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C-Terminal Region of Protein Kinase CK2α: How the Structure Can Affect Function and Stability of the Catalytic Subunit

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Abstract A novel mutant of the catalytic α subunit of human protein kinase CK2 (CK2α) was designed in an attempt to clarify the role of the carboxylic-terminal segment characteristic of vertebrates, excluding fish. Starting from the sequence alignments, we constructed a phylogenetic tree of the primary structure of CK2α. On this basis, we substituted two distal prolines with alanines (PA 382-384). Theoretical calculations and spectropolarimetry measurements, performed both on native and mutant subunits, indicate an increased content of α-helix after this double amino acidic substitution. In order to clarify the structure/function relationship of the C-terminal region, we verified if the structural change affects the catalytic activity of CK2α. The mutant exhibits slightly increased phosphorylation efficiency, but reduced ability to transfer phosphate in comparison with the native subunit. At last, we compared the thermal stability of the mutant with respect to the native subunit and we tested the proteolytic degradability. The observation that the PA 382-384 mutant exhibits an increased thermal and proteolytic stability suggests that this mutant could be employed to solve the three-dimensional (3D) structure of human CK2α and to overcome difficulties in crystallizing the native form. J. Cell. Biochem. 92: 270–284, 2004.

Key words: human CK2α; primary and secondary structure analysis; enzymatic activity; thermal and proteolytic stability

Protein kinase CK2 is a serine/threonine kinase, previously called casein kinase 2 (CK2), because of its first documented substrate [Hathaway and Traugh, 1979]. CK2 is an ubiquitous and pleiotropic enzyme, which plays a crucial role in many cellular pathways, such as cell proliferation and differentiation [Munstermann et al., 1990; Perich et al., 1990], tumorigenesis [Issinger, 1993], stress response [Litchfield and Luscher, 1993], and cell survival [Ahmed et al., 2002].

In light of the involvement of this enzyme in crucial physiological and pathological events, considerable efforts have been made to solve its three-dimensional structure. In the majority of organisms, native CK2 is a heterotetramer ($\alpha_2\beta_2$) composed of two regulatory (β) and two catalytic (α or α′) subunits. On the contrary, no β subunit exists in maize CK2, which represents the only catalytic subunit with a solved third-dimensional (3D) structure [Niefind et al., 1998; Battistutta et al., 2000]. The human and the vegetal proteins differ for the lack of a tail of 54 residues at the C-terminus. Recently [Niefind et al., 2000; Niefind et al., 2001] the three-dimensional structure of a variant human subunit has been solved by X-ray crystallography. The authors employed a truncated form of the α subunit, because the well-known degradability of the native form made it impossible to crystallize the entire subunit. This variant exhibits a molecular weight of approximately

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5 kDa, because of the lack of 54 residues at the C-terminal portion. In contrast, the native protein has a molecular weight of 45 kDa. It is widely documented that during purification, the human subunit undergoes a characteristic degradation, which produces two electrophoretic bands (at 45 and 40 kDa, respectively) [Sarno et al., 1996]. Therefore the truncation at the C-terminus reduced the intrinsic degradability of this subunit and it allowed crystallization and definition of its 3D structure.

Despite these advances in the knowledge of human CK2, information on the carboxyl terminus of the α subunit remains rather poor. Whereas the amino terminus is known to be highly conserved across the evolution, the C-terminus shows a greater variability [Tapia et al., 2002]. This portion has been demonstrated to be a phosphorylation target, where four specific sites (Thr-344, Thr-360, Ser-362, and Ser-370) are recognized by the protein kinase p34 ^{Cdc2}. This phosphorylation reaction is 'proline-directed' because it requires proline to be placed immediately next to the target amino acid [Bosc et al., 1995]. Moreover, Messenger and co-workers [Messenger et al., 2002] reported as just this carboxyl terminus interacts with the peptidyl-prolyl isomerase Pin1 with a phosphorylation-dependent mechanism. Based on all these observations, we postulated the importance of the proline residues positioned at the C-terminus of the α subunit in terms of CK2 phosphorylation. Anyway, not all the prolines are proximal to phosphorylable residues therefore further investigations are necessary to better clarify their role.

Historically, the creation of novel mutants was used to clarify the regions of the primary sequences where important functional sites are localized (i.e., sites for substrate recognition, for cofactor and inhibitor binding, etc.). However, the problem can be seen from another point of view: a mutation of primary sequence can be used to clearly understand protein structure by modifications of its biochemical parameters [Chaillot et al., 2000]. This approach is usually applied to proteins with unsolved 3D structure by postulating a hypothesis and verifying its correctness on the designed mutant. We followed this approach in order to clarify the biological role of the last 54 amino acids of human CK2α. We modified the primary sequence of the α subunit in this region in an attempt to improve the protein stability. In particular, we introduced two selected amino acid substitutions at the C-terminus and expressed and purified the mutant protein. According to our expectations, we obtained a variant form of CK2α with improved features of thermal and proteolytic stability, which could be employed for trials of crystallization in place of the native subunit, which is highly degradable.

MATERIALS AND METHODS

Materials

Chemicals were supplied from Sigma Aldrich (Milan, Italy). Reagents for bacterial growth were purchased from Fluka (Milan, Italy). T4 DNA ligase and *Taq* polymerase were supplied by Stratagene (La Jolla, CA), restriction enzymes and dNTPs by Boehringer-Mannheim (Monza, Italy). *Escherichia coli* strains BL21 and JM109, nitrocellulose membrane, and Sephacryl S-200 gel filtration column were purchased from Pharmacia Biotech (Uppsala, Sweden). Primers for sequencing and mutagenesis were synthesized by TibMolBiol (Genoa, Italy). Phosphocellulose P-11 ion-exchange resin was supplied by Millipore (Bedford, GB). Heparin-agarose resin, protease inhibitors, trypsin, and low range molecular weight markers were obtained from Sigma Aldrich. Prestained markers were supplied by Biorad (Milan, Italy). [γ-^{32}P] ATP (specific radioactivity 1,000–6,000 cpm/pmol) was supplied by DuPont-New England Nuclear. The 'Casein kinase 2 assay' kit was purchased from Upstate Biotechnology (Lake Placid, NY), anti-CK2α monoclonal antibody from Calbiochem (San Diego, CA), and anti-mouse horseradish peroxidase-conjugated IgG1 from Santa Cruz Biotechnology (Santa Cruz, CA).

Bacterial Expression of the Recombinant Human CK2α

Plasmid pT7-7, containing cDNA encoding human CK2α subunit, was supplied by Polo Nazionale Bioelettronica (Roma, Italy). Recombinant plasmid was introduced into BL21 (DE3) *E. coli* cells [Vergani et al., 2002] and transformed cells were inoculated in LB medium (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 100 μg/ml ampicillin). After overnight growth at 37°C with vigorous shaking, 10 ml of the culture were inoculated into 1 L of prewarmed LB medium and grown until OD_{600}

reached 0.6. After inducing protein expression by the addition of 1 mM Isopropyl-β-D-thiogalactopyrsanoside (IPTG), cells were incubated for 5 h at 30°C, then harvested and stored at −80°C until use.

Site-Specific Mutagenesis and Purification of CK2α

The PA 382-384 mutant was generated by oligonucleotide directed mutagenesis, as previously described in details [Grasselli et al., 2002]. The substitution of two prolines with alanines was carried out using the 'Quick change site-directed mutagenesis' system (Stratagene, La Jolla, CA). Two synthetic oligonucleotidic primers (forward: 5′-cccttgggatggctgttgcagctgccgctggc-3′; reverse: 5′-gccagcggcagctgcaacagccatcccaagggg-3′) were used to modify the coding sequence. The recovered clones were sequenced and the positive ones were used to transform BL21 (DE3) competent *E. coli* cells. After expression, both the native and the mutant recombinant proteins were purified near to electrophoretic homogeneity following a modified protocol [Grankowski et al., 1991; Grasselli et al., 2002]. Pellet from 2 L of culture was resuspended in buffer A (Tris-HCl, 25 mM, pH 8.5; 2-Mercaptoethanol, 7 mM). Bacterial cells were sonicated and after a brief centrifugation, the supernatant was recovered and loaded onto a phosphocellulose P-11 ion-exchange chromatography. Then a linear gradient was applied (200–1,200 mM NaCl) and the positive fractions were loaded onto a heparin-agarose affinity chromatography. A linear gradient (100–1,000 mM NaCl) was applied and the eluted fractions were loaded onto a 1.6 × 100 cm Sephacryl S-200 gel filtration column. The fractions, corresponding to the correct molecular weight and checked by immune-detection, were pooled and dialyzed extensively versus Storage buffer and stored at −80°C.

Protein Determination

The amount of total proteins recovered from each step of purification was evaluated by Bradford assay [Bradford, 1976] using bovine serum albumin as standard, both for native and mutant recombinant protein. At the end of the entire purification process, the concentration of the purified protein was estimated by UV spectrophotometer, using the molar extinction coefficient ($\varepsilon_{280} = 60{,}495\ M^{-1}\,cm^{-1}$) [Jakobi and Traugh, 1995].

Gel Electrophoresis and Immunoblotting

The purified α subunit was resolved in 12.5% SDS–PAGE gel [Laemmli, 1970] and proteins were visualized in Coomassie brilliant Blue R-250 or alternatively with silver stain method. After electrophoretic separation, proteins were transferred from the gel onto a nitrocellulose membrane according to a standard procedure [Towbin et al., 1979]. Recombinant protein was detected by using a primary monoclonal antibody selective for residues 70–89 of the human CK2α sequence. After washing, anti-mouse horseradish peroxidase-conjugated IgG1 was used as a secondary antibody.

Phylogenetic Analysis of CK2α Primary Sequences

The CK2 sequences were obtained from the 'Swiss-Prot Protein Knowledgebase or TrEMBL', handled and stored with the help of the Lasergene program EditSeq (1994 release from DNAstar, Madison, WI) and Megalign (1994 release; DNAstar). MEGA program (Molecular Evolutionary Genetics Analysis 1.01) was used for statistical and phylogenetic analysis [Kumar et al., 1993]. Phylogenetic trees were constructed by neighbor-joining method [Saitou and Nei, 1987] using p-distances as measure of the extent of sequence divergence.

Absorption Spectroscopy

Absorption spectra were recorded in the wavelength range 220–320 nm as a function of temperature, using a Jasco spectrophotometer model 7800 (Jasco, Japan) equipped with a temperature controller. Samples of mutant and native recombinant CK2α (10 μM in 15 mM sodium phosphate buffer, pH 8.0) were measured in 1 cm path-length quartz cell. The spectra were recorded in the thermal range 25–85°C every 10 degrees with increments of 1 degree per min.

Circular Dichroism Spectroscopy

Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter (Jasco, Japan) equipped with a Peltier thermostatic cell holder. All spectra were recorded in 0.05 cm path-length quartz cell under nitrogen atmosphere, using the following parameters: time constant 4 s, scanning speed 20 nm/min, bandwidth 2 nm, sensitivity 10 mdegree, step resolution 0.5 nm [Bartolucci et al., 1997]. Photomultiplier

voltage did not exceed 600 V in the spectral region measured. The instrument was calibrated with a standard solution of (+)-10-camphosulphonic acid. Each spectrum was averaged five times in the wavelength range 250–180 nm. Samples were prepared in 15 mM phosphate buffer, pH 8.0 at a protein concentration of about 0.1 mM. All the acquired spectra were corrected for the baseline and normalized to the amino acid concentration in order to calculate the mean residual molar ellipticity (degrees cm^2/decimoles).

The melting curves of recombinant proteins were calculated by acquiring the CD spectra at increasing temperatures (from 25 to 85°C) with steps of 10 degrees and increments of 1 degree per min.

Secondary Structure Estimation

The percentages of secondary structures of the two proteins were estimated by dedicated softwares [Hennessey and Johnson, 1981; Johnson, 1990]. The best results were supplied by the program CONTIN [Provencher and Glöckner, 1981]: in order to calculate the different secondary conformations [Kabsch and Sander, 1983] this method analyzes a CD spectrum as a linear combination of 16 original proteins, plus poly-L-glutamate [Sreerama and Woody, 2000].

Secondary Structure Prediction

The percentages of the main secondary structures occurring in the native and mutant subunits were predicted by theoretical calculations using the GOR IV algorithm [Garnier et al., 1996]. This algorithm is the fourth version of a secondary structure prediction method based on the information theory. GOR IV allowed us to establish the structural code relating the amino acid sequence and the secondary structure of a protein. This database uses as data source a pool of 267 protein structures having a resolution better than 2.5 Å and with a length greater than 50 residues.

Phosphorylation Assay

Phosphorylation of the synthetic peptide RRR-DDDSDDD [Messenger et al., 2002] was performed using the 'Casein kinase 2 assay' kit. The reaction started with the addition of the CK2α subunit to the following reaction mixture: 20 mM MOPS pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 13.5 mM magnesium chloride, and 100 μM [γ-^{32}P] ATP (specific radioactivity 1,000–6,000 cpm/pmol). The phosphorylated substrate was separated from the residual [γ-^{32}P] ATP using P81 phosphocellulose paper and ^{32}P incorporation into substrate was quantified by Counter scintillation (Lumac-LSC B.U., Olen, Belgium). The assay was linear for incubation times of up to 30 min and incorporation of up to 20% of total ATP. The phosphotransferase activity of 0.5 μg of the purified subunit was tested at 30°C upon increasing the substrate concentration (from 50 to 200 μM). CK2 auto-phosphorylation and background were subtracted from each measurement. Kinetic constants for ATP were determined from Lineweaver-Burk plots by linear-regression analysis. Data reflect the average of at least five different assays for each substrate concentration [Sarno et al., 1996]. One unit of enzyme corresponds to the amount of enzyme that incorporates 1 nmol phosphate per minute into the substrate at 30°C [Grankowski et al., 1991].

Controlled Proteolysis Assay

Limited and controlled proteolytic digestions of both mutant and native α subunits were performed using increasing concentrations of trypsin. Thus 10 μg of CK2α was incubated at 25°C in presence of 0.1, 0.2, 0.5, and 1 μg of proteolytic enzyme in 50 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$. The reaction was stopped after 2 min by the addition of 5 mM phenyl-methyl-sulfonyl-fluoride (Sigma). The digestion products were resolved on 11% SDS–PAGE and detected both by Coomassie and silver staining of the gel [Meggio et al., 1986]. The relative amount of each digestion product was evaluated by measuring the integrated optical density of the corresponding electrophoretic band by using Image J 1.6 free software (National Institute of Health, Bethesda).

RESULTS AND DISCUSSION

Alignment and Phylogenetic Analysis of CK2α Subunit

The phylogenetic analysis involved 25 eukaryotic CK2α sequences (the Swiss-Prot Protein Knowledgebase or TrEMBL) and it agrees well with the traditional evolutionary relationships among the taxa considered. All the analyzed sequences correspond to α subunits, except for the α′ from *Xenopus*. Because the similarity of

this α′ with other α sequences was higher than with the corresponding human α′, we included it in our cluster. We also included the α′ subunit from *Danio rerio*.

The sequence alignment allowed us to identify the conserved and the variable regions of the protein (Fig. 1). The amino-terminal region is highly conserved probably because it represents the portion of the molecule that interacts with the activation loop and maintains the catalytic pocket in active conformation [Sarno et al., 2002]. In contrast, the carboxyl-terminal region is more variable: the major difference between vertebrates and the others considered organisms consists in the length of this portion [Tapia et al., 2002] which seems to be typical for vertebrates, excluding zebra-fish (*Danio rerio*). In *Homo sapiens* and in all of the upper vertebrates examined, this region contains 54 residues (337-391), except for *Xenopus laevis*, which has an adjunctive serine at position 344 (Fig. 1). It is intriguing that the calculated mass of this additional tail (4.986 kDa) corresponds exactly to the 5 kDa peptide, spontaneously cleaved during purification [Sarno et al., 1996]. Finally, we noted that in *Homo sapiens* about 50% of the C-terminal residues are prolines, which indicates an increment of the proline content during evolution.

On the basis of the CK2α sequence alignment, we constructed a phylogenetic tree (Neighbour Joining; p-distance) (Fig. 2). This enzyme is present in many distant taxa, from lower eukaryotes to mammalians. Analyzing evolution of the proline content, our attention was called by P384, which is the most distal proline present in *Homo sapiens*. In *X. laevis*, this proline is substituted by an alanine. Starting from this observation, we designed a human mutant where this proline is substituted by alanine. Because proline residues usually act as helix interrupters, we tried to enforce this effect by substituting another adjacent proline (Pro 382), which is instead highly conserved during evolution.

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S. pombe          ---------------------------------------------------- 332
Y. lipolytica     ---------------------------------------------------- 339
P. tetraurelia    ---------------------------------------------------- 332
S. cerevisiae     ---------------------------------------------------- 372
H. sapiens        SMPGGS-TPVSSANMMSGISSVPTPSPL-GPLAGSPVIAAANPLGMPVPAAAGAQQ 391
O. cuniculus      SMPGGS-TPVSSANMMSGISSVPTPSPL-GPLAGSPVIAAANPLGMPVPAAAGAQQ 391
R. norvegicus     GMAGGS-TPVSSANMMSGISSVPTPSPL-GPLAGSPVIAAANSLGIPVPAAAGAQQ 391
M. musculus       SMAGGS-TPVSSANMMSGISSVPSPSPL-GPLAGSPVIAAANSLGIPVQAAAGAQQ 391
G. gallus         NMPGGS-TPVSSASMMSGISSVPTPSPL-GPLAGSPVISATTTLGMPVPAAAGAQQ 391
X. laevis         NMPSGSSTPVSSASMMSGISTVPTPSAL-GSLAGSPVISATNTLGTPVAAAAGATQ 392
S. frugiperda     NS-------------------PTP----------------NALQGPISTTE---- 353
D. melanogaster   NQQ---------------------------------------------------- 336
H. pulcherrimus   TQPNTSTSATNSSSASIDTKARPSGTIPGAPLIPTPTAQTVSPIPNPSPAAAVAP- 393
C. elegans        EQADGQ-------------------------------GASNSASSQSSDAKIDGA 360
B. rerio          ------------------------------------EGALAISSSTTT------ 348
O. sativa         PQ------------------------------------------------------ 333
Z. mays           PQ------------------------------------------------------ 333
T. aestivum       AQ------------------------------------------------------ 333
N. tabacum        TQ------------------------------------------------------ 333
A. thaliana       SQ------------------------------------------------------ 186
D. discoideum     ---------------------------------------------------- 337
T. parva          ---------------------------------------------------- 420
L. mexicana       AENGGGGASVCNEENEH-KRAPRIES------------------------------ 371
L. chagasi        AENGGGGASVCNEENVHEKRVPRIES------------------------------ 360
T. brucei         --------AECTARIREKKRGNVVTE------------------------------ 349
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Fig. 1. Primary structure analysis of the carboxyl-extremity of different CK2α subunits. Variations in the carboxyl-terminal portion (residues 351-391) of 25 eukaryotic α subunits: the two prolines 382 and 384 substituted are underlined. We listed the accession number of each sequence with the respective organism source: P19138 *Homo sapiens*, P19139 *Rattus norvegicus*, P33674 *Oryctolagus cuniculus*, Q60737 *Mus musculus*, P21868 *Gallus gallus*, P28020 (α′) *Xenopus laevis*, Q9NI95 *Hemicentrotus pulcherrimus*, O76484 *Spodoptera frugiperda*, AAL39698 *Drosophila melanogaster*, P18334 *Caenorhabditis elegans*, Q90457 (α′) *Danio (Brachydanio) rerio*, Q9AR27 *Oryza sativa*, Q9ZR52 *Zea mays*, Q94IG2 *Triticum aestivum*, Q93XN0 *Nicotiana tabacum*, Q02720 *Dictyostelium discoideum*, P28547 *Theileria parva*, P40231 *Schizosaccharomyces pombe*, Q92390 *Yarrowia lipolytica (Candida lipolytica)*, Q966Z2 *Paramecium tetraurelia*, Q963F4 *Trypanosoma brucei*, P15790 *Saccharomyces cerevisiae*, Q9GRT0 *Leishmania mexicana*, O77008 *Leishmania chagasi*, Q94F17 *Arabidopsis thaliana*.

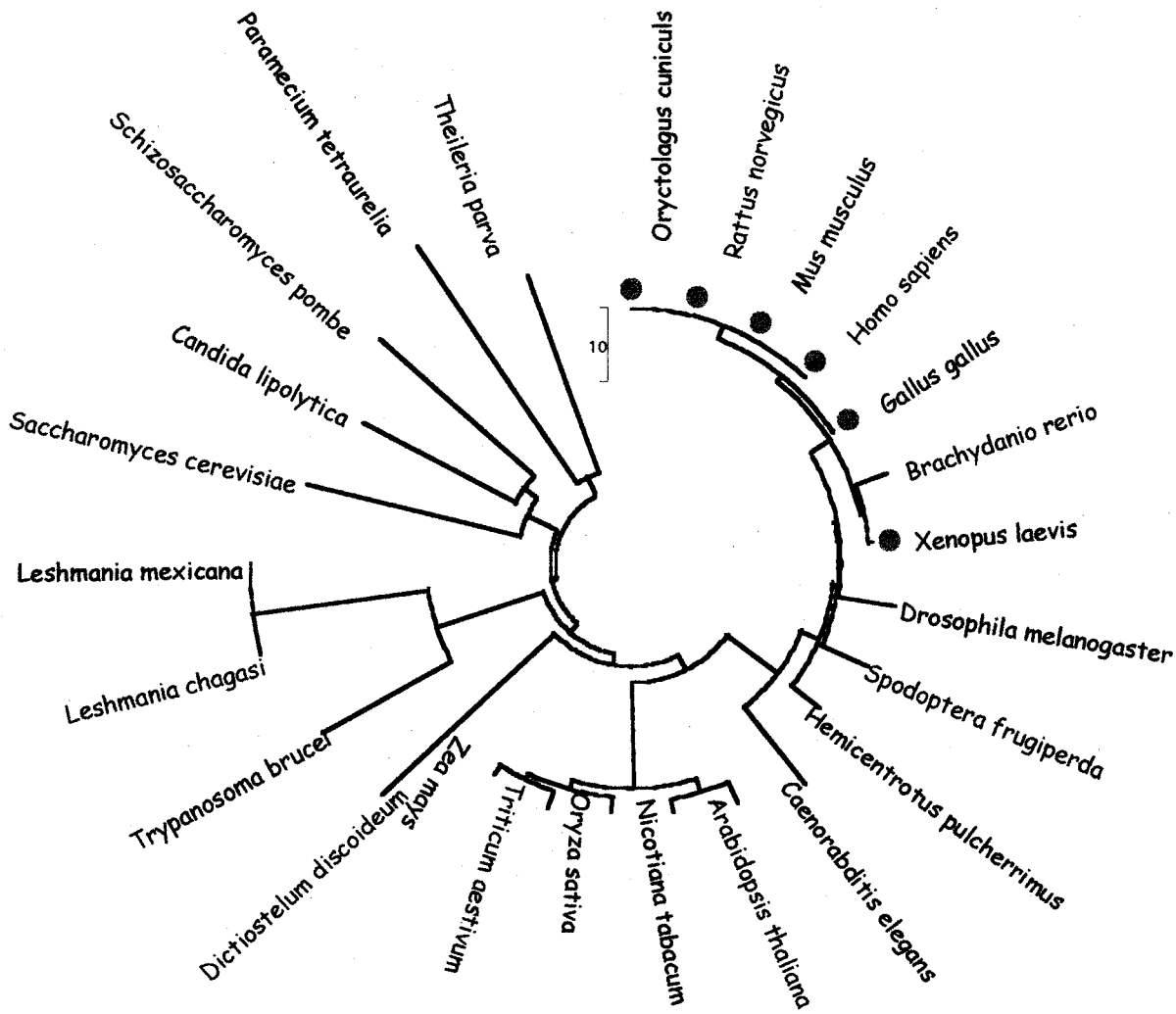

Fig. 2. Phylogenetic tree of CK2α subunit. Neighbor-joining tree (p-distance) illustrating the relationships among the primary sequences of the above 25 α subunits. The tree was constructed on the basis of the primary structure alignments as described in the text. The grey dots indicate the sequences with the adjunctive C-terminal tail.

Secondary Structure Prediction

The native α subunit was subjected to GOR IV prediction: the results of this theoretical calculation gave for its tail a high probability of an unfolded conformation. The high content of prolines at the C-terminus of the catalytic subunit could explain the inability of the tail to assume a stable folding in vitro, even under physiological ionic conditions. According to our hypothesis, GOR IV predicted that the double amino acid substitution should partially revert the random coil structure of the C-terminal tail (Table I). In fact, an increasing α helix content of about 3% (from 30.95% to 33.76%) was predicted to occur in the PA 382-384 mutant. In our opinion, the lack of β sheet in CK2α, which has been inferred from GOR IV predictions, should be due to the protein clusters available on this

TABLE I. Theoretical Prediction of the Percentages of the Main Secondary Structures Occurring in Native and PA 382-384 Mutant Subunits as Calculated by GOR IV Algorithm

Secondary structure	Native subunit (%)	Mutant subunit (%)
α-helix	30.95	33.76
Extended strand	21.74	21.74
Random coil	47.31	44.50

program. Anyway, this discrepancy with the experimental data becomes negligible because all our data are relative to a fixed control represented by the native recombinant subunit.

Electrophoretic Characterization

The native and the mutant subunits were compared on the basis of their electrophoretic patterns: the features of the two proteins were checked on 12.5% SDS–PAGE (Fig. 3A) and confirmed by immunoblotting (Fig. 3B).

The native subunit displays the classical doublet, rather than a single 45 kDa band, which is indicative of the proteolysis occurring during the purification procedures [Sarno et al., 1996]. According to our expectations, the mutant subunit is resolved mostly as a predominant 45 kDa band, whereas the degradation band at 40 kDa is reduced. We assumed this band to derive from loss of a 5 kDa peptide spontaneously cleaved during purification of the human enzyme [Niefind et al., 2000, 2001].

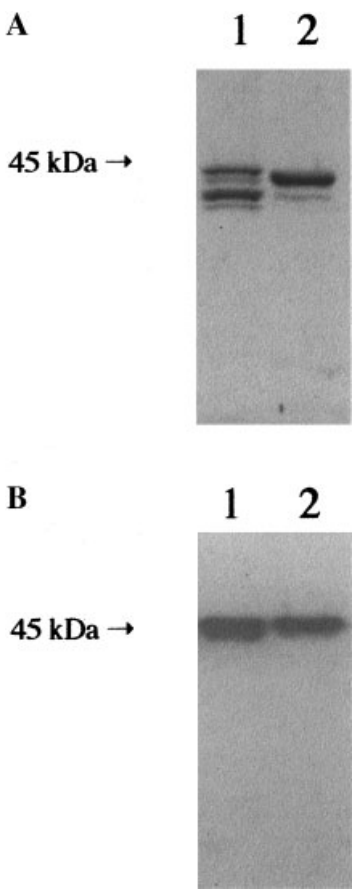

Fig. 3. Comparison between mutant and native recombinant α subunits. Mutant and native subunits were separated by electrophoretic run on 12.5% SDS–PAGE: (**lane 1**) native subunit; (**lane 2**) mutant subunit. The detection was performed both by Coomassie staining (**A**) and by Western blot (**B**), as described in Materials and Methods section.

The electrophoretic analysis points to the expected result: substitution of the two distal prolines with alanines makes the mutant protein less degradable during the purification procedure.

Characterization of the Secondary Structure of Native and Mutant Subunits

The secondary structure of the two subunits has been analyzed by means of Circular dichroism spectroscopy. Our strategy in fact was to verify if the substitution of two prolines at the C-terminus could allow the protein to assume a helical folding in this region, detectable by comparing the CD spectra of the native and the mutant subunits. The far-UV CD spectra of the native and mutant α subunits were acquired in 15 mM phosphate buffer, pH 8 (Fig. 4). When we compare the two spectra, we appreciate a marked difference in the region around 222 nm, which is considered indicative of the α helix content of a protein. Each spectrum was then analyzed by CONTIN program (Table II), which estimated a higher content of α-helix of the mutant with respect to the native subunit. The percentage of helical structure increases from 27% to 30% after Pro → Ala substitutions. This result fits very well with the theoretical prediction previously carried out by GOR IV algorithm, which calculated a similar increase of the helical folding as a consequence of the two substitutions (Table I).

We can conclude that both the theoretical and the experimental approach point to propagation of the helical folding along the C-terminus as a consequence of the double amino acid substitution.

Functional Characterization of the Mutant Versus Native Subunit

In order to understand if a more ordered folding of the C-terminal tail led to differences in the catalytic activity of the enzyme, we carried out a functional assay of both forms of CK2α. The V_{max} and K_m values, as well as the overall phosphorylation efficiencies expressed by the V_{max}/K_m ratios [Sarno et al., 1996], are summarized in Table III. Figure 5 shows the double reciprocal plots used to calculate the kinetic constants both for the native and the mutant subunits.

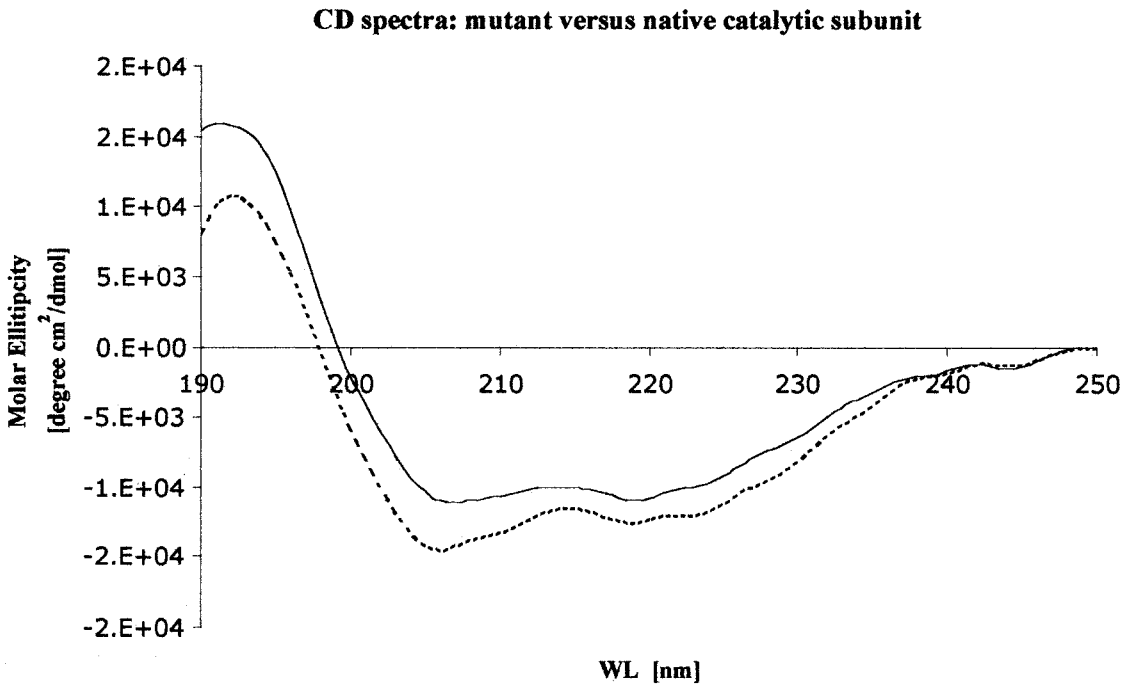

Fig. 4. Circular dichroism spectra of mutant versus native α subunit. Standard Circular dichroism spectra were acquired for the native (dashed line) and the mutant (continuous line) α subunits. Both the samples were analyzed in 15 mM phosphate buffer, pH 8.0, as described in Materials and Methods section.

The mutant protein preserves its catalytic activity, but its ability to transfer the phosphate group is reduced with respect to the native one. We measured the following constants for the native α subunit: $K_m = 260$ μM, $V_{max} =$ 0.345 μmol phosphate $min^{-1} mg^{-1}$, specific activity = 0.071 μmol phosphate $min^{-1} mg^{-1}$. These values are comparable to those previously measured by other authors [Grankowski et al., 1991] for the catalytic subunit alone. When the same assays were performed on the mutant subunit, we observed reduction of all the kinetic constants: K_m decreases to 30 μM, V_{max} to 0.060 μmol phosphate $min^{-1} mg^{-1}$, and the specific activity to 0.026 μmol phosphate $min^{-1} mg^{-1}$.

We wish to emphasize that the double Pro → Ala substitution produces slight improvement of phosphorylation efficiency, which increases from 1.33 in the native subunit to 2.0 in the mutant. It should be noted that even if the mutant exhibits a phosphorylation efficiency very similar to that of the native subunit, the fundamental kinetic constants are very different. In the mutant, the marked decrease of K_m is counterbalanced by drastic reduction of V_{max}. In order to analyze these differences, we performed the ANOVA test ($f = 10.2$, $P = 0.0002$) and the results indicate a highly significant difference between the native and the mutant.

Proteolytic Degradability

As reported above, the substitution of two prolines with alanines induces α helix propaga-

TABLE II. Secondary Conformation of Native and PA 382-384 Mutant Subunits Estimated on the Basis of the Far-UV CD Spectra

CK2α	α-helix (%)	β-sheet (%)	β-turn (%)	Remainder (%)
Native subunit	27 ± 1.3	40.0 ± 2.2	7.0 ± 1.7	26.0 ± 1.9
PA 382-384 mutant subunit	30.0 ± 1.8	17.0 ± 3.3	15.0 ± 1.6	39.0 ± 3.5

The values represent the percentages of the main secondary structures supplied by CONTIN algorithm. The experiments have been repeated at least five times for every sample. For each percentage the corresponding error has been reported.

TABLE III. Kinetic Constants for the Model Substrate Measured for Native and Mutant α Subunits

Kinetic constants	Native subunit	Mutant subunit
K_m [μM]	257.14	29.13
V_{max} [μmol phosphate $min^{-1} mg^{-1}$]	0.345	0.060
V_{max}/K_m [μmol phosphate $min^{-1} mg^{-1} \mu M^{-1}$]	$1.34e^{-3}$	$2.07e^{-3}$
Specific activity [μmol phosphate $min^{-1} mg^{-1}$]	0.071	0.026

The values have been calculated by the double reciprocal plots as described in Materials and Methods section. Each value is the mean of at least five different experiments tested with the support of the statistical analysis ANOVA ($f = 10.2$; $P = 0.0002$).

tion along the C-terminus of the protein. We supposed that a more folded extremity could make the protein less sensitive to proteolytic degradation. To test this hypothesis, we performed a controlled proteolysis assay, both on the native and mutant α subunits.

When the digestion products were visualized on 11% SDS–PAGE (Fig. 6), several considerations could be made. Firstly, on both the proteins trypsin digestion reduces, up to complete disappearance, the two main bands at 45 and 41 kDa, which are characteristic of the human CK2α. An opposite trend is observed for the two lower molecular weight bands (39 and 37.2 kDa), which increase upon the trypsin digestion.

According to our predictions, the mutant is less susceptible to trypsin digestion than the native subunit. In fact at low trypsin concentrations (below 0.5 μg tot), the mutant preserves almost completely the 45 kDa band, which disappears early in the native protein. In fact, digestion with 0.2 μg of trypsin cuts the native subunit in three bands. In contrast the mutant

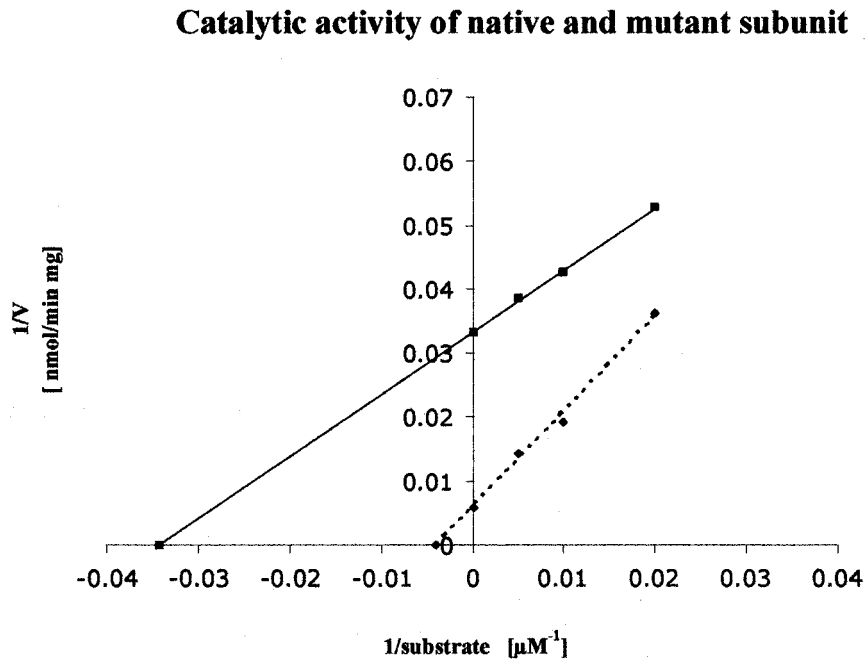

Fig. 5. Functional characterization of mutant versus native α subunit. The catalytic assays performed on native (dashed line) and mutant (continuous line) α subunits were represented by Lineweaver-Burk plot: the reciprocal of phosphorylation rate [nmol phosphate $min^{-1} mg^{-1}$] versus the reciprocal of substrate concentration [μM^{-1}]. Both the plots fit very well with the linear regression analysis ($R^2_{Mutant} = 0.9964$; $R^2_{Native} = 0.9998$).

is almost completely intact: the densitometric analysis shows that the 45 kDa band represents about 70% of the total sample, with just a light (23%) band at 39 kDa. When trypsin concentration increases up to 0.5 μg, the band at 39 kDa becomes evident also in the mutant, but the intact form is still present. We wish to underline that the band at 41 kDa (the 'natural' degradation product of the catalytic subunit) is less abundant in the mutant also in native conditions. At the highest trypsin concentration (1 μg), the uncleaved form completely disappears in the

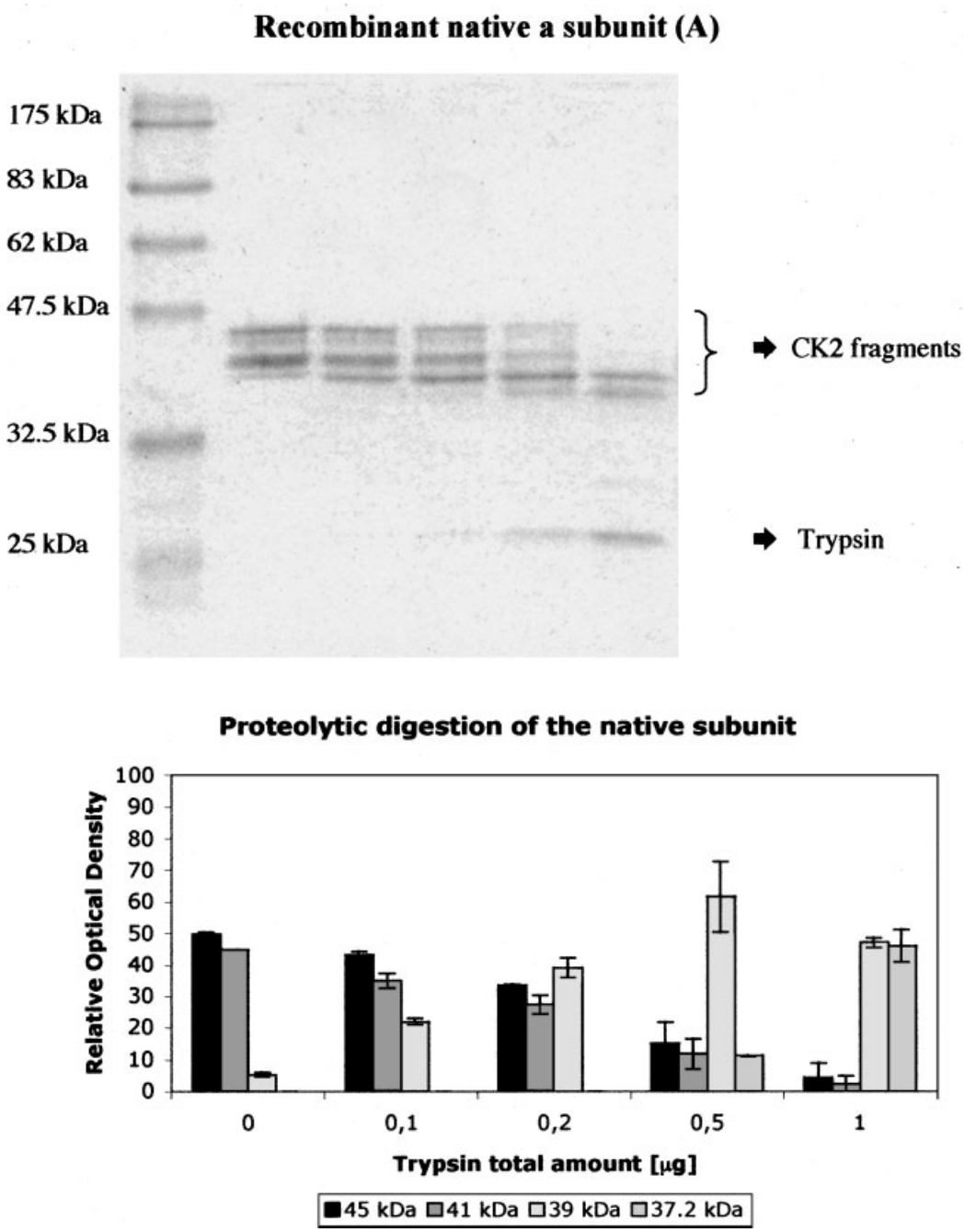

Fig. 6. Limited proteolytic digestion of native and mutant α subunits. The digestion products produced by trypsin digestion of both CK2α subunits were analyzed by electrophoretic run on 11% SDS–PAGE and detected by Coomassie staining. For each sample, we acquired the densitometric profile by means of Image J software. The histogram reports the relative optical density of the main bands appearing in every lane both for native (**A**) and mutant (**B**) subunits.

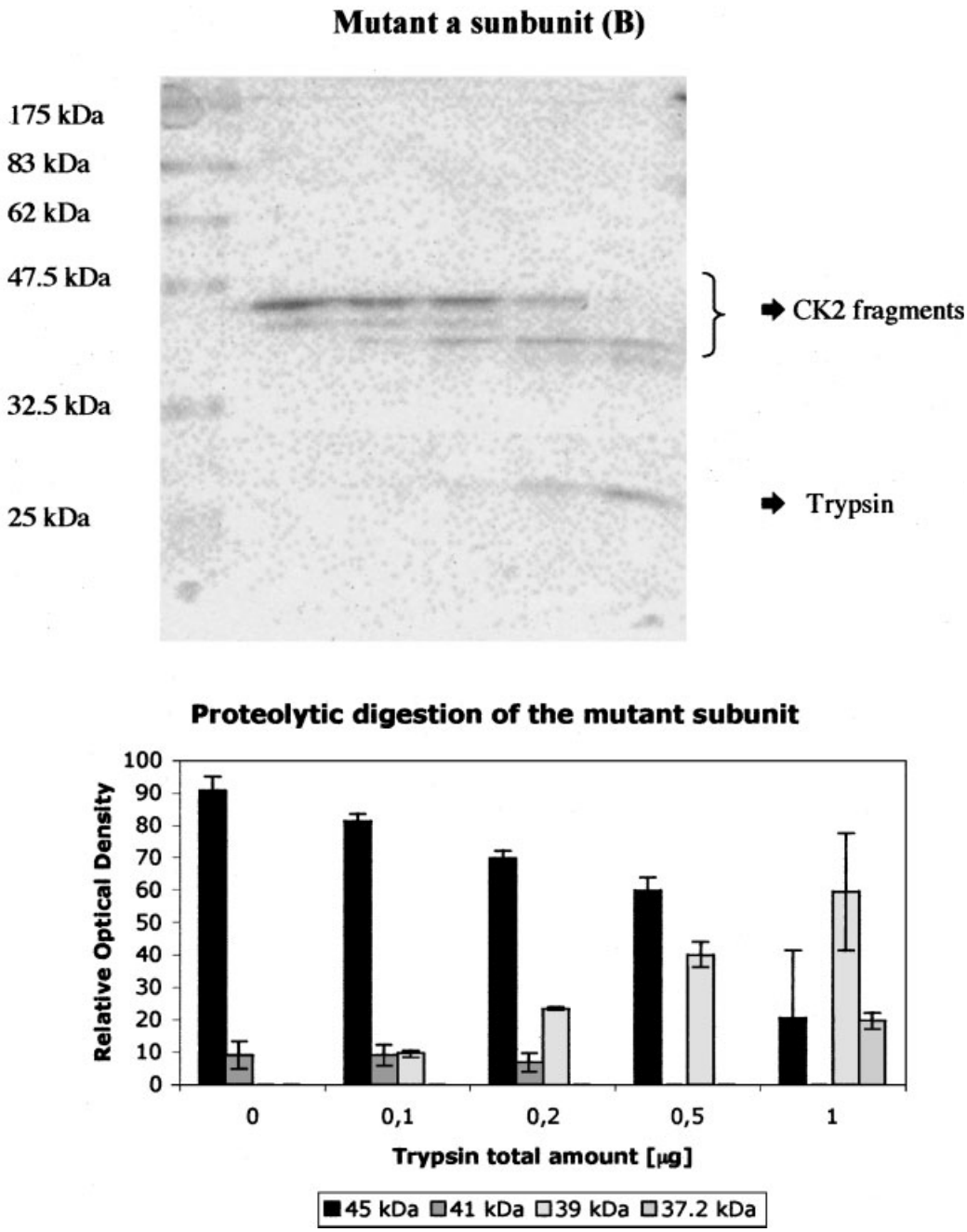

Fig. 6. (*Continued*)

native subunit, whereas it is still present (20%) in the mutant protein.

Thermal Denaturation of Mutant Versus Native Subunit

In order to understand if a more ordered structure at the C-terminus affects thermal stability of the entire subunit, we recorded the denaturation profile of the mutant versus the native enzyme. We performed this analysis by different spectroscopic techniques, which allowed us to evaluate the changes in the secondary conformation induced by the temperature gradient. This integrated approach is suitable to verify if the physical–chemical properties of the mutant subunit are altered with respect to the native one.

By UV spectroscopy, we recorded absorbance values at 280 nm and expressed them as a percentage of the reference value (absorbance of

the sample at 25°C). Both subunits show a decrement of the absorbance at 280 nm upon the temperature increase (Fig. 7A), but the slope of the curve is lower for the native subunit than for the mutant one. These data indicate that the native protein has a minor thermal stability than the mutant one.

In CD spectra molar ellipticity at 222 nm is usually kept to be representative of the α helix content of the protein. Therefore, we plotted

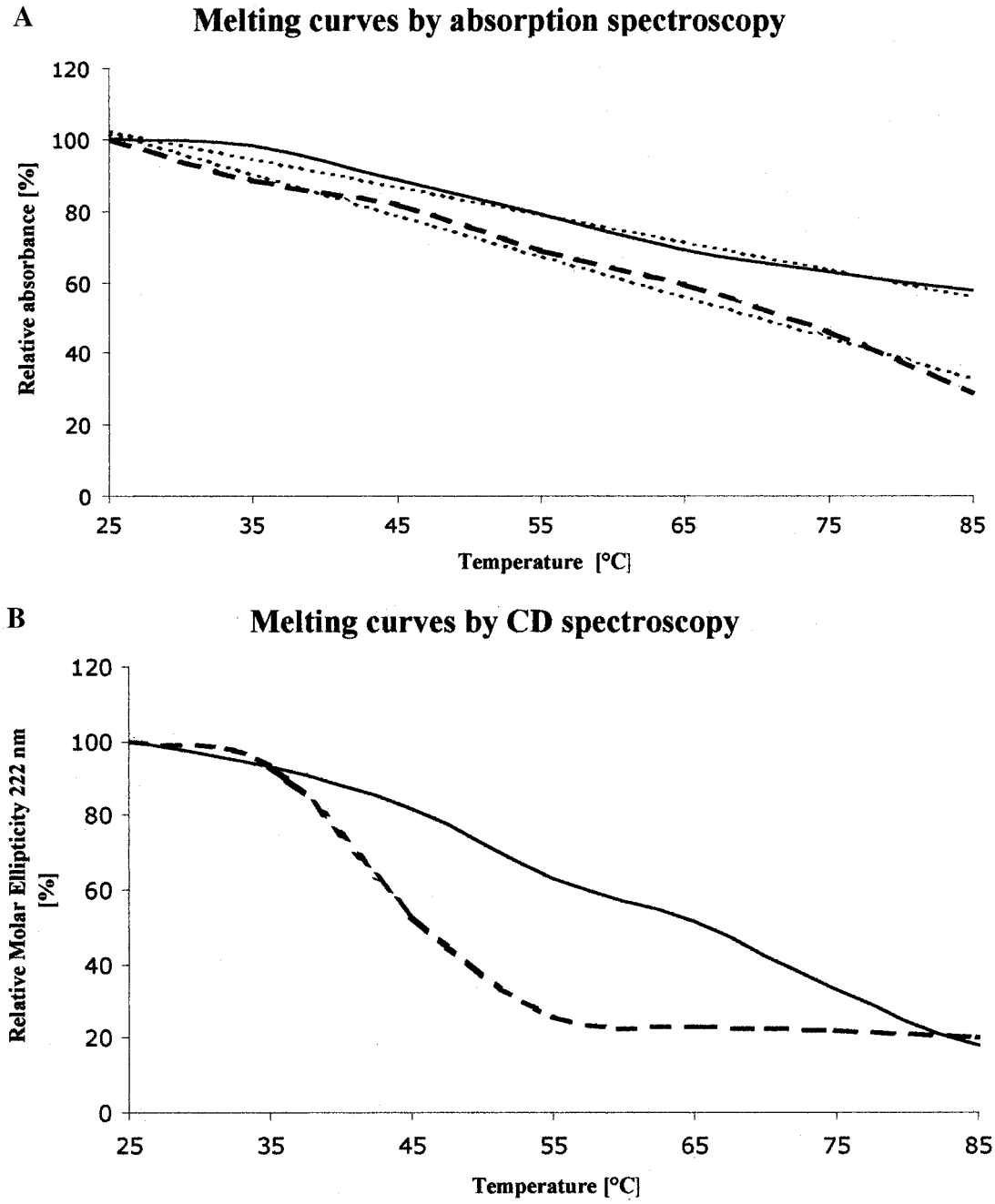

Fig. 7. Thermal denaturation profile of native and mutant CK2α subunit. **A**: Absorbance at 280 nm was recorded at increasing temperatures for native (dashed line) and mutant (continuous line) subunits. Both the curves show a linear decrease of the absorbance as a function of the temperature and fit well with the linear regression analysis. Anyway, we observe significant differences in the coefficient of the lines, pointing to a higher stability of the mutant protein with respect to the native one. **B**: Molar ellipticity at 220 nm was measured at increasing temperatures for native (dashed line) and mutant (continuous line) subunits. While the melting curve of the native subunit can be fitted by a sigmoid curve, that of the mutant is fitted by a linear regression ($R^2 = 0.985$).

the molar ellipticity values at increasing temperatures as percentage of the reference (corresponding to 25°C). These data confirm the results obtained by UV spectroscopy. As the temperature increases from 25 to 85°C, the CD spectra change considerably, showing a gradual decrease in the molar ellipticity at 222 nm. Also in this case, the mutant subunit appears to loose its secondary structure less than the native one (Fig. 7B).

This result confirms our prediction: the substitution of the two prolines at the C-terminus led to an α helix propagation in this region, making secondary conformation of the protein more stable to the temperature gradient.

CONCLUSIONS

Protein kinase CK2 is an important enzyme implicated in many cell-cycle events. It is required during cell-cycle progression at G2/M transition and checkpoint control [Vilk et al., 1999; Ford et al., 2000; Sayed et al., 2001]. Its activity has been found to be consistently enhanced in several solid tumors and leukemias [Munstermann et al., 1990; Roig et al., 1999]. The majority of studies on CK2 concerned its α subunit, with particular attention paid to the structure/function relationship of the N-terminal extremity, which is responsible for the catalytic activity.

The C-terminal extremity has been disregarded for a long time. Only recently, its importance has been recognized [Tapia et al., 2002], in particular for its involvement in regulating CK2 phosphorylation activity. Despite their importance, the mechanisms of CK2 regulation are not fully clarified, anyway phosphorylation represents one of the principal mechanisms for its modulation. In fact, phosphorylation of human CK2α is cell-cycle depending [Litchfield et al., 1992]. In mitotic cells, for example, the catalytic subunit is selectively phosphorylated at four sites localized within the 60 C-terminal residues. All these phosphorylation sites are 'proline-directed phosphorylation sites' because they require the presence of an adjacent proline residue [Bosc et al., 1995].

The aim of our study was to clarify the structural and functional features of the carboxyl terminus of the CK2α, starting from a phylogenetic investigation. In fact, the last 54 amino acids are not present in all the organisms, but they are typical of almost all vertebrates. Moreover, composition of this additional tail changed during evolution becoming richer in prolines.

Our strategy started from the hypothesis that the substitution of two distal prolines with alanines in the carboxyl tail of the human α subunit should allow helical folding in this region. This portion of the protein is not present in maize, the unique native CK2 with a fully solved 3D structure, and up to now its structure could be only predicted. Our theoretical calculations supplied for this tail a high probability of being in a random coil conformation. When we substituted two distal prolines with alanines, the theoretical prediction indicated us an increased α helix content.

Starting from this analysis, we created a PA 382-384 mutant of CK2α and we characterized it in structural and functional terms. According to the theoretical predictions, the double amino acid substitution produced a mutant with an increased helix content, as measured by the CD analysis.

When the catalytic activity was tested by phosphorylation assays, the mutant appeared to preserve its functionality, even if it was reduced with respect to the native protein. In particular, the K_m of the mutant is lower, indicating higher affinity for the substrate, whereas its V_{max} is markedly reduced. The combination of these effects yielded about similar phosphorylation efficiency for mutant and native subunits. Modifications of the kinetic constants in the mutant subunit could be explained by the presence of a rigid helix at the C-terminal extremity. If the enzyme conformation is less flexible the reaction velocity could be reduced, but the affinity for the substrate could increase. Recently, it has been demonstrated that residues near the C-terminal portion (Phe 324, Ile 327) interact with hydrophobic residues linked to the catalytic domain and a deletion of the C-terminal residues reduces the enzyme activity [Tapia et al., 2002]. Therefore, it is not surprising that a double substitution in the same region might affect the catalytic activity of the subunit.

Proceeding with our hypothesis, we supposed that the propagation of the helical folding towards the C-terminus should make the protein more stable. The thermal denaturation profiles acquired by different and integrated techniques on both the subunits confirm an

increased thermal stability of the mutant. In fact, formation of a helical region, also if this is very short, at the C-terminal extremity should require a higher temperature to be denatured.

We supposed that a more ordered extremity should produce a further effect on the protein. The mutant should be protected from proteolytic digestion, which is typical of the native subunit under purification and storing conditions. When controlled proteolysis was carried out on both subunits, the mutant showed improved biological stability, which confirms our predictions. We chose trypsin as proteolytic enzyme because it starts digestion from the C-terminus of the protein, therefore representing an optimal probe for testing this portion of the protein. The improved proteolityc stability of the mutant can be explained by supposing the double amino acid substitution produces two different effects. The first one may be related to the more ordered conformation of the C-terminus, which could somehow protect against trypsin action. It is well known that unfolded regions are usually more sensitive to proteolytic degradation. The second effect could be due to the steric masking of one or more specific sites of trypsin digestion, due to the change of protein conformation.

In conclusion, the changes introduced in the primary sequence of the C-terminal region affect the structure and function of the entire CK2α, with its biophysical and biochemical features changing according to our expectations. In particular, our attempt to stabilize the human form of the catalytic subunit has been successfully obtained.

A last consideration could be made with regards to the physiological significance of the fragility of the C-terminus tail, which represents a highly phosphorylable region involved in the enzymatic regulation. Can its intrinsic fragility be a mechanism to control CK2 activity via its degradation?

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