Protein Engineering vol.12 no.2 pp.119–127, 1999

Structural interpretation of site-directed mutagenesis and specificity of the catalytic subunit of protein kinase CK2 using comparative modelling

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The catalytic subunit of protein kinase casein kinase 2 (CK2α), which has specificity for both ATP and GTP, shows significant amino acid sequence similarity to the cyclin-dependent kinase 2 (CDK2). We constructed site-directed mutants of CK2α and used a three-dimensional model to investigate the basis for the dual specificity. Introduction of Phe and Gly at positions 50 and 51, in order to restore the pattern of the glycine-rich motif, did not seriously affect the specificity for ATP or GTP. We show that the dual specificity probably originates from the loop situated around the position His115 to Asp120 (HVNNTD). The insertion of a residue in this loop in CK2 α subunits, compared with CDK2 and other kinases, might orient the backbone to interact with the base A and G; this insertion is conserved in all known CK2α. The mutant ΔN118, the design of which was based on the modelling, showed reduced affinity for GTP as predicted from the model. Other mutants were intended to probe the integrity of the catalytic loop, alter the polarity of a buried residue and explore the importance of the carboxy terminus. Introduction of Arg to replace Asn189, which is mapped on the activation loop, results in a mutant with decreased k_{cat}, possibly as a result of disruption of the interaction between this residue and basic residues in the vicinity. Truncation at position 331 eliminates the last 60 residues of the α subunit and this mutant has a reduced catalytic efficiency compared with the wild-type. Catalytic efficiency is restored in the truncation mutant by the replacement of a potentially buried Glu at position 252 by Lys, probably owing to a higher stability resulting from the formation of a salt bridge between Lys252 and Asp208.

Keywords: casein kinase 2/comparative modelling/mutagenesis/protein kinase/protein phosphorylation/structure prediction

Introduction

Protein kinases CK2 (casein kinase 2) comprise a ubiquitous family of enzymes in eukaryotes. They are usually composed of catalytic subunits (α and α′) of molecular mass 40000–43000 and regulatory subunits (β) of about 26000 (Pinna, 1990; Issinger, 1993; Allende and Allende, 1995) arranged as heterotetramers of $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ composition. Higher order aggregates depend on the salt conditions (Chester *et al.*, 1995; Valero *et al.*, 1995). Members of the CK2α family are most closely related to the cyclin-dependent kinases (CDKs) in terms of similarity in the amino acid sequences. However, the amino acid sequences of the regulatory subunits, CK2β, show no significant similarity to those of various cyclins, which are regulators of CDKs, except for the presence of a short stretch similar to the 'cyclin destruction box'.

There are several characteristics that make CK2 especially interesting. First, it phosphorylates more than 100 different protein substrates, including enzymes that participate in nucleic acid synthesis, transcription and translation factors, oncogenes and tumour suppressor proteins, cytoskeleton proteins and proteins that participate in signal transduction (Allende and Allende, 1995). Second, CK2 may be involved in regulation of cell division; it can act as an oncogene in transgenic mice (Seldin and Leder, 1995) and decreases in CK2 activity due to injection of antibodies (Pepperkok *et al.*, 1994), antisense oligonucleotides (Lorenz *et al.*, 1993) or temperature-sensitive mutants (Hanna *et al.*, 1995) arrest cell division in the G1/S and G2/M restriction points of the cell cycle. Third, CK2 can use GTP as well as ATP as phosphate donor.

The cloning and expression of the α, α′ and β subunits of CK2 from several species have provided a basis for studies on the structure and function of this enzyme. Detailed studies of mutations and deletions of the β subunit of CK2 indicate that the acidic and proline residues in the region between amino acids 55 and 64 cause an inhibition of the catalytic activity of the α subunit (Boldyreff *et al.*, 1993; Hinrichs *et al.*, 1995). On the other hand, the carboxyl region of β is responsible for its interaction with α and the amino terminus for dimerization of β (Boldyreff *et al.*, 1994; Kusk *et al.*, 1995). Mutations in the α subunit have been useful in defining the basic stretch of amino acids (74–80) as the site of interaction with heparin and other polyanionic inhibitors (Gatica *et al.*, 1994). Furthermore, the residues involved in peptide substrate recognition (Sarno *et al.*, 1996) and in β activation (Jacobi and Traugh, 1995) have been explored.

Crystal structure analyses of protein kinases (Cox *et al.*, 1994) have demonstrated that members of this very large superfamily have very similar three-dimensional structures. Co-crystallization of protein kinases with nucleotides, peptide substrates and inhibitors have permitted the identification of the regions of the kinases involved in the catalysis and in substrate interaction (Knighton *et al.*, 1991a,b, 1993; Bossemeyer *et al.*, 1993; Zheng *et al.*, 1993) and in the activation of the enzyme (Johnson *et al.*, 1996). Jacobi and Traugh (1992) examined the ATP/GTP specificity of CK2α and observed an increase in the affinity to ATP together with a reduction in GTP affinity after mutating a valine (at position 70) to Ala and a tryptophan (at position 176) to Phe. However, the changes observed were small, indicating that other residues must contribute to the nucleotide specificity in addition to those identified by this mutagenesis work (Bossemeyer, 1994).

In this work, we used site-directed mutagenesis and comparative modelling based on the known structure of cyclin-dependent kinase 2 (CDK2) to explore different regions of the molecule that are involved in the catalytic function of the α subunit of CK2.

Materials and methods

The recombinant α subunits of CK2 from Xenopus laevis

The cDNAs coding for the wild-type α subunit of CK2 from *X.laevis* and for the mutants ΔN118, H115F, N189R, Δ331 and Δ331 E252K were subcloned by digestion with *Nde*I and *Sal*I in a pT7.7 vector, modified to express a (His)6 tag at the amino end of the recombinant protein (pT7-7H6), in order to facilitate their subsequent purification using a nickel-chelating column (Cosmelli *et al.*, 1997). The mutant Y50F S51G was subcloned in the vector pT7-7, expressed and purified as described for CK2α. The expression and purification of the CK2β subunit from *X.laevis* were performed as described previously (Hinrichs *et al.*, 1993). The nucleotide sequence of the inserts coding for each of the modified proteins was determined by the dideoxynucleotide method (Sanger *et al.*, 1977).

Preparation of the mutants ΔN118, H115F and N189R

The deletion mutant ΔN118 was generated by overlapping primer extension PCR (Ho *et al.*, 1989) using initially the oligonucleotide pairs (5′ TTCGAACATGTCAACACAGATTTTAA 3′) and the T7 primer (5′ AATACGACTCACTATAG 3′), and with (5′ TTAAAATCTGTGTTGACATGTTCGAA 3′) and the reverse T7 primer (5′ ATTGGTAACTGTCAGACCAAG 3′). For the last amplification reaction the T7 and reverse T7 primers were used with the mixture of the products of the first two PCR reactions. The products were digested with *Nde*I and *Sal*I and subcloned into pT7-7H6 and the complete sequence was confirmed by sequencing.

Mutants H115F and N189R were prepared as given for ΔN118 using the same T7 primers and the appropriate oligonucleotides for the initial overlap extension PCR reactions were as follows:
mutant H115F: 5′ CTTGTCTTCGAATTTGTCAACAATACA 3′ and 5′ TGTATTGTTGACAAATTCGAAGACAAG 3′;
mutant N189R: 5′ CAACACGGACACGGTACTCCTGT 3′ and 5′ ACAGGAGTACCGTGTCCGTGTTG 3′.

Deletion mutants Δ331 and Δ331 E252K

The deletion mutant, Δ331, eliminating 60 amino acids at the carboxy terminus was obtained from wild-type CK2α clones in pT7-7 by PCR amplification using the following primer pairs: (5′ ATATCATATGTCAGGACCTGTGCCAAGT 3′) that contains *Nde*I restriction site and (5′ ATATCTGCAGCTACTGGTCCTTTACGATGGGATAG 3′) that contains *Pst*I restriction site.

Another clone, obtained after PCR amplification, had a random mutation that changed a codon coding for Glu252 to a codon that specified Lys. These mutants, Δ331 E252K and Δ331, were expressed in *Escherichia coli* and purified and kinetic constants were defined.

Preparation of the mutant F50 G51

The mutation of Y50 S51 to F50 G51 in the CK2α subunit was generated by overlapping primer extension PCR (Ho *et al.*, 1989). Initial PCRs were conducted with the following sets of primer pairs: 5′ CAAAGACTTCACCGAATTTGCCCCG 3′ and the T7 primer and with 5′ CGGGGCAAATTCGGTGAAGTCTTTG 3′ and reverse T7 primer.

For the last amplification reaction of the full-length cDNA, the T7 and reverse T7 primers were used with the mixed products of the initial amplification.

Assay of the CK2α phosphorylating activity and determination of the kinetic constants

The phosphorylating activities of CK2α and its mutants were assayed essentially as described previously (Hinrichs *et al.*, 1993). The assay mixture in a total volume of 30 μl contains 8 mM $MgCl_2$, 50 mM HEPES (pH 7.5), 0.5 mM dithiothreitol, 50 mM [γ-^{32}P]ATP (500–2500 c.p.m./pmol) or 150 mM [γ-^{32}P]GTP (2000–3000 c.p.m./pmol), 5 mg/ml dephosphorylated β-casein, 50 mM KCl and usually 1–2 pmol of CK2α subunit or its mutants. The incubation was carried out at 30°C for 10 min.

The reaction was terminated and the samples were processed on phosphocellulose filters. The peptide RRRDDDSDDD was used instead of casein where specified.

Initial velocity measurements were performed in duplicate under the standard conditions except for the varied substrate concentrations (ATP, 2–500 μM; GTP, 3–500 μM). Casein and peptide concentrations were varied from 0.05 to 15 mg/ml and 0.01 to 1 mM, respectively. The values presented have been corrected for those obtained in the absence of enzyme and are representative of the results obtained from two or three independent experiments performed with the same preparation of enzyme and where the standard deviation ranges from 5 to 14%. The apparent K_M and k_{cat} were calculated using the Microcal software.

Relationship among the catalytic subunits of CK2 isoforms

We used the multiple sequence alignment program MALIGN (Johnson *et al.*, 1993) in order to align all known sequences of the α and α′ subunits of various isoforms of CK2. The evolutionary distances between all pairs of members within the family were predicted from sequence dissimilarity scores provided by MALIGN. The program KITCH (Felsenstein, 1985) was used to generate phylogenetic relationships represented as dendrograms.

Extrapolation of the crystal structures of CDK2 to an α subunit

Sequences of protein kinases of known structure available in the Brookhaven protein data bank (Bernstein *et al.*, 1977) were aligned on the basis of structural features such as solvent accessibility, secondary structure and side-chain–main-chain hydrogen bonding patterns using the program COMPARER (Šali and Blundell, 1990; Zhu *et al.*, 1992). Sequences of catalytic subunits of CK2 were aligned with those of protein kinases by matching structural templates derived from aligned kinase structures. This analysis confirmed that CK2α is closer to the CDK2 than any other kinase of known structure (Table I). For this reason we primarily used the structure of CDK2 to model the α subunit of CK2.

The COMPOSER suite of programs (Blundell *et al.*, 1988; for a recent review, see Srinivasan *et al.*, 1996a), available in the SYBYL software (Tripos, St Louis, MO), was used for comparative modelling of the α subunit from *Xenopus* CK2. The active (Russo *et al.*, 1996a) and partially active forms of CDK2 (Jeffrey *et al.*, 1995) structures are bound to cyclin that results in significant structural changes in CDK2. These changes are unlikely to happen in CK2α as no cyclin binding

Table I. Percentage sequence identity and similarity[a] between protein kinase CK2α and protein kinases of known crystal structure[b]

Protein kinase with known structure	Percentage sequence identity with CK2α	Percentage sequence similarity with CK2α
Cyclic AMP-dependent protein kinase	21.6	61.5
Cyclin-dependent kinase 2	31.4	69.5
Casein kinase 1	14.3	57.0
Phosphorylase kinase	25.1	62.8
Calmodulin-dependent kinase 1	25.3	63.6
Insulin receptor kinase	18.8	59.7
FGF-receptor kinase	19.5	60.0
src family–hck kinase	16.2	59.7

[a]Percentage sequence similarity (N.Srinivasan, R.Sowdhamini and T.L.Blundell, unpublished results) is calculated by weighting amino acid replacements using a 20×20 mutation matrix.
[b]Only those structures with atomic coordinates available at the time of this work are considered.

takes place in CK2α. Further, the active form of CDK2 involves a phosphorylation at the activation loop that is not reported to happen in CK2α. Hence the three-dimensional model of the sequence of *Xenopus* CK2α was based on the inactive (de Bondt *et al.*, 1993; Schulze-Gahmen *et al.*, 1995, 1996) form of CDK2. The regions of CDK2 with no insertions or deletions (when aligned with the α subunit) are defined as structurally conserved regions (SCRs). These were similar in sequence to the α subunit and were largely composed of helical and extended strand regions defining the core of the protein. The substituted side-chains of CK2 were modelled on the basis of the orientation of the equivalent side-chain in CDK2 and also using a large number of rules relating side-chain and main-chain conformations derived from known 3D structures (Sutcliffe *et al.*, 1987).

While the core regions of CK2α are similar in sequence to those of CDK2 and can be modelled on the basis of CDK2, the loop regions involving insertions and deletions in the alignment pose a problem. Equivalent segments from known structures of other protein kinases can be used to minimize the errors in modelling such loops (Srinivasan and Blundell, 1993).

When no suitable loop was available from a homologue, a template matching procedure was used to rank candidate loops from other proteins (Topham *et al.*, 1993). For each loop, distances between three Cα atoms at either side of the loop were calculated. The database of known structures was then searched for fragments with the required number of residues in the fragment and distances that match those from flanking SCRs. Candidate loops were ranked by the template matching procedure of Topham *et al.* (1993). In this procedure, a structural template for every candidate loop is generated which characterizes the amino acid residue types that can be accommodated in a given position of the candidate loop. The amino acid sequence of the loop to be modelled was matched with the template. Conformational compatibility of the sequence to the model for a given fragment was thus assessed and ranked. The loop segment was modelled on the best template that has no short contacts with the rest of the model.

Energy minimization

The models were energy minimized in SYBYL using the AMBER force-field (Weiner *et al.*, 1984). During the initial cycles of energy minimization the backbone was kept rigid and side-chains alone were moved. Subsequently all atoms in the structure were allowed to move during minimization. This approach kept disturbance of the backbone structure to a minimum. Energy minimization was performed until all short contacts and inconsistencies in geometry were rectified. During the initial stages of minimization, the electrostatic term was not included as the main objective was to relieve steric clashes and to rectify bad geometry. The electrostatic term was invoked only at an advanced stage of the minimization.

Modelling of complexes

The binding of MnATP or MnGTP in the binding site of CK2α was modelled by using the structures of CDK2 complexed with ATP. The model of the uncomplexed form of CK2α was superimposed on the crystal structure of CDK2 and the position of ATP in CDK2 transferred to the model of CK2α. Due to the buried nature of this binding site, no major adjustments in the structure were necessary.

Numbering of the sequence of *Xenopus* α subunit is followed in this paper. The numbering of other kinase structures is used only where appropriate.

Results and discussion

Sequence alignments and phylogeny of CK2 α subunits

A search for the sequences of CK2 α subunits in SWISSPROT using BLAST and FASTA resulted in 18 hits for α (or α′). A multiple sequence alignment of homologous α subunits (data not shown) shows that the sequence identity varies between 50 and 99%, implying a highly conserved sub-family of protein kinases. Apart from the conserved hydrophobic residues many of the polar residues are also well conserved. Some of the conservation is probably for functional reasons. The region between 46 and 53 is characterized by a conserved sequence GRGKYSEV. This is characteristic of the ATP binding signature sequence L/I/V–G–X–G–X–F/Y/M–S/G–X–V and is conserved generally within the protein kinase family (Hanks and Hunter, 1995). The residue before Gly46 is variable but can be L, I or V. The occurrence of Y at 50, one of the two inhibitory phosphorylation sites, is characteristic of the CDK2 family of kinases. A similar role for this Tyr in the CK2 family is so far unknown.

The phylogenetic relationship, derived from dissimilarity scores of pairwise sequence alignments, is represented as a dendrogram in Figure 1. The variants of α subunits from lower organisms, such as yeast, cluster separately from α subunits of plant and mammalian origin. Variants from higher organisms are clustered in a recently evolved branch. While the α′ variants from higher organisms, such as chicken and human, cluster in a branch distinct from that of their α counterparts, the α′ variants from yeast and *Arabidopsis thaliana* are outliers that are clustered closer to their α counterparts.

Use of comparative modelling to understand selectivity and mutations

We have considered sequence, structure and functional state variations among many of the known protein kinase structures that we have aligned on the basis of tertiary structural properties, relationships and functionally important residues (N.Srinivasan and T.L.Blundell, unpublished results). All known sequences of α and α′ subunits of CK2 have been aligned with known protein kinase structures to obtain the final alignment between CDK2 and the *Xenopus* CK2 α subunit (Figure 2). The sequence *identity* between the two is about 31.4%, which is at the threshold of the ‘twilight zone’.

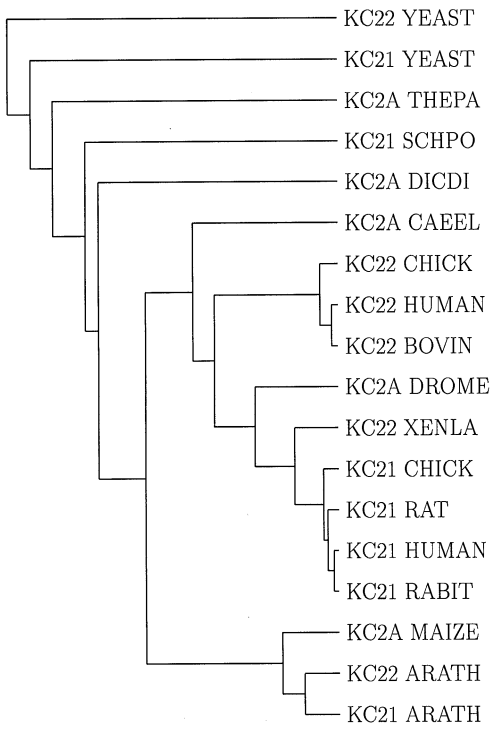

Fig. 1. Dendrogram depicting the relationship between various isoforms of α and α′ subunits of CK2. The 'distances' between various sequences are based on the dissimilarity scores in the multiple sequence alignment. The second part of the codes represents the source of the protein. The second part of the code and actual source are as follows: YEAST, *Saccharomyces cerevisiae*; THEPA, *Theileria parva*; SCHPO, *Schizosaccharomyces pombe*; DICDI, *Dictyostelium discoideum*; CAEEL, *Caenorhabditis elegans*; CHICK, *Gallus gallus*; HUMAN, *Homo sapiens*; BOVIN, *Bos taurus*; DROME, *Drosophila melanogaster*; XENLA, *Xenopus laevis*; RAT, *Rattus norvegicus*; RABBIT, *Oryctolagus cuniculus*; ARATH, *Arabidopsis thaliana.*

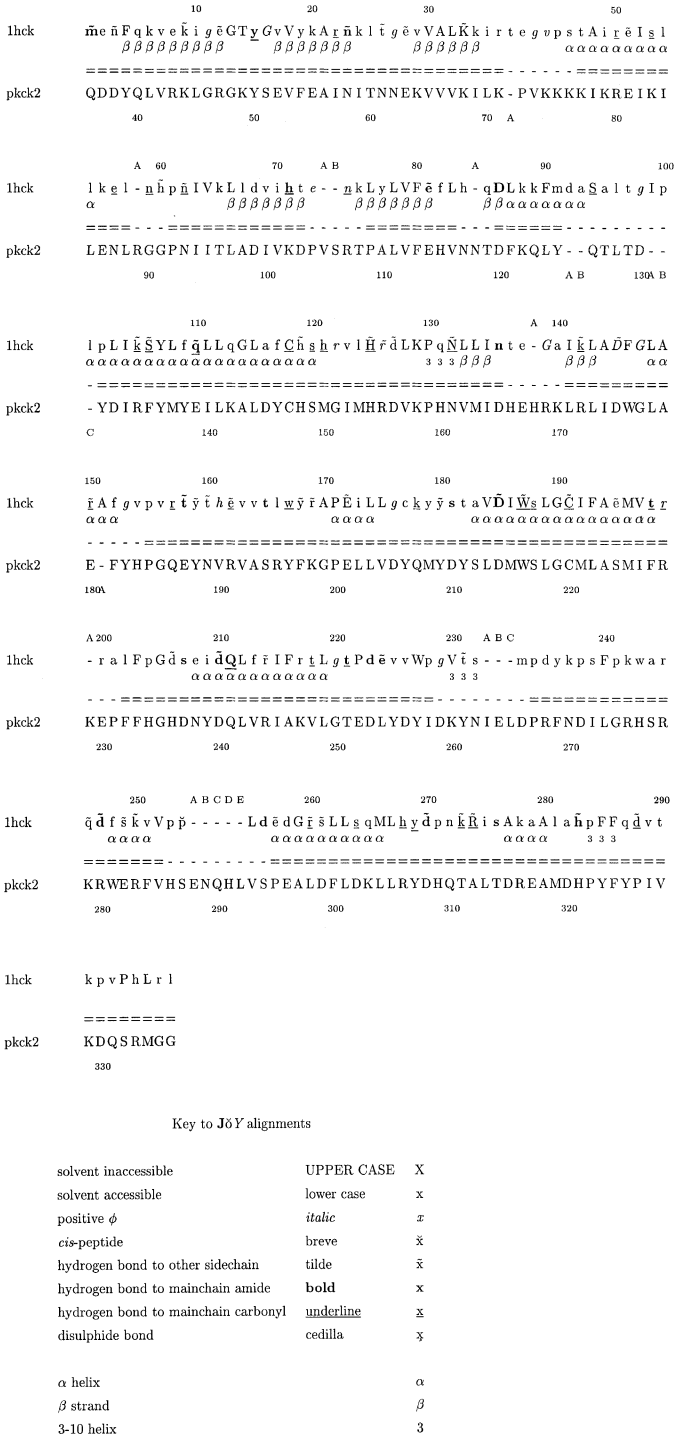

Fig. 2. Alignment of amino acid sequence of *Xenopus* CK2α with the inactive (1hck) form of CDK2. CDK2 numbering and *Xenopus* CK2α numbering are shown at top and bottom, respectively. The structurally conserved and variable regions used in the generation of the tertiary model of CK2 are indicated in the alignment by = and –, respectively. Figure produced using the program JOY (Overington *et al.*, 1990).

However, if the similarity of the residue substitution at each position is scored, the overall sequence *similarity* is about 69.5%, reflecting the fact that even in mismatch positions in the alignment most substitutions involve similar residues. As can be seen from the alignment, the similarities in the helical and β-strand regions are reasonably high. While most regular secondary structural elements of CDK2 seem conserved in CK2α, some helical regions of CDK2 are likely to be shorter in CK2α. The PSTAIRE helix of CDK2, which is known for conformational heterogeneity depending upon the activation state of CDK2, is replaced in CK2α by a sequence motif rich in lysine which is likely to interact with negatively charged ligands of CK2α (Hu and Rubin, 1990; Gatica *et al.*, 1994; Vaglio *et al.*, 1996).

Active, inactive, partially active and inhibited forms of CDK2 have been defined by X-ray analyses (de Bondt *et al.*, 1993; Jeffrey *et al.*, 1995; Russo *et al.*, 1996a,b). Some of these are phosphorylated. They provide information about structural variations in CDK2 and give clues to their functional relevance. The most significant variations include the movement of the N-terminal β-sheet-rich domain relative to the C-terminal α-helix-rich domain, the melting of the PSTAIRE helix in the active or partially active form and changes in the structure of activation loop (T-loop) where a permissive phosphorylation site (Thr160) is located. CK2α differs from CDK2 in not having a PSTAIRE helix and phosphorylation site(s) at the activation loop and hence the structural changes observed in different forms of CDK2 are likely to be less extensive in CK2α.

We have generated a model of the α subunit of *Xenopus* CK2α on the basis of the inactive form of CDK2 which is bound to ATP, unphosphorylated and not bound to cyclin (de Bondt *et al.*, 1993; Schulze-Gahmen *et al.*, 1996). Most residues of the activation loop of CDK2 have an equivalent

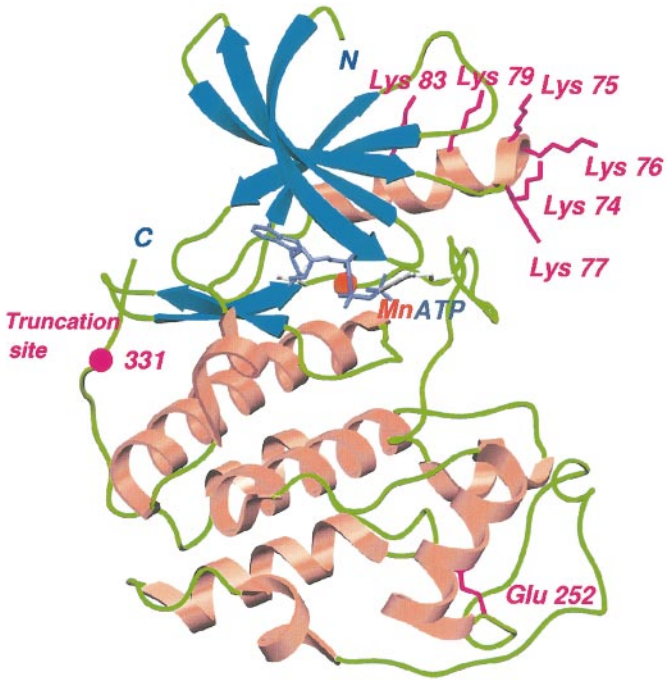

Fig. 3. Overall fold of the model of CK2. The α-helices, β-strands and loops and the bound MnATP are shown in different colours. The sites of truncation in the deletion mutant and the positions of lysine cluster and Glu 252 are indicated. Figure produced using the software SETOR (Evans, 1993).

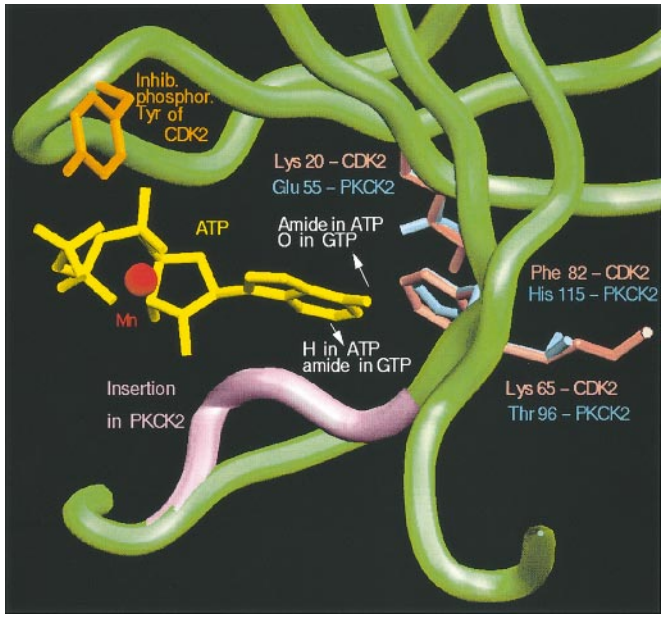

Fig. 4. Close-up of the MnATP binding region. The modelled loop with an insertion is shown superimposed on the backbone of CDK2. Key residue substitutions are highlighted. PKCK2 in this figure refers to protein kinase CK2. Figure produced using the software SETOR (Evans, 1993).

in CK2α and hence provide a good model for CK2α. However, the loop prior to this region has a residue deleted in CK2α and so is modelled by fitting a segment obtained from a search of all known protein structures. The models generated by COMPOSER were energy minimized to eliminate short contacts and to optimize bond lengths and angles and peptide planarity. The model based on the active form of CDK2 is shown in Figure 3.

The region 74–77 of CK2α is made up of four lysine residues in tandem (Figure 2) and these residues are conserved among most members of this family. This region forms a loop in the structure and is followed by a helix equivalent to the PSTAIRE helix of CDK2 that is known for its variation of conformation in different forms of the enzyme. Lysine residues also occur in the positions 79 and 83 which are in the helix. Interestingly, Lys75, Lys79 and Lys83 are situated on one face of the helix and hence the gross directionalities of these side-chains are the same. Significantly, this Lys-rich cluster occurs close to the activation loop, 178–192. The region following the activation loop involves Lys198, which is implicated to play an important role in substrate recognition (Sarno *et al.*, 1997). Figure 3 shows the positions of these lysine residues in the CK2α model. Potential variability of conformation of the activation loop and the helix (equivalent to PSTAIRE) containing the lysine cluster is further support for an involvement of many of these positively charged residues in recognizing acidic ligands (Gatica *et al.*, 1994). However Lys79 and Lys83 are not likely to be involved in heparin binding as suggested by Vaglio *et al.* (1996). The precise conformation of this region in the active form of CK2α is likely to be different from that in CDK2 as the orientation of the side-chain ligands of CK2α should be appropriate for recognizing substrates. Most of the lysine residues occurring in this region of CK2α are conserved among the members of CK2 family, implying a common key role of these residues in CK2α variants. This region may also play a role in the association of CK2 with cell nucleus (Rihs *et al.*, 1991). In addition Lys122 and Lys158 occur close to the activation loop and hence these could play a role in the recognition of the acidic ligands.

Specificity of CK2 for ATP and GTP

Most protein kinases use ATP exclusively as the phosphate donor substrate; however, CK2 can use GTP effectively in addition to ATP for this purpose. The glycine-rich signature sequence of protein kinases associated with ATP binding is present in *Xenopus* CK2 in the form of GRGKYS. The serine in this motif is substituted by glycine in many of the protein kinases. However, this feature is unlikely to explain the dual specificity of CK2, as much of the glycine-rich region is situated proximal to the phosphate group, rather than the adenine, in the tertiary structures of protein kinase complexes. Proximity of this residue to the phosphate group is also observed in the structures of CDK2 and is likely to be conserved in CK2α (Figure 2).

The main-chain polar atoms of Glu81 and Leu83 in CDK2 form hydrogen bonds with adenine. These interactions are suggested to be characteristics of adenine accommodation in protein kinases (Kobayashi and Go, 1997). There is a local structural similarity between the adenine recognition site in protein kinases and in DD-ligase, despite completely different tertiary structures (Kobayashi and Go, 1997). This region in protein kinases is located in a linker between a β-strand and an α-helix. Most significantly, in CK2α there is an insertion of a residue in this region (Figure 4); in *Xenopus* CK2α the sequence is HVNNTDFK (residues 115–122), with one of the asparagines as the likely insert (Figure 2). In the regions flanking this insertion (Figure 2), Leu87 of CDK2 is replaced by Phe121 in *Xenopus* CK2 and Phe121 is absolutely conserved within the CK2 family. Leu87 of CDK2 is buried and so

replacement by Phe in CK2α is likely to alter the local structure which is sensitive to the activation state of CDK2.

The NH_2 group of ATP is replaced by an oxygen in GTP and a hydrogen attached to a ring carbon in ATP replaced by NH_2 in GTP. In order to hydrogen bond with NH_2 of GTP, additional polar groups from CK2α are required, probably from main-chain atoms at the insertion at 118 or the side-chain atoms of the asparagines into contact with GTP. The loop conformation at this insert may vary to accommodate either ATP or GTP. This insertion occurs in all the members of CK2 family and occurs as an Asn–Asn motif in an overwhelming majority. In some cases one of the asparagines is replaced by a Ser, Asp or Lys, all of which have a preference for loop regions in proteins, frequently with positive ϕ conformation (e.g. Srinivasan *et al.*, 1991), suggesting that a characteristic orientation of polar groups in the main chain is maintained. The position of ATP/GTP could slightly vary depending upon whether ATP or GTP is present. The conformation of the loop containing insertion of an Asn residue may also vary depending upon whether ATP or GTP is bound. Hence the prediction of a precise interaction between this loop and the base (A or G) is difficult.

The replacements Phe82 of CDK2 by His115 in CK2α, Lys65 of CDK2 by Thr96 in CK2α and Lys20 of CDK2 by Glu55 in CK2α, which occur in the vicinity of the ATP/GTP binding site, will result in a significantly different charge distribution; but this can play only a minor role in the dual specificity of CK2α as not all are well conserved in the CK2 family.

In p34 cdc2 kinase there is an insertion (compared with CDK2 and other kinases of known structure) of aspartate in the region equivalent to the base-recognizing loop region in CK2α. Hence the loop conformation in cdc2 kinase could at least partly resemble the conformation of the equivalent region in CK2α. Interestingly, p34 cdc2 kinase is reported to have specificity to both GTP and ATP, but with a lower affinity for GTP (Erikson and Maller, 1989)

The family of GTPase corresponding to the elongation factor Tu and G proteins contain the sequence motif NKXD which is required for GTP interaction. Hwang and Miller (1987) mutated the Asp 138 of the Tu sequence to asparagine and observed a change in the specificity of the protein for the nucleotide triphosphate from GTP to XTP. The replacement of the Asp138 by Asn would allow the amide of the asparagine side-chain to stabilize, apparently by hydrogen bond formation with the N-1 of the xanthine base and its exocyclic 2-keto group (for a discussion, see Schimmel, 1993). The close resemblance to the G protein sequence motif, found in the CK2α in the region NNTD (117–120), supports the idea that this region is involved in the recognition of the guanine base.

Subsequent to the completion of our modelling work, Shah *et al.* (1997) reported their successful attempt to switch the specificity of Rous sarcoma virus tyrosine kinase from ATP to N^6-(cyclopentyl)-ATP. Their mutagenesis work involved replacement of both Val323 and Ile338 of Rous sarcoma virus tyrosine kinase by Ala. The engineered kinase showed specificity to N^6-(cyclopentyl)-ATP which is not recognized by the wild-type. These two mutated residues are equivalent to Val64 and Phe80 of CDK2 and occur just preceding the asparagine insertion region of CK2α. This work, in accordance with our deduction, emphasizes the importance of this region in determining the specificity to phosphotransfer substrate and implies that the Gly-rich region is not a determinant of the specificity to the base.

Mutation at the phosphate binding region

In order to explore the structural features responsible for the specificity of CK2α for ATP and GTP, we constructed a mutant involving two mutations, Tyr50Phe and Ser51Gly in the glycine-rich signature sequence region of *Xenopus* CK2α (Y50F S51G). The resulting sequence, GRGKFG, more closely resembles the consensus sequence in most protein kinases than does the glycine-rich motif in the wild-type of CK2α. Ser 51 is absolutely conserved in the CK2 family and Tyr 50 is conserved in all except one variant where it is replaced by a Phe. If the basis of dual specificity does reside in the glycine-rich region, this mutant should be more specific to ATP than to GTP like most kinases.

The results of K_M and k_{cat} measurements for this mutant are presented in Table II. There is a significant increase in the apparent K_M for ATP whereas there is an insignificant change for GTP. However, k_{cat} decreased for both ATP and GTP, to 20 and 6%, respectively, of the values obtained for the wild-type, resulting in an appreciable decrease in the catalytic efficiency (k_{cat}/K_M), especially for ATP. No significant change is seen in the apparent K_M for a peptide substrate or for casein.

This mutant binds the β subunit and is activated by the β subunit to an extent similar to that of the wild-type. Comparison of the kinetic constants of the holoenzyme indicates similar changes caused by the mutation except that the effects both on K_M and k_{cat}/K_M are not pronounced in the tetramer.

In the inactive form of CDK2, the accessibility pattern of the GTYG motif in the glycine-rich region is buried–buried–exposed–buried (Figure 2). Extrapolating the accessibility patterns to CK2α, it appears that Ser 51 in the wild-type can be buried in the unactivated form of CK2α. There are two aspects of the mutation Ser51 → Gly that are important to consider. First, the buried Ser in the inactive form is likely to play an important role in forming a hydrogen bond with a polar group in the phosphate or the protein. As can be seen in Figure 4, the sites of mutation are proximal to the phosphate group but not to adenine. Disruption of a buried polar interaction should alter the structure significantly as buried and hydrogen bonding polar residues are well conserved within the family of homologous protein structures and often play a key role in maintaining the integrity of the fold (Overington *et al.*, 1990). Second, replacement of a buried serine by glycine would mean a significant reduction in the volume and such disruptions are likely to alter the packing in the buried region and induce conformational changes in the vicinity which are difficult to predict. Replacement of Tyr50 by Phe also contributes to reduction in the volume.

Although the presence of Ser at position 51 instead of Gly in CK2α might be required for the integrity of the structure of the ATP/GTP binding pocket, Ser is unlikely to be involved in an interaction with adenine or guanine. Instead, serine is situated proximal to the phosphate group and it might interact with phosphate and/or with another buried polar group in the protein. Substitution by glycine at position 51 could then result in a rearrangement of the polar groups in the vicinity of the phosphate and this could induce a conformational change at the loop which recognizes the base and hence alters the shape of the ATP/GTP binding pocket. Predictions of the exact conformational changes that would be caused by the mutation are difficult, because the loop with an insertion of asparagine

Table II. Kinetic constants of CK2 α and its mutants[a]

	CK2 α subunit						CK2	
	Apparent K_M (μM)		k_{cat} (s^{-1})		k_{cat}/K_M (s^{-1} M^{-1})		Protein substrate: apparent K_m	
	ATP	GTP	ATP	GTP	ATP	GTP	Casein (mg/ml)	Peptide (μM)
WT	13.4	24.4	6.9	5.9	8580	4030	1.9	177
Y50F S51G	35.4	16.7	1.4	0.37	659	369	1.1	333
N189R	28	29.5	1.3	0.6	765	339	1.1	178
H115F	4.6	23	0.73	–	2644	–	2.0	210
Δ331	19.7	45	2.1	2.5	1777	911	N.D.	256
Δ331 E252K	20.5	39	6.3	6.4	5000	2744	N.D.	256

[a]Experimental conditions are given in Materials and methods.

in CK2α is not conserved in CDK2. The results of the mutagenesis suggest that one or more of the interactions with ATP is lost owing to the change in the shape of the binding pocket, whereas orientations of the polar groups that recognize guanine are less affected in the mutant.

Mutations in the region binding adenine/guanine (H115 VNNT D120)

Three-dimensional modelling of CK2α proposed an involvement of the region 115–120 in the recognition of ATP/GTP as substrate. In order to test this proposition, two mutants were produced. In one of the mutants, His115 was changed to Phe (H115F) whereas the other mutant had a deletion at Asn118 (ΔN118). The H115F mutant showed no significant changes in the K_M for either nucleotide, casein or peptide substrates, although a notable decrease in k_{cat} was observed. Significant conformational changes in this loop of the mutant resulting in instability of the active conformation of the mutant may result in such reduced k_{cat} values. The recombinant CK2α subunit containing the deletion ΔN118 was observed to be very unstable and it was impossible to determine the catalytic properties on this subunit alone. The presence of the regulatory CK2β subunit was required to stabilize the kinase activity and, therefore, the recombinant holoenzyme mutated in the α subunit was used for these studies. As shown in Table III, this mutant has a significant increase in the apparent K_M for GTP and a less significant effect on ATP affinity. No changes were observed with casein or peptide as substrate.

Mutations that alter the integrity of the activation loop

A characteristic feature of the active form of many protein kinases is the charge neutralization of the phosphorylated residue, at the activation loop, by nearby conserved basic residues (Johnson *et al.*, 1996). Structure-based alignment of some of the sequences of protein kinases and the sequence of CK2α in a local region is shown below:

cAPK	Y	R^{165}	D	L	...	V	T^{197}	L
CDK2	H	R^{126}	D	L	...	Y	T^{160}	H
CK1	Y	R^{130}	D	I	...	K	N^{177}	L
CK2α	H	R^{155}	D	V	...	Y	N^{189}	V

For example, in cyclic AMP-dependent protein kinase (cAPK) Arg165, which precedes the catalytic base, Asp166, is one of the three basic residues neutralizing phosphorylated Thr197. In CDK2, Thr160 is one of the two permissive phosphorylation sites and it is topologically equivalent to Thr197 of cAPK. In partially active or active forms of CDK2 Thr160 could interact with Arg126, which is equivalent to Arg165 of cAPK.

Asn189 in CK2α is equivalent to the permissive phosphorylation site Thr160 in CDK2. In addition, this site is also equivalent to nonphosphorylatable Asn177 of CK1 wherein Asn177 interacts with Arg130, which precedes the catalytic base Asp131. We have mutated Asn189 in the T-loop of *Xenopus* CK2α to Arg (N189R) resulting in an active enzyme. However, this mutation did not affect the apparent K_M for ATP, GTP, casein or peptide substrates (Table II). On the other hand, k_{cat} is decreased 5-fold for ATP and 10-fold for GTP.

A superposition of active, inactive and partially active forms of CDK2 shows that relative orientations of the phosphorylatable Thr160 and the interacting Arg126 differ. While in the active form of CDK2 Thr160 interacts with Arg126, in the inactive form the entire T-loop, including Thr 160, is swung away from the Arg126. In the unphosphorylated, cyclin-bound form of CDK2, Glu162 occupies the position of the phosphate (if Thr160 is phosphorylated) on Thr160, so that charge neutralization takes place. This implies that even in unphosphorylated or non-phosphorylatable situations the transformation from inactive to active form of a kinase would tend to bring the arginine close to the Thr (or Asn in the case of CK1 and CK2).

Although no phosphorylation occurs in the activation loop of CK1, the structural features of CK1 (Xu *et al.*, 1995; Longenecker *et al.*, 1996) at the activation loop are similar to CDK2 and cyclic AMP-dependent kinase (Johnson *et al.*, 1996). However, the hypothesis that Asp189 of CK2α plays a similar role to that of Asn177 of CK1 is not substantiated by the results obtained with the N189R mutant, which, although catalyticaly less efficient than the wild-type enzyme, retains measurable activity and similar affinity for all of the substrates analysed (Table II).

Truncation mutants and CK2α Δ331 E252K

Truncation of the α subunit at amino acid 331 (Δ331) leads to an active enzyme that has an apparent K_M for the substrates tested that is not appreciably different from the full-length protein (Table II). The catalytic efficiency of the enzyme, however, is diminished to about one-third of that of the wild-type. The location of this truncation in the tertiary structure is indicated in Figure 3.

These results suggest that the last few residues of CK2α (from 331) are not participating significantly in the activity of the protein as they do in cAPK, protein kinase C and few other protein kinases (e.g. see Srinivasan *et al.*, 1996b). Their contribution could be to maintain the integrity of the catalytic region. Further truncation at positions 285 or 185 of CK2α leads to inactive enzymes, as expected since these truncations

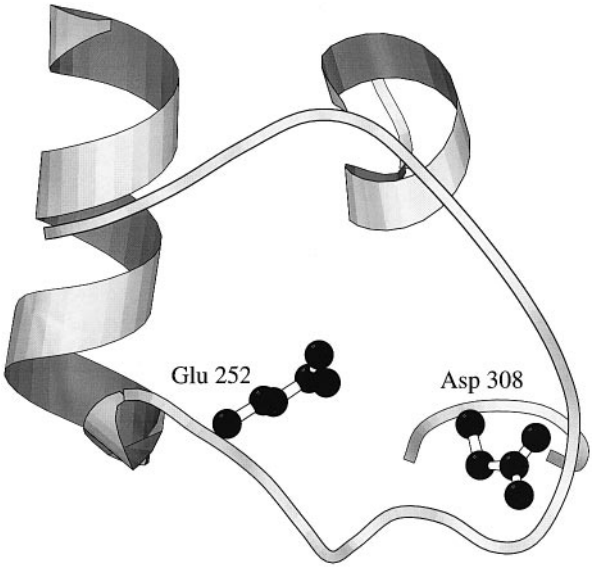

Fig. 5. A close-up of the mutation site, Glu252, in the model. Side chain of Asp308 which could form a salt-bridge with Lys252 in the mutant is shown with Glu252. Figure produced using the software MOLSCRIPT (Kraulis, 1991).

eliminate one or several of the conserved domains common to protein kinases (M.Antonelli, unpublished results). The negative effect on k_{cat} of the truncation mutant (at amino acid 331) does not occur in a naturally occurring shorter α-type subunit such as α′ subunits from zebrafish (348 residues) or human/chicken (350 residues). Indeed, the k_{cat} of the α′ subunit of zebrafish is similar to that of the full length (7.2 min^{-1}) (Antonelli *et al.*, 1996). The sequence similarity and conservation tend to be poor at the amino- and carboxy-terminal regions of homologous protein kinases leading to ambiguity in defining precise boundaries of the kinase domains (Hanks and Hunter, 1995). Hence it is possible that reduction in length of a longer variant may not lead to the same structural changes as in a shorter, natural homologue.

Interestingly, the fall in the catalytic activity caused by the truncation in the *Xenopus* variant is reverted by the mutation Glu252Lys (mutant Δ331 E252K). This truncated mutant regains the k_{cat} value of the full-length enzyme and has all the other properties, including apparent K_M and activation by the β subunit, as in the wild-type enzyme.

Glu252 occurs at the helix-rich domain of CK2 in an otherwise conserved loop following an α-helix (Figures 2 and 3). Glu252 is replaced by Pro222 in CDK2, which is buried in both the active and inactive forms. Hence Glu is expected to be buried in the structure of CK2α. The tertiary structure is particularly sensitive to the local structure and interactions of such buried polar residues and hence it might have an effect on the activity. In fact, Asp308 is the only polar residue close to Glu252 (Figure 5) and these are unlikely to form a stable interaction. Substitution of Glu252 by Lys, a positively charged residue, should result in a more favourable salt bridge with Asp308. This could lead to an increased stability of the structure, compensating for the loss of interactions of the carboxy termini with the core and may contribute to restoring kinase activity.

Comparison of the model with the crystal structure of CK2α from maize

Subsequent to the submission of this paper, the crystal structure of CK2α from *Zea mays* at 2.1 Å was published (Niefind *et al.*, 1998). The recombinant enzyme in the presence of magnesium ion and ATP crystallized in the active conformation. The N-terminal region (which lacks similarity with CDK2 and hence not modelled in our study) interacts with the activation loop and this feature is unique among the protein kinases of known structure. The active centre is occupied by an ATP molecule with ill-defined electron density. Part of the ATP is disordered. However, the position of ATP is found to be slightly shifted, compared with other protein kinases.

Table III. Kinetic constants of recombinant CK2 holoenzyme and mutant CK2 α2 ΔN118 β2[a]

	K_M (μM)	
	ATP	GTP
CK2 α2 β2	24	37
CK2 α2 ΔN118 β2	49	146

[a]Experimental conditions are given in Materials and methods. The standard assay was used except that 100 mM KCl, 5 mg/ml substrate casein and 3 pmol of β subunit were added. A 3-fold stimulation of the mutant α activity by β subunit was observed before purification, at which point the β subunit was added to the preparation for stabilization.

We compared our model of CK2α (*Xenopus*) with the crystal structure of CK2α (*Zea mays*). The location and relative orientation of various helices and β-strands in the model match well with the crystal structure. The most pronounced differences occur in two regions: the activation loop and a variable loop region in the C-terminal lobe. It is interesting that the conformation of the activation loop in the crystal structure is very similar to that in the phosphorylase kinase, which also lacks a permissive phosphorylation site in the activation loop. However, the conformation of the activation loop in our model was based on that of the inactive form of the CDK2 structure. The position of ATP in the model is also based on the CDK2 structure and differed slightly from the ATP position in the crystal structure of CK2α.

Almost all the deductions made based on the model are found to be consistent with the crystal structure. In the crystal structure, the Lys-rich region in the N-lobe folds as a helix as predicted by modelling, and this contributes to the formation of the Lys cluster as predicted. The predicted interaction between Arg155 and Asn189 is present in the crystal structure although it is weak. The loop containing an insertion of Asn (in the region 117–118) is close to the base of ATP and an alteration in the loop could influence the location and specificity to the base.

Conclusions

The combined approaches of three-dimensional structure modelling and site-directed mutagenesis of the catalytic subunit of *X.laevis* CK2 allowed us to define structural features that affect the catalytic activity and substrate specificity of this enzyme.

The structural modelling exploited the significant sequence similarity between CDK2 and CK2α in order to address the molecular basis of the ability of CK2α to use both ATP and GTP as phosphate donors. The analysis suggests that asparagine residues at 117 and 118 could influence this dual specificity. The mutant with one of the asparagine residues in the insertion deleted showed reduced affinity to both GTP and ATP. However, the affinity for GTP is much lower than for ATP, in agreement with the modelling studies. Our analysis also provides insights into the design of new mutations that could

affect nucleotide affinity. The study of certain site-specific mutations in the Gly-rich signature sequence suggests further sites that could affect the dual capacity of CK2α to use both GTP and ATP as substrates. We used the analysis of known kinase structures to test the postulated importance of the neutralization of Arg155 by residues in the activating loop such as Asn189. Finally, the effects of truncation and substitution mutations in the carboxyl end of the enzyme have been explained by the tertiary structure.

Acknowledgements

The authors thank Drs Schomburg, Pinna and Niefind and their colleagues for making the crystal structure of CK2α (*Zea mays*) available before public release. This research was supported by a grant from the Wellcome Trust to T.L.B. and by grants from the Council for Tobacco Research, the International Centre for Genetic Engineering and Biotechnology (ICGEB) and FONDECYT, Chile, to J.E.A. Two of us (N.S. and V.D.) are supported by fellowships from the Wellcome Trust.

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Received February 26, 1998; revised October 30, 1998; accepted November 11, 1998