An inactive mutant of the α subunit of protein kinase CK2 that traps the regulatory CK2β subunit

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Abstract **Protein kinase CK2 (casein kinase 2) is a ubiquitous Ser/Thr protein kinase involved in cell proliferation. Mutation of the α subunit of the *Xenopus laevis* CK2 to change aspartic acid 156 to alanine (CK2αA^{156}) resulted in an inactive enzyme. The CK2αA^{156} mutant, however, binds the regulatory subunit as measured by retention of β on a nickel chelating column mediated by (His)$_6$-tagged CK2αA^{156}. Addition of CK2αA^{156} also caused β to shift sedimentation in a sucrose gradient from a β_2 dimer (52 kDa) to an $\alpha_2\beta_2$ tetramer (130 000 kDa). CK2αA^{156} can trap the β subunit in an inactive complex reducing the stimulation of casein phosphorylation caused by addition of β to wild-type α. This competitive effect depends on the ratio of α/αA^{156} and on the amount of β available. Since β inhibits the phosphorylation of calmodulin by CK2α, the addition of CK2αA^{156}, in this case, increases calmodulin phosphorylation by the α and β combination. These results suggest that CK2αA^{156} may be a useful dominant-negative mutant that can serve to explore the multiple functions of CK2β.**

Key words: Protein kinase CK2; Casein kinase II; CK2α subunit mutant; CK2β subunit; Dominant-negative mutant

1. Introduction

Protein kinase CK2 (also known as casein kinase II) is a ubiquitous Ser/Thr protein kinase known to phosphorylate many important protein substrates (reviewed in Refs. [1–3]). CK2 is composed of catalytic subunits α and α′, which although closely analogous, are coded by different genes. The CK2 subunits α (M_r 44 000) and α′ (M_r 40 000) are active by themselves but their activity and stability are markedly altered by the presence of the β subunit which binds to form heterotetramers of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ and $\alpha'_2\beta_2$ composition [4,5]. With the majority of the substrates, the addition of β subunit increases the phosphorylating activity of α; however, in the phosphorylation of calmodulin by α, the β subunit acts as a negative regulator [6,7].

There is much evidence indicating that CK2 is involved in the process of cell division. The levels of CK2 in growing and tumor tissues is greatly enhanced [8]. Introduction of the CK2α gene in transgenic mice demostrated its capacity to act as an oncogene complementary to myc [9]. The microinjection of CK2 antisense RNA or antibodies indicates that CK2 is required for G1/S and G2/M cell cycle transition in cultured mammalian cells [10,11]. Similar conclusions have been reached using thermosensitive mutants of the CK2α subunit in yeast [12]. Very recently, yeast two-hybrid analysis has been used to demonstrate the interaction of CK2β with A-Raf [13,14] and with mos [15]. Both A-Raf and mos are cytoplasmic oncogene products that have Ser/Thr protein kinase activity that phosphorylate and activate MEK and are therefore involved in the transduction of the stimuli that trigger cell division. CK2 also binds to p53 and p21$^{WAF/CIP}$ [16,17] which are well characterized repressors of cell division. Despite this evidence pointing to a prominent role for CK2 in many key cell processes, the question as to the mechanisms that physiologically regulate CK2 activity remains open.

A number of studies have dealt with the structure and function of this enzyme through site directed mutagenesis. These studies have indicated that the carboxyl end of the β subunit is important for its interaction with the α subunit and that the acidic cluster of amino acids (55–64) of this subunit down-regulates the enhancing capacity of β for α [18,19]. Mutations in the α subunit have pinpointed the region responsible for polyanionic inhibitor binding to the basic region including amino acids 71–78 [20] and provided information as to the residues involved in peptide substrate recognition [21]. Other mutations in α subunit have explored the mode of activation used by β subunit and the interaction with ATP and GTP [22].

The important structural information derived from the known tridimensional structure of other members of the family of protein kinases has made it possible to predict the function of some amino acid residues present in the CK2α structure. Thus, the aspartic acid residue in position 156 of CK2α occupies a position similar to aspartic acid 166 in the cyclic AMP-dependent protein kinase (PKA), where this aspartic acid plays a key role in the catalysis of phosphoryl group transfer [23].

In the present study we report results obtained with a *Xenopus laevis* CK2α mutant in which aspartic acid 156 is changed to alanine. As expected from the homology to PKA, the mutant protein has no phosphorylating activity. The mutants does, however, retain high affinity for the CK2β subunit. The CK2αA^{156} mutant can efficiently compete with the wild-type CK2α for the regulatory β subunit and may therefore be a useful "dominant negative" type of mutation that can serve to explore the cellular function of CK2 subunits in vivo.

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Abbreviations: CK2α and CK2β, the α and β subunits of protein kinase CK2; CK2αA^{156}, mutant of recombinant CK2α in which D^{156} is changed to A

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2. Experimental procedures

2.1. Materials

Nucleotide oligomers and peptides were synthesized by Oligopeptido, the core facility of the University of Chile. [γ-^{32}P]ATP was purchased from ICN. Restriction enzymes and Taq polymerase were obtained from Promega. GSH-agarose was from Pharmacia and nitrilotriacetic acid (NTA) agarose from Invitrogen. Glutathione, dephospho-β-casein, calmodulin, thrombin and remaining reagents were from Sigma.

2.2. Methods

2.2.1. Preparation of pT7-7H6. To facilitate expression and purification of CK2α and mutant subunits, a vector including a $(His)_6$ tag was prepared by inverse PCR of plasmid pT7-7 using the primers: (5′-ATATATATATACATATGATGATGATGATGCATATCTC-CTTCTTAAAGTT-3′) and (5′-TATATACATATGGCTAGAATT-3′). The product was digested with *Nde*I and self-ligated to reestablish the plasmid. Effective incorporation of the tag was confirmed by DNA sequencing.

2.2.2. Preparation of CK2αA^{156}. The point mutation was introduced into wild-type CK2α cDNA clones in pT7-7 by PCR overlapping primer extension [24]. Initial PCRs were conducted with the following primer pairs: (5′-TAATGCACAGAGCTGTGAAACC-TC-3′) and the T7 primer (5′-AATACGACTCACTATAG-3′) and with (5′-GAGGTTTCACAGCTCTGTGCATTA-3′), and reverse T7 primer (5′-ATTGGTAACTGTCAGACCAAG-3).

For the last amplification reaction the T7 and reverse T7 primers were used.

The product was digested with *Nde*I and *Sal*I and subcloned into pT7-7H6. The complete sequence of the mutated recombinant construct was confirmed by sequencing.

2.2.3. Expression and purification of recombinant CK2 subunits. The cDNAs coding for the CK2 subunits α and β were obtained as previously described [25]. CK2α′, subcloned in pT7-7, was expressed as described [26]. CK2α and CK2αA^{156} were ligated into the expression vector pT7-7H6 and expressed in *E. coli* strain BL21 (DE3). These latter proteins were purified by passing the supernatant fraction of bacterial extracts through a 3 ml nickel-chelate NTA-agarose column, previously equilibrated with buffer A (50 mM Tris-HCl pH 8, 20 mM β-mercaptoethanol, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), washed with the same buffer and eluted with a linear gradient, 10–200 mM imidazole in buffer A with 10% glycerol. The elution of CK2α was checked by activity and SDS-gel electrophoresis while the elution of the inactive CK2αA^{156} was followed by electrophoresis and Western blot using a polyclonal antibody specific for CK2α [27]. The purified preparations of CK2α and α′ used had a specific activity of 67 and 42, nmol ^{32}P incorporated into casein/min per mg, respectively.

The recombinant CK2β subunit, subcloned in pGEX-2T vector, was expressed as previously described [25]. The fusion protein was purified by chromatography of GSH-agarose, treated with thrombin and rechromatographed on GSH-agarose.

2.2.4. Preparation of native CK2 holoenzyme. Stage VI oocytes were isolated from *Xenopus laevis* ovary and extracts prepared by hand-homogenizing 50 oocytes in 1 ml of buffer B (50 mM HEPES, pH 8.0; 10 mM $MgCl_2$; 1 mM dithiothreitol; 10 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride and 10% glycerol). Homogenates were centrifuged at 14 000 rpm (Sorvall Microspin 24S rotor) and the supernatant solution was adjusted to 0.25 M NaCl and applied to a 0.5 ml column of phosphocellulose equilibrated with a 0.25 M NaCl in buffer B. After washing with 10 vol. of the same solution, CK2 was eluted with 0.5 ml of 0.7 M NaCl in buffer B.

2.2.5. Preparation of [^{32}P]CK2β subunit. CK2β (40 pmol) was incubated in the presence of 2.7 μCi of [γ-^{32}P]ATP, 0.8 pmol of CK2α, 50 mM HEPES pH 7.5 and 10 mM $MgCl_2$, in a final volume of 60 μl. Incubation was for 30 min at 30°C. The reaction mixture was cooled to 4°C and applied to a 0.1 ml column of nickel NTA-agarose, which retains the $(His)_6$-tagged CK2α subunit and a stoichiometric amount of CK2β. The column was washed with a minimum of equilibration buffer A to remove the unbound phosphorylated CK2β subunit which was measured by precipitation in 10% trichloroacetic acid and collecting precipitates on GF/A filters for counting in a scintillation system. A typical preparation contained 10 000 cpm/pmol of CK2β.

2.2.6. Analysis of CK2 subunits on sucrose density gradients. CK2 subunits alone or in the combinations indicated were adjusted to 0.3 M NaCl and 0.1% Triton X-100 preincubated for 45 min at 4°C and 200 μl applied to a 5 ml lineal gradient of 5–20% sucrose containing buffer C (50 mM HEPES, pH 7.5, 10 mM MgCl, 0.3 M NaCl, 0.5 μM dithiothreitol and 0.1% Triton X-100), followed by centrifugation at 40 000×g for 13 h at 4°C. Fractions were collected and aliquots analyzed for acid precipitable radioactivity as given above.

2.2.7. Protein kinase assay of the recombinant subunits and native holoenzyme. The standard assay mixture contains in a total volume of 30 μl: 50 mM HEPES pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (500–2000 cpm/pmol) and either 5 mg/ml casein or 0.3 mM peptide substrate RRREEEESEEE. CK2 subunits were added as indicated. Incubations were carried out for 10 min at 30°C and the reaction was terminated by applying 25 μl to P81 phosphocellulose filter paper, as described [25].

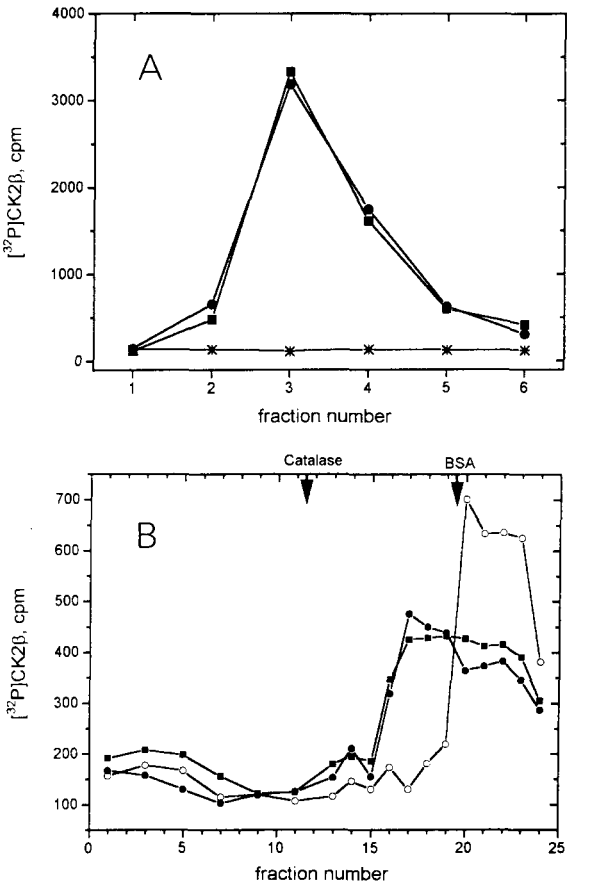

Fig. 1. Binding of CK2αA^{156} to the CK2β subunit. A: 1 pmol each of CK2α (●), CK2αA^{156} (■), or buffer A alone (×) were applied to separate 0.2 ml columns of NTA-agarose and the columns were washed extensively with buffer B to remove any unbound protein. [^{32}P]CK2β (20 000 cpm) prepared as described in Section 2.2 was passed through each column and then washed with 3 ml of buffer B. The resulting complexes were eluted with 200 mM imidazole in buffer B. Fractions of 0.2 ml were collected and 0.1 ml analyzed for radioactivity by precipitation with 10% trichloroacetic acid and retention of protein of GFA glass fiber filters. B: sedimentation profiles were obtained with sucrose gradients (5–20% in buffer C), centrifuged as given in Section 2. The gradient tubes contained 1 pmol of [^{32}P]CK2β (~10 000 cpm) to which was added 5 pmol of CK2α (●), 5 pmol of CK2αA^{156} (■) or buffer C alone (○). Fractions were analyzed for radioactivity as given in A. Protein standards with known sedimentation coefficients were catalase (232 kDa), and bovine serum albumin (BSA; 67 kDa) indicated by arrows.

3. Results

3.1. CK2αA^{156} binds to the CK2β subunit

The recombinant CK2αA^{156} mutant and the wild-type CK2α subunit were expressed in bacteria as $(His)_6$-tagged proteins and purified on a nickel-chelate NTA-agarose columns. The mutant protein was shown to have the same molecular size and antigenic properties as the *Xenopus laevis* CK2α subunit (not shown). However, when CK2αA^{156} was analyzed for activity with casein or the specific substrate peptide, it was completely inactive in its phosphorylating capacity.

Considering the importance of subunit interaction for the regulation of CK2 activity, the interaction of this mutant form with the CK2β subunit was analyzed. It was found that CK2αA^{156} can efficiently bind the β subunit. In Fig. 1A it is seen that $(His)_6$-tagged CK2αA^{156} can mediate the retention of [^{32}P]CK2β on an NTA-agarose affinity column and that the radioactivity of β is eluted with imidazole in the same position as that seen for the wild-type $(His)_6$-tagged CK2α subunit. The elution of active CK2α was determined by assay of the column fractions and the position of the α and β subunits was confirmed by SDS-polyacrylamide gel electrophoresis (not shown).

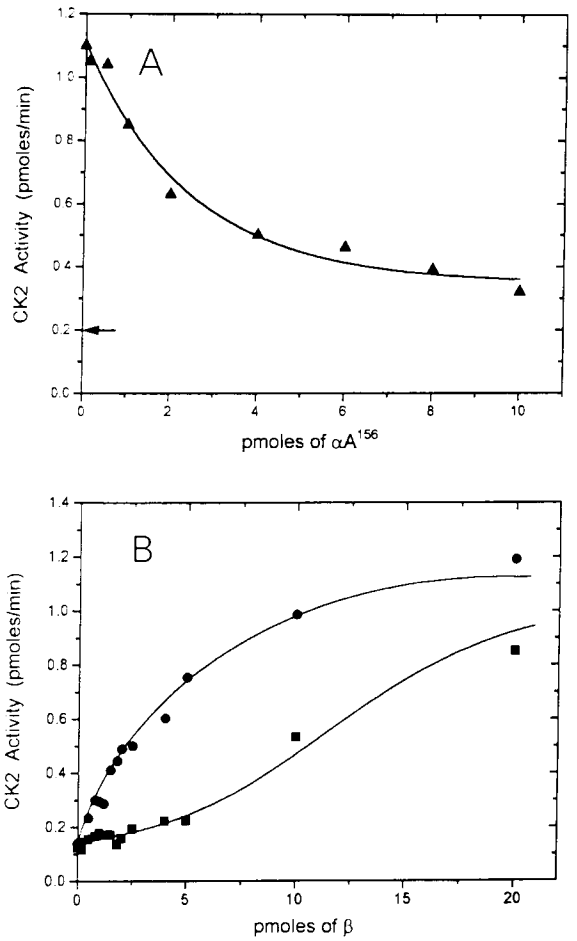

Fig. 2. CK2αA^{156} inhibits the activation of the CK2α subunit by CK2β. A: 1 pmol of each CK2α and CK2β was assayed in the presence of varying concentrations of CK2αA^{156}, under standard conditions, with β-casein as substrate. The basal activity of CK2α in this experiment was 0.2 pmol/min of [^{32}P] incorporated into protein (arrow); the maximal activation by CK2β was 5.5-fold (1.1 pmol/min). B: CK2α subunit (1 pmol) was assayed in the presence (■) or absence (●) of a 6-fold excess of CK2αA^{156} and with different concentrations of the CK2β subunit, using the standard conditions given in the Section 2 with β-casein as substrate.

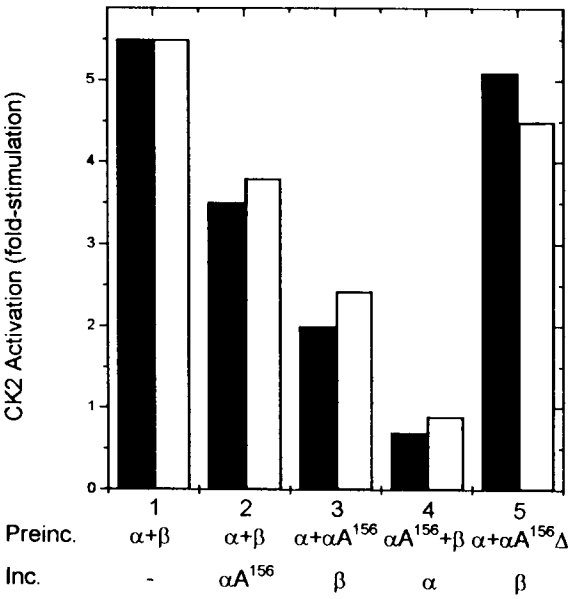

Fig. 3. Effect of preincubation of α and β subunits. CK2 subunits (1 pmol of α, 6 pmol of CK2αA^{156}, and 1 pmol of β) were preincubated (preinc.) in the combinations indicated in the figure for 10 min at 0°C before adding the third component (inc.) and then assayed under standard conditions using β-casein (closed bars) or synthetic peptide (open bars) as substrates. In 5, the αA^{156} Δ indicates that this subunit was heated at 100°C for 5 min before the assay.

An alternative method to demonstrate the interaction of CK2αA^{156} with CK2β was the use of sedimentation analysis in sucrose gradients, where a shift in sedimentation of the β subunit would be expected if such interaction does occur. The [^{32}P]CK2β subunit, in the absence of other proteins, sediments as a dimer with a molecular mass of 52 kDa (Fig. 1B). The addition of either CK2αA^{156} or CK2α causes a large fraction of the labeled β subunit to shift to a sedimentation value calculated as 130 kDa, which corresponds to the expected value for the $\alpha_2\beta_2$ tetramer.

3.2. Competition of CK2αA^{156} with CK2α for the CK2β subunit

The capacity of CK2αA^{156} to bind β can also be observed by its inhibitory effect in the protein kinase assay, in which the mutant subunit is added simultaneously with the α and β subunits. In Fig. 2A an assay is described in which casein phosphorylation is measured and it is seen that increasing amounts of CK2αA^{156} inhibit the activation of the wild-type CK2α by β approaching complete inhibition of the effect when a 10-fold excess of mutant is present. From the molar amounts of the wild-type and mutant subunits used, it can be estimated that the affinity of CK2αA^{156} for β is approximately 50% of the affinity of the wild-type protein. Similar experiments carried out with the recombinant wild-type CK2α′ subunit demonstrate that αA^{156} can also compete with this isoform for the β subunit (not shown). Fig. 2B shows that when CK2αA^{156} is present in the assay system in 6-fold excess over CK2α and increasing amounts of CK2β are tested for activation of α, the mutant subunit does not itself inhibit CK2α

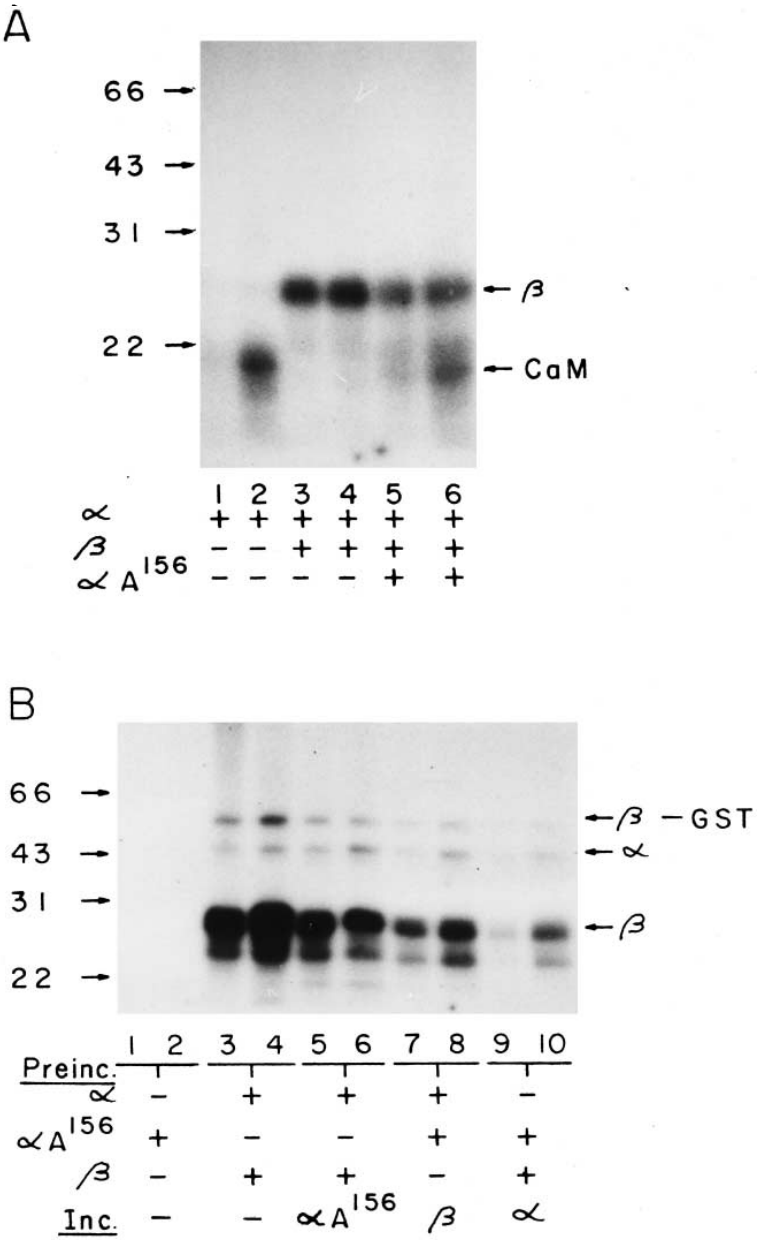

Fig. 4. CK2αA^{156} reverses the inhibitory effect of CK2β on calmodulin phosphorylation and inhibits autophosphorylation of CK2β by CK2α. A: the standard kinase assay was used except that incubations were for 10 min (odd numbers) and 30 min (even numbers) and 60 pmol of calmodulin was used as substrate. Subunit amounts were: 1 pmol CK2α, 5 pmol CK2α^{156} and 1 pmol CK2β, where indicated. Phosphorylated proteins were resolved on a 13% SDS polyacrylamide gel and visualized by autoradiography. The migration of calmodulin and β subunit are indicated by arrows. B: CK2 subunits, 1 pmol CK2α, 5 pmol CK2 αA^{156}, and 1 pmol β, were preincubated and incubated in the combinations indicated below the figure. Preincubations were for 10 min at 0°C. The resulting phosphoproteins were analyzed as in A. Arrows indicate the migration of the individual subunits and a contaminating fusion protein glutathione transferase CK2β. The migration of size standards are given in kDa.

basal activity and significant activation becomes evident only when the level of β is roughly equivalent to the amount of the mutant protein. Further increase of the CK2β concentration permits proportionaly increased activation of the phosphorylation reaction.

In the previous experiments, the CK2β subunit was added to the reaction mixture which already contained a mixture of the native and mutant α subunits, so that the competing subunits might form their respective complexes simultaneously. It was pertinent therefore to determine whether preincubation of CK2β with either of the CK2α subunits would affect the activation reaction. In Fig. 3, in which the activity is assayed with the peptide substrate and with casein, it can be observed that preincubation at 0°C of the α and β subunits (condition 2) results in a lower inhibition than when both α and αA^{156} react with β at the same time (condition 3). Conversely preincubation of αA^{156} with β increases significantly the competitive inhibitory effect (condition 4). Heating the αA^{156} at 100°C for 5 min (condition 5) destroys its inhibitory activity.

It is noteworthy that no inhibition was observed when native CK2 holoenzyme prepared from *Xenopus laevis* oocyte extracts [28] is tested under the standard assay conditions

and with an amount of mutant protein calculated to be an excess of the $\alpha_2\beta_2$ concentration.

The effect of the mutant on the phosphorylation of calmodulin and of the β subunit itself was also studied. As seen in Fig. 4A, the presence of the CK2αA^{156} reverses the inhibitory effect that β has on calmodulin phosphorylation by the wild-type α subunit of CK2. In Fig. 4B we see that addition of CK2αA^{156} decreases the phosphorylation of the β subunit and that preincubation of αA^{156} with β diminishes even further this phosphorylation.

4. Discussion

The mutation of aspartic acid 156 of the α subunit of protein kinase CK2 to alanine results in complete loss of catalytic activity. This result confirms the recurrent observation that all kinases which share the conserved features first described in PKA have very similar structural arrangements that are essential for their catalytic function [29].

The most significant results obtained with CK2αA^{156}, on the other hand, demonstrate the capacity of this inactive mutant to bind the β regulatory subunit of CK2. This capacity to complex β is demonstrated through direct physical interaction studies that show that CK2αA^{156} with a $(His)_6$ tag can mediate the retention of radioactive β in a metal chelate NTA-agarose affinity column. In addition, the CK2αA^{156} subunit in a sucrose gradient can shift the sedimentation of part of the β subunit from the position of a β_2 dimer (52 kDa) to that of an $\alpha_2\beta_2$ tretramer (130 kDa). In both of these experiments the αA^{156} subunit behaves as the wild-type CK2α. Careful studies with the yeast two-hybrid system, that dwelled on the question of the part of CK2α involved in binding to the β subunit, reached the conclusion that the only dispensable regions were the amino and carboxyl terminal non-conserved regions [30].

The capturing of β by the inactive CK2αA^{156} is also shown by its capacity to compete with CK2α for the β subunit. This competition is observed by an inhibition of the phosphorylation of casein or the specific CK2 peptide substrate when the inactive mutant is added to a mixture of wild-type recombinant α and β subunits. The presence of αA^{156} decreases the effect of CK2β when this subunit is at low concentrations but the mutant does not alter the activity of α. The effect of αA^{156} on the phosphorylation activity is clearly due to competition for β with the wild-type subunit since it depends on the α/αA^{156} ratio and on the concentration of β available. Excess of β tends to eliminate the effect of the mutant.

The inhibitory effects of αA^{156} on the phosphorylating activity of the mixture of CK2α and β subunits is not only seen with exogenous substrates but also with the autophosphorylation of the β subunit itself. This result suggests that the phosphorylation of β is mainly due to an intra-tetrameric reaction or that the formation of a complex with the inactive catalytic subunit blocks the access of the autophosphorylation site of the β subunit to the active CK2α.

CK2β acts as a negative regulator of calmodulin phosphorylation by CK2α and phosphorylation of this protein substrate is only seen in the absence of β [6,7]. Accordingly, with this substrate, the addition of αA^{156} to a mixture of α and β causes an activation in the phosphorylation of calmodulin. This result again demonstrates the capacitity of αA^{156} to sequester β and to compete with the wild-type α subunit for this interaction.

Previous studies have shown that there is very little dissociation of the α and β subunits of the native CK2 holoenzyme [31]. Coincident with this observation is the result obtained that indicates that αA^{156} does not inhibit native CK2 purified from oocyte extracts. Also in agreement with this finding is the fact that a short preincubation of α and a limiting amount of β at 0°C considerably reduces the inhibition observed by addition of αA^{156}. Conversely, preincubation of the αA^{156} with β traps more of the regulatory subunit and leaves less available for the interaction with wild-type α. These results also indicate that, under the conditions used, the formation of a stable complex by recombinant α and β is not instantaneous. Even after 10 min of preincubation at 0°C, there is enough β available to interact with αA^{156}, as can be seen by the fact that some inhibition by the mutant is observed in condition 2 of Fig. 3.

The capacity of αA^{156} to scavange β with an affinity of the same order of magnitude as that of wild-type α can make this mutant very useful in exploring the physiological effects of the CK2β subunit in vivo in a similar fashion as dominant-negative mutants have been very important in defining the role of components of signal transducion cascades [32]. In this regard it is relevant to emphasize that CK2β has recently been shown to have a role that transcends its regulation of CK2α activity. The CK2β activation of A-Raf [13,14] and the inhibition of mos [15] by this subunit have been reported to be antagonized by CK2α and presumably would be also blocked by the αA^{156} mutant.

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