitution experiments (Grankowski et al., 1991; Filhol et al., 1991; Birnbaum et al., 1992) would indicate that the β-subunit is required in order to achieve optimal CK2 activity toward most substrates tested, with the only notable exception of calmodulin (Meggio et al., 1992). Thus an increased catalytic activity of the α-subunit upon addition of equimolar amounts of the β-subunit provides one of the best proofs that a functional reconstitution of the enzyme has taken place. The entity of such an increase of activity depends on several experimental conditions, including the nature of the phosphorylated substrate, the presence of salt, and the concentration of Mg^{2+}. Figure 3 shows the results of a representative experiment by which the stimulatory power of each mutant has been systematically evaluated under conditions allowing a 9-fold stimulation of catalytic activity by addition of equimolar amounts of wild type β subunit to the α-subunit (i.e., 12 mM $MgCl_2$, no NaCl, and CaM-peptide as substrate). From the

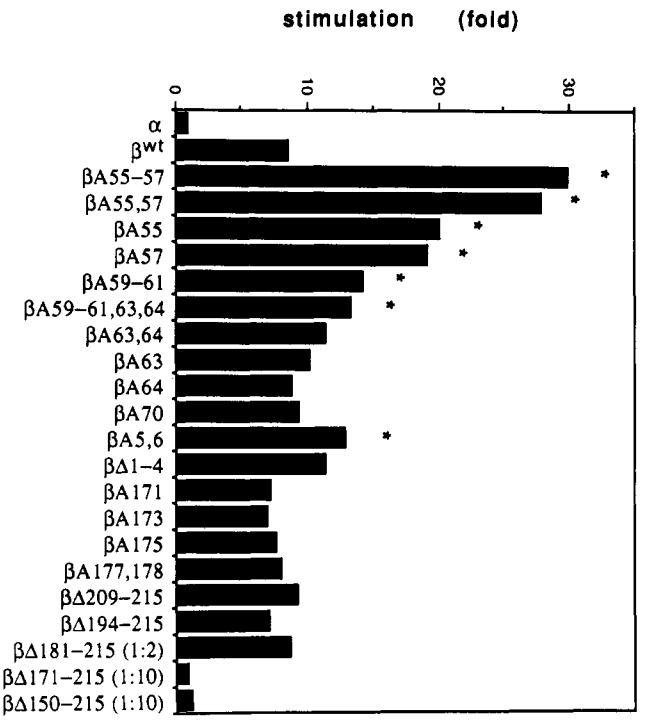

FIGURE 3: Stimulation of the α-subunit catalytic activity by β^{wt} and variably mutated β-subunits. Phosphorylation conditions are described under Materials and Methods except for the omission of NaCl. The calmodulin peptide RKMKDTDSEEEIR was used as substrate. The asterisks denote mutants exhibiting stimulatory activity substantially higher than that of β^{wt}.

data of Figure 3 it is clear that the truncated forms βΔ150–215 and βΔ171–215 (already shown to be unable to reconstitute the holoenzyme (Figure 1)] also fail to stimulate the catalytic activity even if added in 10-fold molar excess. The truncated form βΔ181–215 is conversely as effective as β^{wt} if added in 2:1 molar excess, while the mutants with short N-(Δ1–4) and C-terminal (Δ209–215) deletions are even slightly more effective than the wild type, if added in equimolar amount. The mutants bearing Ala substituted for charged residues in the 171–178 region are as effective as β wild type in stimulating catalytic activity. On the other hand, the Ala for Glu/Asp substituted mutants within the 55–70 region, with the exception of the singly substituted mutants βA^{63}, βA^{64}, and βA^{70} tend to be more effective than β^{wt} itself as stimulators of CK2 activity once added to the α-subunit in equimolar amounts. Such a "hyperstimulation" (denoted by an asterisk in Figure 3) is especially evident if Asp-55 and/or Glu-57 are replaced by alanine. The triple mutation of Asp-59, Glu-60, and Glu-61 and the quintuple mutation of Asp-59, Glu-60, Glu-61, Glu-63, and Asp-64 are less effective in this respect. Hyperactivation is also observed with the doubly substituted mutant $\beta A^{5,6}$. It should be mentioned that the hyperstimulatory properties of βA^{55-57}, $\beta A^{55,57}$, βA^{55}, and βA^{57} are also evident under conditions different from those of Figure 3 as far as magnesium concentration and peptide substrate is concerned, i.e., 2 mM Mg^{2+} and RRRDDDSDDD as a substrate. A scrutiny of these data highlights Asp-55 and Glu-57 as those residues whose replacement with Ala especially reinforces the stimulatory properties of the β-subunit. Also the acidic residues at positions 5,6, 59–61, 63, and 64, however, appear to contribute to some kind of negative regulation of CK2 catalytic activity since their double and triple substitutions by alanine invariably confer stimulatory properties more pronounced than that of wild type β-subunit.

In order to find out more about the observed hyperactivation, kinetic parameters were determined using different peptide substrates. Table II shows that the alteration of kinetic constants induced by wild type holoenzyme reconstitution is depending on the type of synthetic peptide substrate used, e.g., K_m 50 *vs* 62.5 μM with RKMKDTDSEEEIR and 13.6 *vs* 5.5 μM with RRRAADSDDDDD. Using another peptide substrate (RRRDDDSDDD) Grankowski et al. (1991) determined a K_m value for the α-subunit of 240 μM and for the holoenzyme of 80 μM. Irrespective to the influence of the peptide substrate the kinetic constants of Table II show that the higher activity displayed by CK2 holoenzymes reconstituted with the "hyperactivating" mutants $\beta A^{55,57}$ and βA^{55-57} is almost entirely accounted for by increased V_{max} values, whereas the K_m values are not significantly different from those of the holoenzymes reconstituted with either β^{wt} or mutant βA^{70}.

Table II: Kinetic Constants for Variably Reconstituted Holoenzymes[a]

enzyme	K_m (μM)	V_{max} (nmol/min/mg)	efficiency (V_{max}/K_m)
(1) Peptide RKMKDTDSEEEIR			
α	50.0	6.6	0.132
α + βwt	62.5	25.7	0.411
α + βA55,57	86.9	112.7	1.296
α + βA55–57	86.9	110.5	1.271
α + βA70	48.3	26.0	0.538
(2) Peptide RRRAADSDDDDD			
α	13.6	72.7	5.345
α + βwt	5.5	176.7	32.127
α + βA55,57	4.5	303.0	67.333
α + βA55–57	4.5	254.5	56.555
α + βA70	6.1	147.8	24.229

[a] Phosphorylation conditions are described under Materials and Methods except for the omission of NaCl.

Responsiveness to NaCl. Stimulation by 100–200 mM NaCl is a well established property of native CK2 (Meggio et al., 1986). Conversely NaCl inhibits the activity of the isolated α-subunit (Grankowski et al., 1991) as well as of a monomeric form of maize CK2 termed CKIIB (Dobrowolska et al., 1987) and also of recombinant maize CK2-α (Boldyreff et al., 1993). As shown in Figure 4 the addition of equimolar amounts of the β-subunit alters the responsiveness of the α-subunit to NaCl, restoring the normal activation curve observed with the native holoenzyme. As expected the truncated forms of the β-subunit that are unable to reconstitute the holoenzyme, βΔ150–215 and βΔ171–215, also fail to affect the responsiveness of α-subunit to NaCl inhibition. Only eight out of 21 mutants, namely βA^{64}, βA^{70}, βA^{171}, βA^{173}, βA^{175}, $\beta A^{177,178}$, βΔ194–215, βΔ209–215 are similar to the wild type β-subunit, for their capability to subvert the NaCl effect from inhibition to stimulation. The other mutants, with special reference to those that are capable to induce hyperstimulation (see preceding paragraph), display somewhat intermediate properties in that they prevent or reduce inhibition by NaCl without, however, conferring the typical susceptibility to NaCl stimulation observed with the native and wild type reconstituted holoenzyme. Consequently with these mutants the activity of CK2 is only slightly affected by NaCl up to 100 mM (Table III). It should be noted however that due to the different extent of extra-activation afforded by either NaCl (1.5–2 fold, see Figure 4) and Ala for Asp^{55} and Glu^{57} mutations (up to 4-fold, see Figure 3), the holoenzyme reconstituted with hyperstimulating $\beta A^{55,57}$ mutant is still more active than the wild type even in the presence of 100 mM NaCl, as outlined in Figure 5. Consequently the effect of NaCl is just that of reducing, without abolishing, the extent of extrastimulation. The finding however that the favorable effects of "hyperactivating" mutations and of NaCl are not

Table III: Effect of NaCl (100 mM) on CK2 Holoenzyme Activity[a]

enzyme	activity (% of the control in the absence of salts)	enzyme	activity (% of the control in the absence of salts)
1. $\alpha + \beta$A171	153	13. $\alpha + \beta$A63	117
2. $\alpha + \beta$A70	151	14. $\alpha + \beta$A55	113
3. $\alpha + \beta$A70	150	15. $\alpha + \beta\Delta$181–215	100
4. $\alpha + \beta\Delta$209–215	150	16. $\alpha + \beta$A57	97
5. $\alpha + \beta$A175	146	17. $\alpha + \beta$A55–57	96
6. $\alpha + \beta$A177,178	143	18. $\alpha + \beta$A55,57	93
7. $\alpha + \beta$D194–215	139	19. $\alpha + \beta$A59–61	92
8. $\alpha + \beta$A173	135	20. $\alpha + \beta$A59–61,63,64	86
9. $\alpha + \beta$A64	133	21. $\alpha + \beta\Delta$150–215	42
10. $\alpha + \beta$A63,64	124	22. $\alpha + \beta\Delta$171–215	42
11. $\alpha + \beta$A5,6	121	23. α	39
12. $\alpha + \beta\Delta$1–4	117		

[a] Data are drawn from experiments similar to those shown in Figure 4. The mutants are listed according to their decreasing (1) sensitivity to NaCl stimulation and increasing (23) sensitivity to NaCl inhibition.

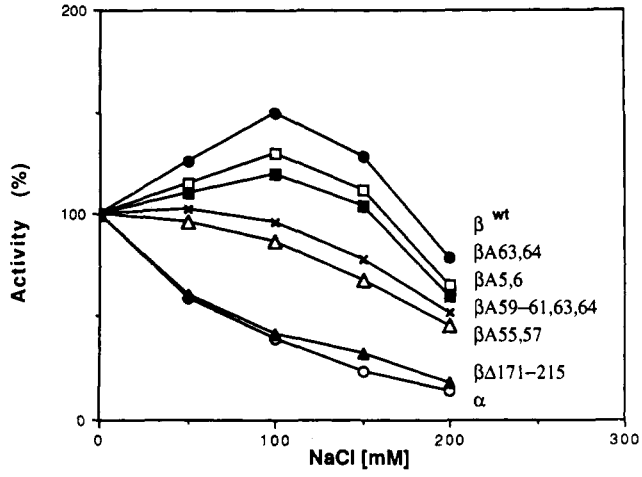

FIGURE 4: Effect of increasing NaCl concentration on the activity of α-subunit after association with variably mutated β-subunit. Phosphorylation conditions are described under Materials and Methods using the peptide RRRDDDSDDD as a substrate. The activity values are normalized assuming as 100% the activity observed in the absence of NaCl. Only a few representative mutants are shown. The effect of 100 mM NaCl on the activity of holoenzymes reconstituted with the other mutants is shown in Table III.

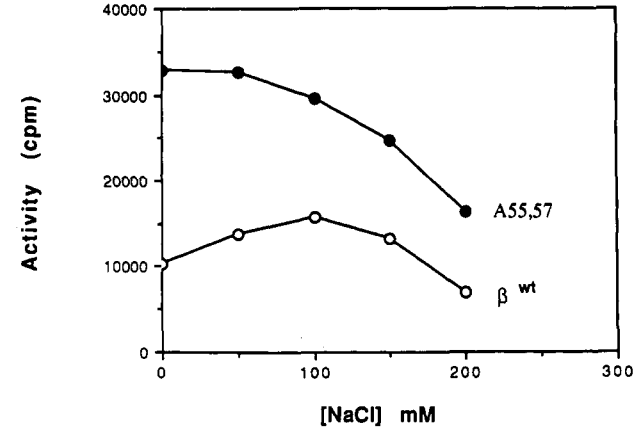

FIGURE 5: Decrease of the hyperactivating effect of βA55,57 by increasing NaCl. CK2 holoenzymes reconstituted with either β^{wt} (○) or βA55,57 (●) were assayed for their responsiveness to increasing NaCl concentration as described in Materials and Methods, using the peptide RRRDDDSDDD as a substrate.

additive may suggest that they occur through at least partially overlapping mechanisms.

Responsiveness to Heparin. Heparin, a powerful inhibitor of CK2, has been shown to interact with the α-subunit at a

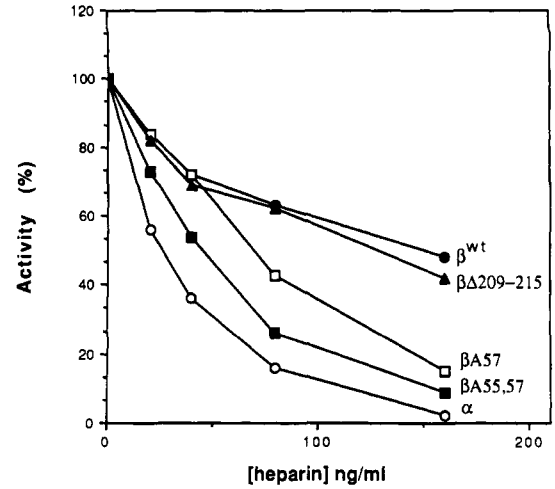

FIGURE 6: Inhibition by heparin of CK2 α-subunit and variably reconstituted CK2 holoenzymes. Phosphorylation conditions are described under Materials and Methods using the peptide RRRDDDSDDD as substrate. The activity values were normalized assuming as 100% the activity in the absence of heparin.

region of the catalytic domains which includes an insert of several basic residues, since the mutation of two such residues reduced the responsiveness to heparin (Hu & Rubin, 1990). Our finding, presented above, that an acidic cluster of the β-subunit is responsible for a kind of intrinsic down regulation of CK2 activity suggested the possibility that the acidic stretch of the β-subunit might interact with the same basic region of the α-subunit which is involved in heparin binding. To check this point the responsiveness to increasing concentration of heparin of the isolated α-subunit and of CK2 holoenzyme reconstituted with either the β^{wt}-subunit or with the hyperactivating mutants of the β-subunit having Ala replaced for Glu-55 and Glu-57 has been examined. As shown in Figure 6 the β^{wt} subunit significantly reduces the inhibition of the catalytic activity by heparin. Such an effect is less pronounced if β^{wt} is replaced by either βA^{57} or $\beta A^{55,57}$. The nonhyperactivating mutant $\beta\Delta$209–215 is conversely as protective as β^{wt}. Such a negative influence of the β-subunit on CK2 responsiveness to heparin inhibition was not observed in previous studies comparing the effects of heparin on CK2 native holoenzyme and on the recombinant α-subunit expressed in *E. coli* (Lin et al., 1991). The different outcome of our experiments can be accounted for by different experimental conditions (e.g., 100 mM NaCl in the assay and synthetic peptide as substrate) and by the dependence of heparin inhibition on CK2 concentration (Meggio et al., 1982) a parameter which can be kept rigorously constant only by performing reconstitution experiments like those described here but not by comparing a native enzyme with a recombinant one.

DISCUSSION

The experiments described in this paper have disclosed two regions of the β-subunit that are functionally relevant for tuning the activity of CK2 holoenzyme. The carboxyl terminal sequence 171–215 includes element(s) which are essential, albeit probably not sufficient, for ensuring a functional association with the α-subunit as outlined by comparing the properties of the two deleted mutants $\beta\Delta$181–215 and $\beta\Delta$171–215: while the former still binds, protects, and activates the catalytic subunit similar to the full length β-subunit, albeit not forming a canonical tetramer, the latter fails to assemble with the α-subunit and consequently neither it protects it against thermal inactivation nor it stimulates its catalytic

activity even if added in large molar excess. The amino acid sequence in this segment (HPEYRPKRPA) is highly conserved from Drosophila to human and it includes in its C terminal moiety three positively charged residues within a predicted loop structure which could well fit in a complementary element present in the α-subunit. Seemingly the overall conformation of this segment rather than the specific properties of individual side chains is required for effective binding of the α-subunit, since single and double substitutions of the charged residues in it with alanine, which reduce the overall positive charge without changing however the predicted secondary structure of this segment, are still compatible with the reconstitution of fully competent CK2 holoenzyme. It should be also mentioned in this connection that the titration curves of CK2 activity as a function of increasing β to α ratio display a sigmoidal rather than linear shape with the truncated mutant $\beta\Delta$181–215 and also with $\beta\Delta$194–215 (not shown), though the latter reconstituted a canonical tetramer when combined with the α-subunit in equimolar amounts. Such a behavior was not observed with $\beta\Delta$209–215 which displayed a hyperbolic titration curve like that with β^{wt}. This could suggest a reduced attitude of the truncated β-subunits to assemble with the α-subunit when their concentration is low, thus supporting the view that, besides the 171–181 crucial segment, also the subsequent part of the C-terminal region up to residue 208 is implicated in the positive interactions with the α-subunit. Pertinent to these observations may be the failure of the product of the Drosophila stellate gene, expressed in *E. coli*, to replace the β-subunit and to associate with the α-subunit (M. P. Bozzetti, F. Meggio, S., Pimpinelli, & L. A. Pinna, unpublished results). This protein in fact, exhibiting a remarkable homology with the N-terminal and central domains of the β-subunit, is lacking a C-terminal stretch of about 30 residues and is totally divergent from the β-subunit in its last part overlapping the 171–180 sequence of the β subunit (Livak, 1990).

Altogether these observations highlight a prominent role of the C-terminal domain of the β-subunit, with special reference to its 171–180 segment, in determining the assembly with the α-subunits.

The acidic residues in the 55–64 sequence, on the other hand, can be substituted with alanine without compromising the binding to the α-subunit, the protection against thermal inactivation, and the increase of catalytic activity which are typical of wild type β-subunit. Some of these acidic residues, however, with special reference to Asp-55 and Glu-57 are clearly responsible for an intrinsic down regulation of CK2 activity since their replacement with Ala gives rise to mutants which hyperactivate CK2 as compared to the β^{wt}-subunit. Such an autoinhibitory effect of β^{wt}-subunit can be partially relieved by high ionic strength, and it may occur through interactions with the α-subunit basic insert 65–86 responsible for heparin binding, considering that the wild type β-subunit under certain conditions significantly protects the α-subunit against heparin inhibition. On the contrary a competitive inhibition by the β acidic stretch at the substrate binding site seems unlikely since hyperactivation is not accounted for by any substantial decrease of K_m values.

The disclosure of the regulatory properties of the β-subunit 55–64 acidic stretch may also account for the inhibitory effect of the β-subunit on the phosphorylation of calmodulin by the α-subunit (Meggio et al., 1992) and provide a clue for the mechanism of action of polybasic effectors, which could bind to this acidic region thus abolishing its negative influence on the catalytic subunit.

It should be finally outlined that the two known phosphoacceptor sites of the β-subunit, including the autophosphorylatable Ser-2 and the $p34^{cdc2}$ phosphorylated Ser-209, can be deleted without substantially affecting the binding, protective, and stimulatory properties of the β-subunit. The observation, however, that $\beta\Delta$1–4 fails to induce stimulation by NaCl, similar to the hyperactivating mutants with substitutions in the acidic cluster 55–64, may suggest that the autophosphorylation site and the autoinhibitory acidic stretch could be functionally and perhaps conformationally related. This point of view would be also consistent with two additional findings, namely (1) the acidic determinants of autophosphorylation (Glu-5 and Glu-6) do contribute to the negative regulation effect since their replacement with Ala promotes hyperactivation, though less pronounced than in the case of Asp-55 and Glu-57 (see Figure 3); (2) the acidic stretch 55–64, albeit remote from the autophosphorylation site (Ser-2/3) along the primary structure, is nevertheless required for the efficient autophosphorylation activity of CK2 (unpublished results), consistent with its proximity to the autophosphorylatable residues, Ser-2/Ser-3, in the folded structure of the β-subunit.

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