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Site-Directed Mutagenesis in the Research of Protein Kinases - The Case of Protein Kinase CK2

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1. Introduction

Protein kinases constitute one of the largest and best-explored superfamilies of mammalian genes. The human genome encodes approximately 518 protein kinase genes, and the majority of their proteins have been characterized to some extent. Many of protein kinases have been implicated in signal transduction pathways that regulate growth and survival of cells, indicating their potential role in cancer (Manning et al., 2002a). Indeed, most cancers are associated with disregulation of protein kinases or by loss or damage of cellular protein kinase inhibitors (Brognard & Hunter, 2011; Johnson, 2009; Pearson & Fabbro, 2004).

A typical protein kinase (based on 478 known enzymes) is characterized by the presence of a highly homologous kinase catalytic domain of about 300 amino acid residues. This domain serves three distinct roles: (a) binding and orientation of the phosphate donor (ATP or, rarely, a different nucleoside triphosphate) in a complex with a divalent cation (usually Mg^{2+} or Mn^{2+}); (b) binding and orientation of the protein substrate; and (c) transfer of the γ-phosphate from the NTP to the hydroxyl moiety of the acceptor residue of the protein substrate.

In response to a variety of regulatory signals, protein kinases phosphorylate specific serine, threonine, or tyrosine residues within target proteins to modify their biological activities. Phosphorylation of several proteins in the cell creates recognition and/or regulatory sites that influence many properties of the target proteins (*e.g.*, catalytic activity, localization, sensitivity to proteolytic degradation, protein-protein interaction, etc). In eukaryotes, Ser/Thr and Tyr protein kinases (Note: The single- and three-letter codes for the amino acids are given in Table 1 in the chapter by Figurski et al.) play a key role in molecular networks controlling the activity of various signaling proteins (Brognard & Hunter, 2011; Cohen, 2002). Ser/Thr and Tyr-protein kinases form the largest protein family in the human genome (Pandit et al., 2004). They constitute about 2-3% of the proteomes of other model organisms, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Manning et al., 2002 a, b; Goldberg et al., 2006; Plowman et al., 1999). Based on the conserved features of catalytic domains of eukaryotic protein kinases, Hanks and coworkers have placed the kinases into various classes, groups, and subfamilies (Hanks et al., 1988).

The first 3D structure of a protein kinase was determined for PKA by X-ray crystallography. It revealed the basic bi-lobed scaffold formed by N- and C-terminal lobes that has been observed in all the protein kinase structures solved to date. The N-terminal lobe of the kinase fold comprises of an anti-parallel β-sheet made of five β-strands (β1 – β5) and a single αC-helix. The C-terminal lobe is larger and is mainly composed of α-helices. The nucleotide- and substrate-binding pockets are located in the cleft between the two lobes. The phosphate groups of ATP are positioned for phosphotransfer by their interactions with conserved residues in the N- and C-terminal lobes. These include a glycine-rich loop characterized by the GXGXXG motif (where X represents any amino acid) between the β1 and β2 strands, a Lys residue localized by a salt bridge formed by a Lys-Glu pair (K72 and E91), and Mg^{2+} ions. The conserved Asn (N171) and Asp (D184) further coordinate the metal ions. The catalytic loop situated in the C-terminal lobe contains aspartate (D166), referred to as the catalytic base that facilitates extraction of a proton from the hydroxyl side-chains of the phospho-sites of the substrates. The activation segment (20–30 residues in length) caps the C-terminal lobe. This segment forms a part of the substrate-binding pocket and shows high structural variation in the active and inactive kinase structures.

The grouping of the protein kinases based on catalytic subunit sequence similarity results in clustering of kinases that share functional features, such as preferred sites of phosphorylation, the mode of regulation and cellular localization. The similarity in the amino acid sequence of the catalytic domains of protein kinases has proven to be a good indicator of other features held in common by the different members of the family.

The diversity of essential functions mediated by kinases is shown by the conservation of approximately 50 distinct kinase families that have been identified in yeast, invertebrates, and mammals. Protein kinases can be clustered into groups, families, and sub-families. These classifications are based on sequence similarity and biochemical function. Among the 518 human protein kinases, 478 belong to a single superfamily whose catalytic domains are related in sequence.

Protein kinases are divided into 10 main groups, which organize diversity and compare genes between distant organisms (Miranda-Saavedra & Barton, 2007). The groups are named as follows: AGC, CAMK, CK1, CMGC, STE, RGC, Other, TK, TK, and Atypical. This classification was first used for characterization of the human kinome (all the kinases encoded in the genome) (Manning et al., 2002) and is based on an earlier classification by Hanks and Hunter (1995).

1.1 The CMGC kinases

This group of Ser/Thr protein kinases was named after the initials of some members (CDK, MAPK, GSK3, and CLK). It includes key kinases involved in growth, stress-response, and the cell cycle, and kinases involved in splicing and metabolic control. The four well characterized subfamilies of this group include the following: cyclin-dependent kinases (CDK) (Liolli, 2010); mitogen-activated protein kinases (MAPK) (Biondi & Nebreda, 2003; Zhang & Dong, 2007); glycogen synthase kinases (GSK) (Biondi & Nebreda, 2003); and cell kinases 2 (CK2), better known as protein kinase CK2 or casein kinase 2 (St-Denis & Litchfield, 2009). The CMGC group also contains other members. These are the following: SR protein kinases [phosphorylating serine- and arginine-rich proteins engaged in regulation of splicing and nuclear transport (Ghosh & Adams, 2011)] and DYRK protein

kinases, *i.e.* dual-specificity tyrosine-phosphorylated protein kinases, presumably involved in brain development (Becker et al., 1998).

1.2 Protein kinase CK2

Protein kinase CK2, formerly called casein kinase II, is a ubiquitous second messenger-independent protein kinase found in all eukaryotic organisms examined (Jensen et al., 2007; Niefind et al., 2009; Litchfield, 2003, Kubiński et al., 2007). This enzyme, which has been studied for over 50 years, is able to phosphorylate more than 300 substrates, on serine, threonine and tyrosine (Meggio & Pinna, 2003; Vilk et al., 2008). As the list of targets for CK2 continues to grow, it is becoming evident that CK2 has the potential to participate in the regulation of various cellular processes. Most of the CK2 substrates reported so far correspond to proteins that participate in cell signaling (Ahmed et al., 2002; Meggio & Pinna, 2003).

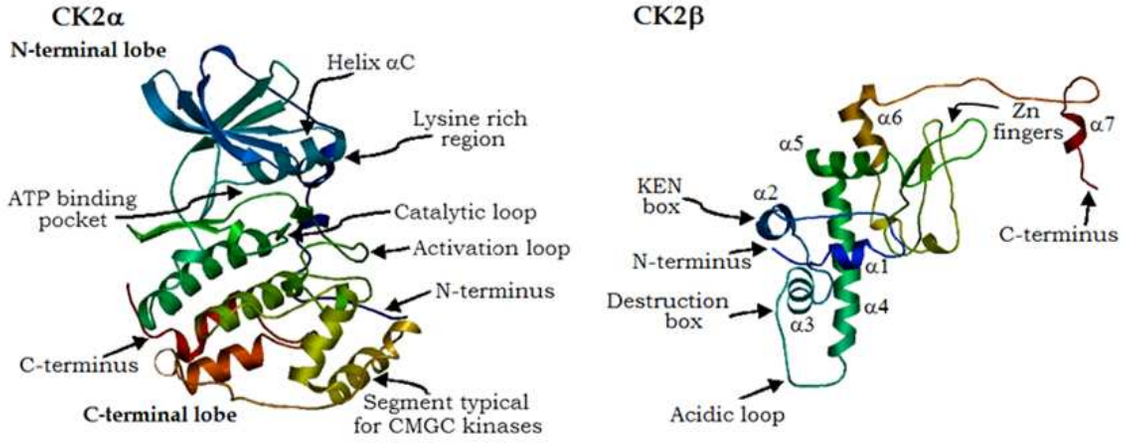

Fig. 1. Model structures of the CK2α and CK2β subunits from *Mytilus galloprovincialis* (Mediterranean mussel) (Koyanou-Koutsokou et al., 2011b)
The structural features of the CK2a and CK2β subunits were elaborated using the SWISS-MODEL Workspace for protein structure homology modeling (Arnold et al., 2006; Kopp and Schwede 2004) and 1ds5D (Batistuta et al., 2000) or 3EED (Raaf et al., 2008) as templates, respectively.

Protein kinase CK2 is distributed ubiquitously in eukaryotic organisms, where it appears as a tetrameric complex composed of two catalytic subunits (α/α′) associated with a dimer of regulatory β subunits (Figs. 1 & 2). The CK2 tetramer exhibits constitutive activity that can be easily detected in most cellular or tissue extracts in the absence of any stimulatory compounds. In many organisms, distinct isoenzymic forms of the catalytic subunit of CK2 have been identified (Glover, 1998; Kolaiti et al., 2011; Kouyanou-Koutsoukou et al., 2011a, b; Maridor et al., 1991; Litchfield et al., 1990; Shi et al., 2001). In humans, only a single regulatory CK2β subunit has been identified; but multiple forms of CK2β have been identified in other organisms, such as *Saccharomyces cerevisiae* (Glover, 1998). Complementary evidence indicates that dimers of CK2β are at the core of the tetrameric CK2 complexes (Graham & Litchfield, 2000; Pinna & Meggio, 1997). Tetrameric CK2 complexes may contain identical (*i.e.*, $\alpha_2\beta_2$ or $\alpha'_2\beta_2$) or non-identical (*i.e.*, $\alpha\alpha'\beta_2$) catalytic subunits (Gietz et al., 1995). Holoenzyme composition may influence CK2 properties, namely nucleotide and protein substrate specificity and sensitivity to effectors (Janeczko et

al., 2011). Protein kinase CK2 holoenzyme and its catalytic subunit alone can use both ATP and GTP as phosphate donors (Issinger, 1993).

The catalytic subunits of CK2α and CK2α′ are the products of separate genes located in different chromosomes. The 330 N-terminal amino acids exhibit over 90% sequence identity. However, the C-terminal sequences are unrelated (Olsten & Litchfield, 2004). The unique C-terminal domains of the catalytic subunits are highly conserved among species (*e.g.*, the amino acid sequences of the C-termini of the catalytic subunits of human and chicken CK2α and CK2α′ exhibit 98% and 97% identity, respectively), indicating a possible functional importance for this domain (Litchfield, 2003).

Although there is no known difference between the catalytic activities of CK2α and CK2α′, there is evidence that they exhibit functional specialization (Duncan & Lichfield, 2008; Faust & Montenarch, 2000). The CK2α subunit is phosphorylated at C-terminal sites (Thr344, Thr360, Ser362 and Ser360) by $p34^{cdc2}$ during cell cycle progression, while CK2α′ is not phosphorylated (St-Denis et al., 2009). Further evidence to support the idea that CK2α and CK2α′ have independent functions in the cell is provided by the different specificities of cellular binding proteins, such as CKIP-1, Hsp90, Pin-1, and PP2A (Olsten et al., 2005).

Despite the many isoforms of catalytic subunits, only one regulatory subunit has been identified for CK2β in mammals (Allende and Allende, 1995). In contrast to the activity of regulatory subunits of other kinases, such as PKA (cAMP-dependent protein kinase) and CDK (cyclin-dependent protein kinase), CK2β does not switch on or off the intrinsic activity of the catalytic subunits (Bolanos-Garcia et al., 2006).

The CK2β regulatory subunit is remarkably conserved among species, but it does not have homology with the regulatory subunits of other protein kinases (Bibby & Litchfield, 2005). The amino acid sequence of the CK2β regulatory subunit is almost identical in *Homo sapiens*, *Drosophila melanogaster*, *Ceratitis capitata* (Mediterranean fruit fly), *Danio rerio* (zebrafish), *Ciona intestinalis* (sea squirt), and *Mytilus galloprovincialis* (Mediterranean mussel) (Kouyanou-Koutsoukou et al., 2011a, b; Kolaiti et al., 2011). It is completely identical in birds and mammals (Maridor et al., 1991; Wirkner et al., 1994). In contrast, the fruit fly *D. melanogaster* has four regulatory subunit genes. They are used for one CK2α (DmCK2α) and three CK2βs (DmCK2β, DmCK2β' and DmCK2βtes) (Jauch et al., 2002). *Zea mays* has three isoforms of the catalytic α-subunit (CK2a-1, CK2a-2 and CK2a-3) and three regulatory β-subunits (CK2b-1, CK2b-2 and CK2b-3) (Riera et al., 2001). *S. cerevisiae* CK2 holoenzyme contains two regulatory β-subunits (β and β′). They cannot substitute for each other, and both of them are needed to form a fully active enzymatic unit (Kubinski et al., 2007).

Results presented by several groups and obtained by the use of a variety of approaches, including X-ray crystallography, have determined that a dimer of the CK2β subunits forms the core of the CK2 tetramer (Chantalat et al., 1999; Sarno et al., 2000; Canton et al., 2001).

The CK2β regulatory subunit is a compact, globular homodimer that shows high amino acid sequence conservation across species. The N-terminal domain (amino acids 1-104) is globular and contains four α-helices (marked as α1-α4 in Fig. 1). Helices α1 (residues 9–14), α2 (residues 27–31) and α3 (residues 46–54) wrap around α4 (residues 66–89) (Bolanos-Garcia et al., 2006). This part of the protein contains autophosphorylation sites, consisting of serines 2, 3, and possibly 4 (Boldyreff et al., 1993). Studies conducted by Zhang and coworkers (2002) indicate that phosphorylation of these sites enhances CK2β stability. The

first 20 N-terminal amino acids of the CK2β regulatory subunit are also involved in the interaction with Nopp140, a protein that binds a nuclear localization sequence and shuttles between the nucleus and the cytoplasm (Li et al., 1997). This part of the protein also contains two motifs that have been previously characterized as motifs that regulate cyclin degradation. The CK2β regulatory subunit has a sequence resembling the nine-amino-acid motif called the destruction box, which plays a key role in the specific degradation of cyclin B at the end of mitosis (King et al., 1996). This motif, located in helix α3, contains three highly conserved residues that conform to the general destruction box consensus (RXXLXXXXN/D) (Bolanos-Garcia et al., 2006). Interestingly, this motif is located on a surface-exposed α3 helix, where it would be available for recognition by the cellular degradation machinery. A signal known as the KEN box, which was found previously in mitotic cyclins and which has been shown to play a role in mediating cell cycle-dependent protein degradation, is also present in CK2β. This degradation motif is characterized by the minimal consensus sequence KEN, but it is often followed shortly by either an N or D residue and is often preceded by another N or D residue. A similar sequence (D_{32}KFNLTGLN$_{40}$) forms helix α2 of the CK2β protein (Bibby & Litchfield, 2005).

The N-terminal part of the CK2β also contains an "acidic loop" between helices α3 and α4. This acidic, surface-exposed region of the protein, encoded by residues 55-64, has been identified as the site on CK2 that binds polyamines, which are known to stimulate CK2 activity *in vitro* (Meggio et al., 1994; Leroy et al., 1997).

The analysis of the CK2β regulatory subunit structure by X-ray crystallography revealed the importance of the zinc finger in CK2β regulatory subunit dimerization (Chantal et al., 1999). The zinc-finger region is characterized by four conserved cysteine residues (residues 109, 114, 137 and 140), which mediate the interaction that allows the CK2β dimer to form the core of the CK2 holoenzyme (Chantal et al., 1999; Canton et al., 2001).

The C-terminal part of the CK2β regulatory subunit (residues 178–205) contains a large loop (residues 178–193) and helix α7 (residues 194–200). Although helix α7 is located away from helices α1-α6, the C-terminal amino acids (190-205) contribute to the formation of the CK2β regulatory subunit dimer (Niefind et al., 2001). This part of the regulatory subunit contains two phosphorylation sites: Thr213, which is phosphorylated by the checkpoint kinase Chk1 (Kristensen et al., 2004) and Ser209, which is phosphorylated *in vitro* and in mammalian cells by $p34^{cdc2}$ in a cell-cycle-dependent manner (Litchfield et al., 1995).

The traditional view of the CK2β regulatory subunit is that it functions as a component of tetrameric CK2 complexes and that it is the regulator of the catalytic CK2α and CK2α' subunits, enhancing their stability, specificity and activity. As an example, the CK2β regulatory subunit stimulates CK2 holoenzyme activity towards certain protein substrates, such as topoisomerase II (Leroy et al., 1999), and inhibits others, like calmodulin (Marin et al., 1999).

It was shown that CK2β does not exist exclusively within stable CK2 complexes. This observation raises the prospect that CK2β has functions that are independent of its role as the regulatory subunit of CK2. For example, overexpression of CK2β in the fission yeast *Schizosaccharomyces pombe* revealed severe growth defects and a multiseptated phenotype, whereas CK2α overexpression had no effect (Roussou & Draetta, 1994).

CK2β seems to interact directly with more than 40 different proteins, including other protein kinases such as A-Raf, Chk1, Chk2, PKC-ζ, Mos and $p90^{rsk}$ (Bibby & Lichfield, 2005; Bolanos-Garcia et al., 2006; Olsen & Guerra, 2008). It was shown that association of the human protein kinases Chk1, Mos, and A-Raf is mediated by the C-terminal region of the CK2β subunit and that these associations involve some residues that interact with the catalytic CK2α subunit (Chen et al., 1997; Lieberman & Ruderman, 2004; Olsen & Guerra, 2008). The interaction between Chk1 and CK2β leads to an increase in the Cdc25C phosphorylation activity of Chk1. Screening of several cell lines has shown that the association between CK2β and Chk1 is also formed *in vivo* (Guerra at al., 2003).

Overexpression of CK2 has been linked to several pathological conditions, ranging from cardiovascular pathologies and cancer progression to neurodegenerative disorders (*e.g.*, Alzheimer's disease, Parkinson's disease, brain ischemia) and infectious diseases (Guerra & Issinger, 2008; Ahmad et al., 2008; Trembley et al., 2009). Various specific, potent small molecule inhibitors of protein kinase CK2 have been developed in recent years, including condensed polyphenolic compounds, tetrabromobenzimidazole/triazole derivatives, and indoloquinazolines (Gianoncelli et al., 2009; Pagano et al., 2008; Raaf et al., 2008). Inhibition of CK2 kinase activity by these compounds display a remarkable pro-apoptotic efficacy on a number of tumor-derived cell lines, indicating a possibility of developing novel antineoplastic drugs (Batistuta, 2009; Duncan et al., 2010; Prudent et al., 2010; Unger et al., 2004).

2. Mutagenesis in studies on protein kinase CK2

Within the last 2 decades, a number of studies have produced mutants of both CK2α and CK2β that provide a valuable, yet incomplete, basis to rationalize the biochemical features of the enzyme, i.e., its constitutive activity, dual-cosubstrate specificity, acidophilic substrate specificity and tetrameric structure (Fig. 2).

2.1 Mutagenesis of the CK2α catalytic subunit

2.1.1 Mutations of CK2α in the regions responsible for constitutive activity

A majority of protein kinases need to be activated. Phosphorylation within the kinase activation loop is the most popular mode of activation. In contrast to other known protein kinases, CK2 has constitutive activity and does not demand activation. In this case, activation is achieved by the interaction between the N-terminal tail and the activation loop in the kinase domain. The role of the N-terminal segment in stable opening of the activation loop was confirmed in mutagenesis studies (Sarno et al., 2001). In particular, the Δ2-12 CK2α mutant, in comparison with the wild-type kinase, displayed an almost complete loss of activity, which was reflected by increased Km values for ATP and the peptide substrate (from 10 to 206 μM and from 26 to 140 μM, respectively). Further experiments revealed that holoenzyme reconstitution restored the activity of the mutant to the wild-type level. This demonstrates an alternative CK2β subunit-dependent mechanism to provide constitutive activity in the case of CK2 holoenzyme (Sarno et al., 2002).

Recently, molecular dynamics (MD) simulation has been carried out in order to explore the role of the CK2α N-terminal segment in the conformational behavior of the kinase (Cristiani

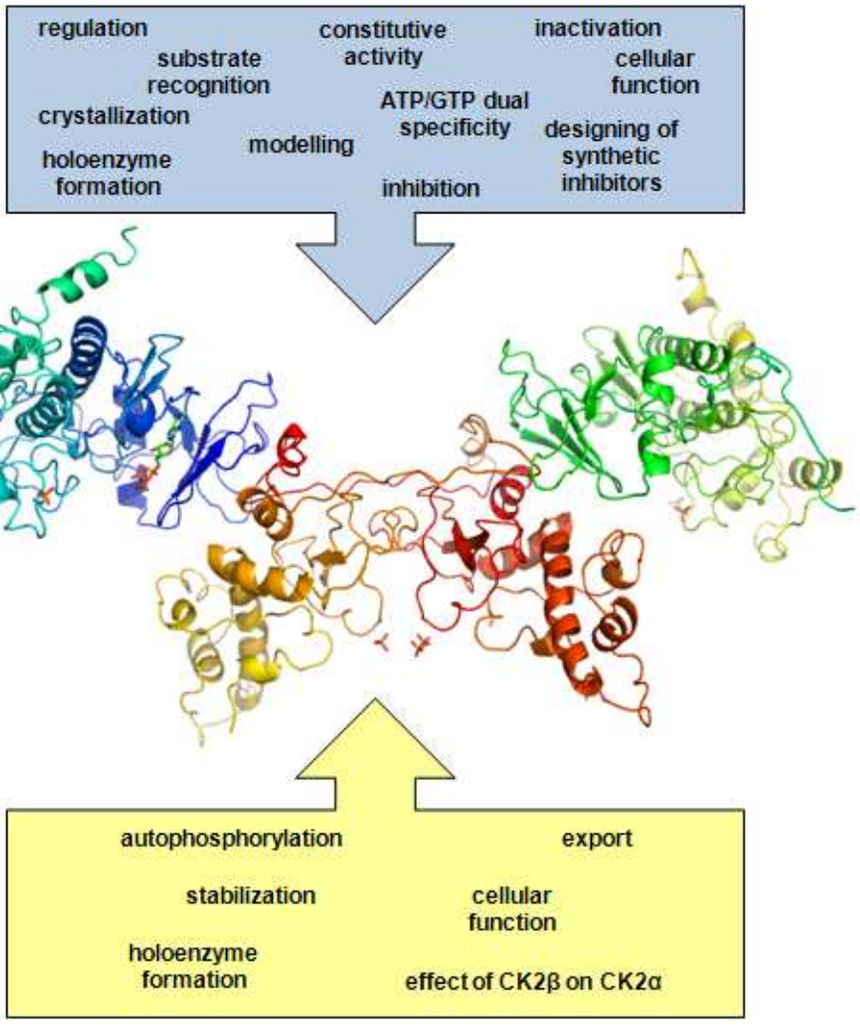

Fig. 2. Multiple applications of mutagenesis in studies on CK2.
The blue box presents various aspects of research using mutagenesis on CK2α; and the yellow box, on CK2β. The model of the human CK2 holoenzyme was developed using the PyMOL software based on the structure of the human CK2 holoenzyme (PDB code 1JWH) from the Protein Data Bank. The catalytic α subunits are presented in blue and green; the regulatory β subunits are in red and yellow.

et al., 2011). Comparison of the αC-helix RMSD (root mean square deviation) values obtained for the Δ2-12 CK2α mutant (*i.e.*, deleted for residues 2 through 12) and the wild-type kinase models show an increase in this parameter for the mutant form of the enzyme. This effect is due to instability of the CK2α conformation in the case of absence of an N-terminal segment and its interaction with the αC-helix. These results are consistent with the data presented by Sarno and collaborators, and they indicate that the complete N-terminal segment is essential for proper conformation and constitutive activity of protein kinase CK2α (Cristiani et al., 2011).

The experiment presented above is an example of the validation of *in vitro* mutagenesis studies with the use of computing analysis, but the opposite direction of studies is also possible. Two CK2α mutants, the triple mutant Y206F/R10A/Y261F and the single mutant Y125F, were constructed *in silico*. MD simulations were then carried out to study the relation

between CK2 conformation and activity (Cristiani et al., 2011). The amino acids substituted in the first virtual mutant are engaged in the most important bonds between the N-terminal segment and other regions of CK2α to maintain kinase activity. The CK2α Y125F mutant is also very useful in studying the influence of Tyr125 on the conformational change of Phe121. According to Niefind and Issinger (2010), Phe121 can assume two different conformations: in and out, which regulate the activity of CK2α. Preliminary MD simulations on the two protein mutant models are very promising. The authors are currently working on the construction of both CK2 mutants. Biochemical characterization of the mutants will be carried out (Cristiani et al., 2011).

2.1.2 Mutation of CK2α in the basic regions

Protein kinase CK2 is characterized by its special aptitude to interact with negatively charged ligands. This ability correlates with the presence of several basic residues in CK2α that are not conserved in a majority of other protein kinases. These residues are located mainly in the "Lys-rich segment" and in the "p+1 loop." The Lys-rich segment (K_{74}KKKIKR_{80}) at the beginning of the αC-helix is a distinctive feature of CK2α (Tuazon & Traugh, 1991; Guerra et al., 1999). Results from mutational studies support the notion that this cluster is involved in substrate recognition, inhibition by heparin, down-regulation by the CK2β subunit and interaction with heat shock protein 90, and nuclear targeting (Guerra et al., 1999; Pinna & Meggio, 1997) (Table 1). CK2α mutants from *Caenorhabditis elegans* and *Xenopus laevis* (K74E/K75E and K75E/K76E, respectively) had lysines replaced by glutamic acid residues, which greatly affected the charge of this region in both mutant enzymes. The changes produced neither a significant increase in the K_m of the CK2α subunit for the casein and model peptide substrates nor changes in the affinity of the mutated CK2α subunit for the CK2β subunit during assembling a fully competent CK2 holoenzyme**.** The same mutations, however, had a significant effect on the affinity of CK2α for heparin and for other polyanionic inhibitors (Hu & Rubin, 1990; Gatica at al., 1994). Complete suppression of heparin inhibition was observed with the quadruple mutated K74-77A CK2α used by Vaglio and collaborators (1996). These authors showed (1) that all the four basic residues at positions 74, 75, 76, and 77 are implicated in heparin binding and (2) that the mutation of all of them was necessary to minimize heparin inhibition. Further mutagenesis studies showed that the additional basic residues cooperated with high heparin binding (apart from the 74-77 quartet). These were mainly Arg191, Arg195 and Lys198 located in the p+1 loop. However, the triple mutant for the three non-Lys-rich segment residues was less effective in heparin inhibition than was the mutant resulting from quadruple mutation of the 74-77 cluster (Vaglio et al., 1996). The triple mutant in which Lys79, Arg80 and Arg83 were changed into alanines did not alter the IC_{50} (concentration needed to give 50% inhibition) value for heparin. However, the mutant did show a reduction in the phosphorylation efficiency of the peptide substrate (and derivatives in which individual aspartyl residues were replaced by alanines). Because of these properties, it was specified that the basic residues in positions 77-83 are mainly involved in substrate recognition, rather than in heparin inhibition (Sarno et al., 1995; Vaglio et al., 1996). These authors concluded that the highly conserved 74-80 basic stretch is composed of two functionally distinct entities: (1) an N-terminal moiety mostly involved in heparin inhibition as well as in down-regulation by the β subunit and (2) the C-terminal part implicated in recognition of the crucial specificity determinant at positions n+3, but irrelevant to heparin.

Extended mutagenesis analysis combined with biochemical characterization provided clear evidence that residues responsible for both substrate recognition and down-regulation of CK2α catalytic activity are located mainly in the Lys-rich loop and p+1 loop spanning sequences 74-83 and 191-198, respectively. This corroborates the concept that the CK2β subunit down-regulates the CK2β by acting as a pseudosubstrate (Meggio et al., 1994; Sarno et al., 1996, 1997a, 1999).

Sarno and collaborators (1997b) analyzed the relative contribution of basic residues, presumably implicated in CK2-substrate interaction, in the recognition of peptide substrates varying in the number and position of acidic determinants. Sixteen derivatives of the optimal peptide substrate RRRA-DDSDDDDD, wild-type CK2 and twelve CK2α mutants defective in substrate recognition were used in the experiments. In the CK2α mutants, different basic residues implicated in substrate recognition were replaced by alanine (*e.g.*, K49A, K74-77A, or K79A/R80A/K83A). The results obtained support the idea that the acidic residues at positions n+1 and n+3 are essential, while additional acidic residues are required for efficient phosphorylation of CK2 substrates. Kinetic analysis with CK2α mutants revealed that Lys48 was implicated in the recognition of the determinant at position n+2. Lys77 interacts with the determinants at n+3 and n+4, while Lys198 recognized the determinant at n+1 (Sarno et al., 1997b). Molecular modeling based on crystallographic data supported these observations. It showed that several of these basic residues are clustered around the active site, where they make contact with individual acidic residues of the peptide substrate, polyanionic inhibitors, regulatory elements present in the β subunit, N-terminal segment of the CK2α, and possibly other proteins interacting with CK2 (Sarno et al., 1999).

2.1.3 Mutations of CK2α in catalytic subdomains

Subdomains II and VII of CK2α involved in nucleotide binding and phosphotransfer are in close proximity to each other in the three-dimensional structure. CK2α differs from more than 95% of other known protein kinases in having Val66 instead of the corresponding alanine within conserved region II and Trp176 instead of the corresponding phenylalanine within region VII (Allende & Allende, 1995). To investigate whether these variant amino acid residues might be responsible for effective GTP utilization, Jakobi and Traugh (1995) mutated both of these residues back to the consensus amino acids. Their results indicated that both single mutants of CK2α and the double mutant CK2α could still use GTP as a phosphate donor. The single and double mutations only altered the relative affinities for ATP and GTP. This finding indicated that at least one other amino acid residue must be responsible for the effective utilization of GTP by CK2. The same authors studied the above-mentioned mutants with respect to the catalytic activity of the reconstructed holoenzyme. The relatively lower affinity for GTP of the holenzyme reconstructed from the mutated CK2α was caused by changes in both the K_m and V_{max} values for GTP and ATP, while for the catalytic subunits, it was a result of changes in the K_m values only. These studies showed that the unique property of the effective utilization of GTP by CK2 was correlated with stimulation of the activity by the regulatory subunits and with the ability to undergo a conformational change upon formation of the holoenzyme.

Srinivasan and collaborators (1999) showed that the dual specificity of CK2 probably originated from the loop situated around the stretch $H_{115}VNNTD_{120}$ in CKα. In their work, they combined site-directed mutagenesis of CK2α with comparative 3D-structure modeling.

Due to significant amino acid sequence similarity (69,5%), kinase CDK2 was chosen to be a good comparative model for CK2α. Based on modeling, a ΔN118 CK2α mutant was constructed. The kinase assay showed decreased affinity of this protein to GTP, in comparison to the wild-type CK2α. The K_m values were 146 and 37 μM, respectively. The results obtained clearly indicate that the adenine/guanine binding region (His115–Asp120) is responsible for the dual specificity of kinase towards phosphate donors (Srinivasan et al., 1999).

The latter study was extended by Jakob and collaborators (2000), who created several mutants of *Xenopus laevis* CK2α with substitutions at positions 118 and 129. They tested them for cosubstrate specificity after their combination with CK2β. The region containing Asn118, known to participate in the recognition of the guanine base, is a part of the sequence $N_{117}NTD_{120}$. This sequence closely resembles the conserved sequence NKXD that is present in G proteins and other GTPases. The study demonstrated that both the CK2α ΔN118 and CK2α N118E mutants produced a 5 to 6-fold increase in the K_m for GTP with little effect on the affinity for ATP.

The mutagenesis by Yde and collaborators (2005) resulted in the first stable and fully active mutant of the human catalytic subunit of protein kinase CK2 that is devoid of dual cosubstrate specificity. The resulting mutant hsCK2α1-335 (human CK2 deleted for the last 56 amino acids) V66A/M163L was designed on the basis of several structures of the enzyme from *Zea mays* in a complex with various ATP-competitive ligands. As structural research revealed the existence of a purine base-binding plane harboring the purine base of ATP and GTP. This plane is flanked in human CK2α by two side-chains of Val66 and Met163, and it adopts a significantly different orientation than it does in other kinase homologues. By exchanging these two flanking amino acids, the cosubstrate specificity is shifted towards strongly favoring ATP. These findings demonstrated that CK2α possesses a sophisticated structural adaptation that favors dual-cosubstrate specificity, a property that may have biological significance.

The mutagenesis studies also provided much insight into the significance of the sequence of the catalytic domain with respect to the CK2α/CK2β interaction. It was reported that CK2α V66A and V66A/W176F were able to interact with CK2β, but this interaction failed to stimulate catalytic activity on the peptide substrate. These results were in contrast to the result with the wild-type α subunit, which was stimulated 4-fold. Nevertheless, the stimulatory response to the cationic modulatory compounds, spermine and polylysine, was the same for holoenzymes reconstituted with the wild-type subunit and all three above-mentioned mutants of the α subunit. The results showed that there must be at least two different interactions between the catalytic α and regulatory β subunit: one that is responsible for stimulation by the β subunit itself and another for mediating the stimulation by polycationic compounds (Jakobi & Traugh, 1992). However, experiments using calmodulin as a substrate for phosphorylation revealed that the insensitivity of the CK2α mutant V66A to CK2β was only apparent. Down-regulation of calmodulin phosphorylation by the CK2β subunit is even enhanced by the V66A mutant. This observation indicated a possible indirect role for Val66 in conferring to the α-subunit a conformation less sensitive to down-regulation (Sarno et al. 1997a).

It is known that the hydrophobic and polar residues of domain II and VII are responsible for the selectivity of a number of specific, potent CK2 ATP-competitive inhibitors, like TBBz

(tetrabromobenzimidazole) and TBBt (tetrabromobenzotriazole) (Sarno et al., 2005a). The importance of the same key residues in the hydrophobic portion of the binding site was corroborated by mutational analysis of residues of the human CK2α. Their side chains contribute to the reduction in the internal size of the hydrophobic pocket adjacent to the ATP/GTP-binding site in CK2 (Battistutta et al., 2001; Sarno et al, 2005). Three of these residues (Val66 or Ile66, Ile174, and Met163) are specific to CK2. They are generally replaced by smaller ones in other protein kinases. Both single and double mutants with substitutions for Val66 and Ile174 gave rise to catalytically active CK2α with altered susceptibility to various inhibitors. However, replacement of Met163 by glycine produced a catalytically inactive mutant (Sarno et al., 2005b). Similar data were obtained with yeast CK2α. Mutants with alterations to V67 and I213 (analogous to V66 and I174 of human CK2α) displayed considerably higher K_i values toward inhibitors TBBz and TBBt and only a slight change in the affinity for ATP (Sajnaga et al, 2008). The structural basis for decreased emodin binding to human CK2α resulting from a single point mutation (V66A) has been examined by molecular dynamics (MD) simulations and energy analysis (Zhang & Zhong, 2010). It was found that the V66A mutation resulted in a packing defect due to a change in hydrophobicity. It led to abnormal behavior of the glycine-rich loop, α-helix, and C-loop. The critical role of Ile66 in cosubstrate binding and selection, besides forcing the nucleotide ligands to adopt different positions in the binding pocket, was also demonstrated in a mutational study (Jakobi et al., 1994; Jakobi & Traugh, 1992, 1995).

Chaillot and collaborators (2000) studied the role of Gly177 in conserved region VII of the catalytic domain, which is close to the active site. It was revealed that the CK2α G177K mutant exhibited improved catalytic efficiency for acid peptidic substrates, probably by establishing interactions with the acidic residues.

The acidic residue Asp or Glu of the catalytic loop (corresponding to Glu170 in PKA and conserved in most Ser/Thr protein kinases) is responsible for the binding of basic residues that specify the protein/peptide substrates. In CK2, the residue is replaced by a histidine (His160). Such a substitution could explain the acidophilic properties of CK2, in contrast to the basophilic properties of PKA and other Ser/Thr kinases. The actual role of the His160 in the determination of the site specificity of CK2 was assessed by Dobrowolska and collaborators (1994). Interestingly, subsequent mutational studies in which His160 was replaced with alanine or aspartic acid ruled out any significant role of this residue in substrate recognition (Sarno et al., 1997b).

A CK2α inactive mutant (D156A) was produced based on structural homology to kinase PKA. The mutant protein was able to compete efficiently with the wild-type CK2α for the regulatory β subunits. Although it does not exhibit kinase activity, the D156A mutant can bind CK2β to form an inactive holoenzyme. Moreover, the mutant abolishes the inhibitory effect of CK2β on CK2α-mediated phosphorylation of calmodulin. These results suggest that CK2α D156A may be a useful dominant-negative mutant for elucidation of the cellular functions of the CK2 regulatory subunit (Cosmelli et al., 1997).

2.1.4 Mutations of CK2α in the glycine-rich loop

The glycine-rich sequence (G-loop) is one of the most critical structures of protein kinases, since it contributes in many ways to enzyme activity. This multifunctional structural

element participates in nucleotide binding, substrate recognition, catalysis, and regulation of activity (Bossemeyer et al., 1994). In their extensive mutational studies combined with biochemical characterization, Sarno and collaborators (1999) confirmed that some basic residues in the glycine-rich loop of the CK2α, particularly Lys49, are implicated in substrate recognition and inhibition by polyanions. Another residue located within this region, Gly48, is involved in binding the ATP phosphate moiety. Replacement of Gly48 by alanine in CK2α affected its catalytic efficiency and specificity. It is thought that alanine causes this phenotype by creating an electrostatic barrier between ATP and the peptide substrate (Chaillot et al., 2000).

2.1.5 Mutations of CK2α in the C-terminal region

The C-terminal region of vertebrate CK2α is composed of 54 amino acids. Knowledge of this segment is rather poor, except for phosphorylation by kinase $p34^{Cdc2}$ and interaction with isomerase Pin1 (Bosc et al., 1995; Messenger et al., 2002). It is known from the publications on crystallization of CK2 that the catalytic subunits are particularly sensitive to degradation, which makes the crystallization process of the entire subunit difficult (Niefind et al., 2000, 2001). Truncation at the C-terminus reduced the intrinsic degradability of CK2α and allowed its crystallization and the determination of its 3D structure. Starting from sequence alignments of C-termini from different CK2αs, Grasselli and collaborators (2004) constructed a mutant carrying the substitution of two distal prolines with alanines (P382A/P384A). Most intriguing was the resistance of the mutant to proteolytic degradation, which makes this protein an excellent candidate for crystallization of the entire CK2α subunit.

Bischoff and collaborators (2011) have recently determined for the first time the structure of the full-length human $CK2\alpha`^{C336S}$ subunit. A point mutation of CK2α` was necessary to prevent covalent dimerization from intermolecular disulfide bridges formed by Cys336. However, these results shed light on the differences between the two catalytic subunits, α and α` (*e.g.*, significantly lower affinity of CKα` towards CK2β relative to that of CK2α).

2.1.6 Mutagenesis of CK2α in other regions

Determination of the structure of the CK2 holoenzyme and individual subunits provided knowledge about the nature and location of the interface between catalytic and regulatory subunits (Niefind et al., 2001). Using structure-guided alanine-scanning mutagenesis combined with isothermal titration calorimetry (ITC), energetic "hot spots" were identified on the surface of CK2α that determine the α/β subunit interaction (Raaf et al., 2011). Three single and one double CK2α subunit mutants were produced, in which individual hydrophobic amino acids located within the CK2α interface were replaced by alanine. The ITC analysis of CK2α mutants revealed that substitution of Leu41 and Phe54 were most disruptive to binding of CK2β. Moreover, the L41A and F54A mutants retained their kinase activity, compared to the wild-type CK2. Based on the results mentioned above, it can be claimed that these residues are suspected of being interaction "hot spots" (Raaf et al., 2011).

The amino-acid sequence and the structure of yeast protein kinase CK2α differ from those of CK2α' and other eukaryotic CK2α subunits. CK2α is unique in containing a 38-amino-acid loop consisting of two α-helical structures situated close to structures engaged in ATP/GTP

and substrate binding (Niefind et al., 2001). Modeling of the tertiary structure of the CK2α showed that, after removing both α-helical motifs, the CK2α subunit assumes a structure that is more similar to that of CK2α' than it is to the structure of intact CK2α. The deletion of the 38 amino acids from CK2α drastically decreases its catalytic efficiency. Its characteristics are similar to yeast CK2α' with respect to sensitivity to salt, heparin and spermine (Sajnaga et al., 2008) (Fig. 3).

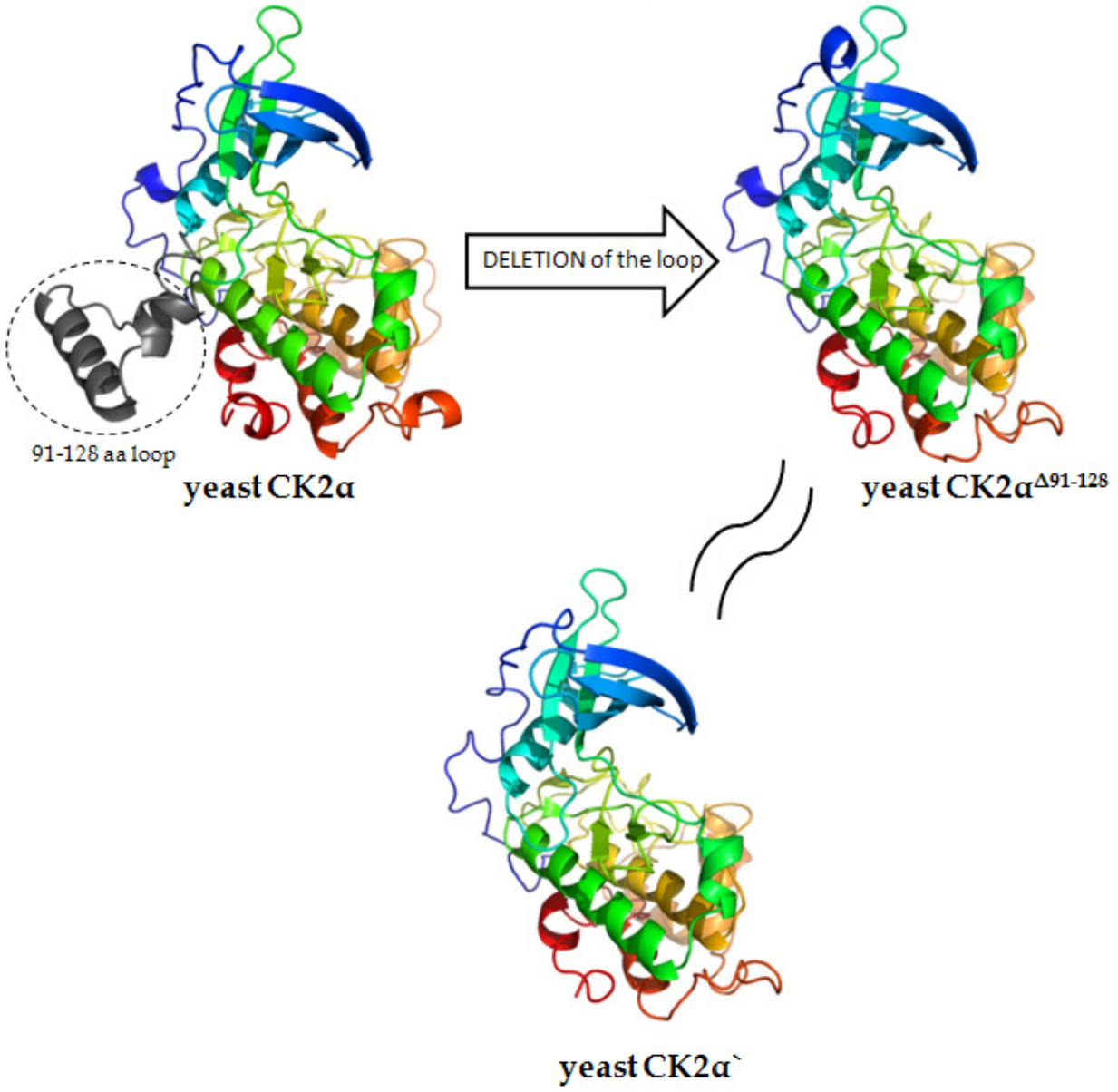

Fig. 3. Conformational consequences of mutagenesis of the yeast CK2α catalytic subunit.

CK2 residues	Location	Mutant	Reference/source
Substrate recognition and inhibition by polyanions			
K49	Subd. I; Gly loop	K49A	Sarno et al., 1999
K74 K75 K76 K77	Subd. II/III, Lys rich loop	K74-77A, K77A	Sarno et al., 1997a, 1998, 1999, Vaglio et al. 1996 Gatica et al., 1994[1]
K79 R80 K83	Subd. III, Helix C	K79A, R80A/K83A	Sarno et al., 1998, 1999
K122	Subd.V, Linker region	K122A	Sarno et al., 1997a, 1999
H160	Subd. VIb, Catalytic loop	H160D	Dobrowolska et al., 1994
R191 R195 K190 K198	Subd. VIII, p+1 loop	R191, 195, K190A K198A	Sarno et al., 1997a, 1998, 1999; Vaglio et al., 1996
Catalytic efficiency and specificity			
G48[2]	Subd. I, Gly loop	G48D	Chaillot et al., 2000
V66 M163	Subd. II Subd. VIb	V66A M163A CK2α [1-335]	Yde et al., 2005

V66 I174	Subd. II Subd. VII	V66A/I174A	Sarno et al., 2005
V66/W176	Subd. II, Subd. VII	V66A/W176F	Jakobi and Traugh, 1992, 1995
N118	Subd. V, ATP/GTP binding region	N118A, $CK2\alpha^{\Delta N118}$	Srinivasan et al., 1999; Jakob et al., 2000
D156	Subd. VIb	D156A	Cosmelli et al., 1997
M163	Subd. VIb	M163G	Sarno et al., 2005
G177	Subd. VII	G117K	Chaillot et al., 2000
N189	Subd. VII, Activating segment	N189R	Srinivasan et al., 1999
Regulation by β subunit			
L41	Subd. I	L41A	Raaf et al., 2011
L54	Subd. I, ATP/GTP binding region	L54A	Raaf et al., 2011
V66 W176	Subd. II Subd. VII	V66A, V66A/W176F	Sarno et al., 1997b Jakobi &Traugh, 1992
Constitutive activity			
M6 –V30	N-terminus	Δ2-12, Δ2-18, Δ2-24, Δ2-30	Sarno et al., 2001, 2002; Cristiani et al., 2011
Y125	Subd. V, Hinge region	$Y125F^{4}$	Cristiani et al., 2011
E180	Subd. VII, Activation segment	E180A	Sarno et al., 2002

E182	Subd. VII, Activation segment	Y182F	Sarno et al., 2002
Stability			
M336-Q393	C-terminus	Δ336-393	Ermakova et al., 2003
P382 P384	C-terminus	P382A P384A	Grasselini et al., 2004
C336[3]	C-terminus	C336S	Bischoff et al., 2011

[a]The residue numbers correspond with those of human CK2α, unless otherwise indicated. The Roman numerals indicate the eleven conserved subdomains present in the catalytic domain of all protein kinases (Hanks & Hunter, 1995). Abbreviations: [1]CK2α from *Xenopus laevis;* [2]CK2α from *Yarrovia lipolytica;* [3]Human CK2α'; [4]*in silico* mutation.

Table 1. Summary of CK2α mutants[a]

The deletion of the loop of amino acids 91-128 from yeast CK2α led to behavioral and structural similarity to CK2α` (Sajnaga et al., 2008). The 3D models of proteins were created using the SWISS-MODEL software based on protein structure templates (PDB code 1ds5D) available in the Protein Data Bank and visualized with the PyMOL software.

Chimeras of different kinases can be easily engineered using recombinant DNA technology and used in studies on the structure and function of kinase. To study the effect of CK2β on the activity of CK1α, Jedlicki and collaborators (2008) generated CK2α/CK1α chimeras that were able to bind tightly to the CK2β regulatory subunit, but maintain the peptide substrate specificity of CK1. This is related to the capacity of the CK2β to regulate the activity of CK2α, as well as other protein kinases, such as A-Raf, C-Mos, and Chk1. It has been shown that a chimera combining a large part of the CK1α kinase with the N-terminal region of CK2α that is responsible for binding CK2β can be stimulated by this subunit. It is possible that such chimeras could be used to test the presence of "the docking site" on the CK2β subunit, which would bring substrate molecules near the catalytic subunits.

2.2 Mutagenesis of the regulatory subunit CK2β

From the primary sequence of the β subunit, it is obvious that the charged amino acids are not equally distributed. The acidic residues are clustered in the N-terminal half, whereas the basic residues are clustered in the C-terminal part of the molecule. Mutational studies have shown that, in contrast to cyclins, which invariably act as indispensable activators of CK2-related CDKs, the CK2β subunit fulfills antagonist functions. The features of CK2β can be explored by generating large synthetic fragments, some of which reproduce the C-terminal moiety and thus stimulate its catalytic activity. Fragments reproducing segments of the N-terminal sequence are inhibitory, which becomes especially evident when calmodulin is the substrate (Marin et al, 1992, 1995; Meggio et al, 1994; Sarno et al, 1997a).

2.2.1 Mutations of CK2β that affect autophosphorylation

The CK2β subunit is known to be autophosphorylated by the catalytic subunit. Autophosphorylation occurs on serine residues at positions 2 and 3 in the amino-terminal region of the molecule. Both these serines fit CK2 consensus specificity requirements (Marin et al, 1992). This finding was corroborated by the fact that the mutant S2,3G (*i.e.*, S2G/S3G) is completely incapable of autophosphorylation (Hinrichs et al, 1993). Deletion of the first four amino acids (CK2β Δ1-4), which eliminated autophosphorylation of CK2β, had no significant effect on the reconstruction of CK2 holoenzymes nor on their catalytic activity, thermostability, and responsiveness to polylysine. Unlike the wild-type CK2β, however, CK2β Δ1-4 failed to confer to the reconstituted holoenzyme the typical responsiveness to NaCl stimulation. These results indicated that autophosphorylation sites are not required on CK2 for conferring a stable structure and full catalytic activity. In contrast an autophosphorylation site is implicated in the NaCl-dependent fine-tuning of CK2 activity (Meggio et al., 1993). Interestingly, the acidic stretch heavily influences autophosphorylation of the β subunit, even though Ser2 is more than 50 amino acids away in the primary sequence (Boldyreff et al., 1994).

2.2.2 Mutations of CK2β that affect binding with CK2α

In order to shed light on the mechanisms by which the CK2β subunits affect the catalytic properties of CK2 and to elucidate the molecular interactions between the catalytic and regulatory subunits of CK2, Boldyreff and collaborators (1992, 1993) generated a number of mutants of the CK2β subunit, which were tested for their ability to functionally replace the wild-type CK2β. These authors showed that deletion of the last 44 residues of the C-end (CK2β Δ171-215) eliminated the capacity to form tetramers with CK2α and to stimulate activity. However, deletion of the last 34 amino acids (CK2βΔ181-215) yielded an active CK2β that had lower affinity for CK2α. Shorter deletions (*e.*g., CK2β Δ194-215) did not affect the interaction between the catalytic and regulatory subunits of CK2. Boldyreff and collaborators demonstrated that deletion mutants in which the last 45 or more amino acids are missing were not able to assemble with the α subunit. These data identified the C-terminal segment of CK2β as essential for association with the CK2α subunit, with special reference to its 171-180 stretch, which is indispensable both to form tetrameric CK2 and to stimulate activity of the CK2α catalytic subunit (Boldyreff et al., 1994). Tight interaction between the CK2α and CK2β subunits, accomplished by the C-terminal part of the CK2β subunit, was also described (Kusk et al., 1995; Marin et al., 1997).

Mutagenesis along with crosslinking and peptide studies have shown that the acidic amino acid stretch of CK2β from residues 55-64 interacts with a corresponding basic stretch of the CK2α subunit. However, these weak electrostatic interactions seem to determine the activity of, but not the formation of, the CK2 holoenzyme (Krehan et al., 1996, Sarno et al, 1997b).

Kusk and collaborators (1995) used mutagenesis of CK2 subunits with a yeast two-hybrid system to explore domains involved in intersubunit contact. [In the yeast two-hybrid system, a peptide or protein is fused to part A of a transcriptional activator. Another peptide or protein is fused to part B. Transcriptional activation of an easily assayed reporter gene occurs only when part A and part B come together. Parts A and B

themselves cannot interact to form the transcriptional activator, nor can either part individually (part A, the part A fusions, part B, and the part B fusions) cause the reporter to be expressed. However, if the fusions interact, part A and part B can come together, and the reporter is activated. This is an indication that the peptides or proteins in the fusions can interact.] A series of plasmid constructs was prepared. They encoded N-terminal or C-terminal truncations of the CK2α and CK2β subunits to indicate which regions of the subunits were engaged in CK2 holoenzyme formation in yeast cells. The data revealed that the regulatory CK2β subunit has a modular structure. An N-terminal domain (residues 20-145) is responsible for homodimerization (CK2β/CK2β). A C-terminal domain (residues 152-200) is necessary for heterodimerization (CK2α/CK2β). Amino acid residues 1 to 20 in the N-terminus and 351 to 391 in the C-terminus of CK2α are dispensable for interaction with the regulatory subunit.

2.2.3 Mutations of CK2β that affect the activity of CK2α

The modulation of CK2α subunit activity by CK2β has a stimulatory effect on most substrates. However, when calmodulin is used as the substrate, the CK2β subunit almost completely inhibits the activity of the catalytic subunit (Guerra et al., 1999). This inhibition can be overcome by addition of polylysine (Meggio et al, 1992). Mutagenesis studies on the CK2β subunit revealed an acidic stretch (amino acids 55-64) that is responsible for the inhibitory effect and for the stimulation by polylysine (Meggio et al., 1994). Interestingly, mutants of CK2β bearing substitutions at positions 55, 57, and 59-64 to alanine produced up to 4-fold more active holoenzyme after assembling with the catalytic α subunit than did the wild type. At the same time, these mutants were refractory to the stimulatory effect of polylysine. This finding revealed that the acidic N-terminal cluster of CK2β, especially Asp55 and Glu57, is involved in intrinsic down-regulation of CK2 basal activity and has been implicated in responsiveness to various effectors (Boldyreff et al., 1993, 1994).

Other data provided by Hinrichs and collaborators (1995) demonstrated that Pro58 located in the center of the acidic segment also constitutes an important structural feature affecting the function of down-regulation of CK2β towards the catalytic subunits. The effect of a mutation of proline to alanine resulted in an effect that was similar to mutation of the acidic residues alone. It produced hyperactive CK2β subunits that stimulated the CK2α activity to a greater extent than did the wild-type CK2β subunit.

2.2.4 Mutations of CK2β that affect export of the holoenzyme

It is known that protein kinase CK2 is present in not only the cytoplasm, nuclei, and several other cell organelles, but also on the external side of the cellular membrane (Kubler et al, 1983). Rodrigez and collaborators (2008) have studied the role of CK2β in the export of the holoenzyme to the extracellular membrane through deletion and point mutations. The region of CK2β between amino acids 20 and 33 was found to be necessary, but not sufficient, to allow the catalytic subunits to function as an ectokinase. An important function of this region is fulfilled by Phe21 and Phe22, which anchor the loop of the 20-33 sequence. Another key element of this region is constituted by the acidic residues in positions 26-28. They are exposed to the medium, free to interact with other proteins (Bolanos-Garcia et al, 2006).

2.2.5 Mutation of CK2β that affects its stability

Overexpression of CK2 catalytic subunits leads to increased cell proliferation and transformation, while overexpression of the regulatory CK2 subunit is associated with decreased proliferation in yeast and mammalian cells (Li et al., 1999; Lebrin et al., 2001; Vilk et al., 2001). Moreover, CK2β is physiologically expressed at a higher level than CK2α, and the excess of the regulatory subunit is rapidly ubiquitinated and degraded in a proteasome-dependent manner (Luscher & Litchfield, 1994; Zhang et al., 2002). To protect CKβ from the degradation machinery and to stabilize it, six surface-exposed lysine residues were mutated to arginine (French et al., 2007). The 6KR mutant functioned as normal CK2β, but it was not sensitive to proteasome inhibition. The physiological role of mutagenesis-mediated CK2β stabilization was also examined with the use of cell proliferation assays. A significant decrease in proliferation was observed in cells expressing the 6KR mutant when compared to wild-type CK2β. The authors suggest that the stabilized form of the CK2 regulatory subunits can be utilized to inhibit cell proliferation in cancer cells (French et al., 2007).

2.3 Mutagenesis of CK2 substrates

Protein kinase CK2 is a multi-substrate enzyme with a large number of cellular partners. In 2003, Meggio and Pinna updated the list of 307 CK2 substrates with 308 sites phosphorylated by CK2 (Meggio & Pinna, 2003). This number is now out-of-date, as novel CK2 protein substrates are discovered every year. A *bona fide* CK2 substrate may possess one or several phosphoacceptor sites affected by CK2, but an analysis of the initial amino acid sequences of possible CK2 partners may show a dozen or so putative CK2 sites. Site-directed mutagenesis is a useful tool to create CK2 substrate mutants. Such proteins are produced (1) to indicate precisely the phosphorylatable amino acid, (2) to study the physiological significance of CK2-mediated phosphorylation of a given protein substrate, or (3) to confirm the physiological relevance of CK2-mediated phosphorylation. Presented below are several examples of the mutagenesis of CK2 substrates.

Mdm2 is a cellular oncoprotein that down-regulates the growth suppressor protein p53 (Barak et al., 1992). Computer analysis of the amino acid sequence of Mdm2 revealed 19 putative CK2 phosphorylation sites. Three Mdm2 mutants with deletions at codons 1-114, 93-285, and 271-491 were produced to exclude sites that are not affected by CK2. The phoshorylation assays revealed that only the central part of Mdm2 is phosphorylated. Based on further detailed analysis of the remaining CK2 consensus sites, Ser269 was chosen to be the most promising. Using overlap extension PCR (see section 2.7 in the chapter by Sturtevant), the Mdm2 point mutant S269A was constructed and the relevant CK2 phosphorylation site was finally discovered (Götz et al., 1999).

In some protein substrates, putative CK2 phosphorylation sites are located close to one another, and thus several point mutants had to be produced to score them. The consensus sequence analysis of the N-terminal domain of the human transcription factor Tcf-4 indicated multiple sites that fit the motif for CK2 phosphorylation. No CK2-mediated phosphorylation was detected on the Tcf-4 fragments comprising amino acids 1-30 and 1-49. Thus, the best candidates for CK2-affected amino acids were the serine residues located in the Tcf-4 peptide T_{54}NQDSSSDSEAERRP$_{68}$. Three Tcf-4 mutants, one triple point mutant (S58A/S59A/S60A) and two single point mutants (S58E and S60E) were made to help indicate the phosphorylatable amino acid. *In vitro* phosphorylation assays revealed that all three adjacent serines are modified by CK2 with different efficiencies (Miravet et al., 2002).

Sic1 is a yeast protein that specifically inhibits Clb/Cdk activity in the G1 phase, so that DNA replication is suppressed (Verma et al., 2001; Nash et al., 2001). Moreover, Sic1 undergoes multistep phosphorylation. Therefore, Sic1 phosphorylation occurs at several positions. One looks like the CK2 consensus site. CK2-mediated phosphorylation of Sic1 within the Q_{199}ESEDEED sequence was confirmed both *in vitro* and *in vivo* in *Saccharomyces cerevisiae* cells (Coccetti et al., 2004, 2006). Mutations of the CK2 consensus site on Sic1 (S201A and S201E) alter the coordination between cell growth and division. They also change the level and time-course of S-Cdk kinase activity. These mutation data strongly support the physiological relevance of Sic1 phosphorylation for inhibitory activity (Coccetti et al., 2004).

The regulatory effect of CK2 activity on the Wnt signaling pathway is widely known (Pinna, 2002; Litchfield, 2003). Kinase phosphorylates and interacts with β-catenin and thus enhances the stability and transcriptional activity of β-catenin (Song et al, 2003; Seldin et al, 2005). The AKT/PKB kinase is also a well-known CK2 substrate and interacting partner. CK2-mediated phosphorylation at Ser129 causes AKT hyperactivation (Di Maira et al, 2005; Guerra, 2006). CK2 may link the two pathways..

To elucidate the roles of CK2 in the Wnt and AKT/PKB signaling pathways, the AKT phosphorylation-deficient mutant (S129A) was overexpressed in an embryonic cell line. The β-catenin-dependent transcriptional activity was analyzed. The data obtained indicate that blockage of AKT phosphorylation by CK2 impairs β-catenin activity and decreases its stability. Therefore, CK2-mediated AKT phosphorylation at Ser129 is a necessary step in the up-regulation of the β-catenin transcriptional activity in human embryonic kidney cells (Ponce et al., 2011).

Besides phosphorylation of numerous cellular proteins, CK2 directly interacts with many of them forming protein-protein complexes (Litchfield, 2003). Both catalytic and regulatory CK2 subunits can interact with different proteins, independently of the holoenzyme (Bibby et al., 2005). Wee1 kinase, involved in cell cycle progression, is one such CK2 protein partner. The Wee1 kinase is a key inhibitor of cyclin-dependent kinase (CDK1) and mitotic entry in eukaryotes. Several deletion mutants of the Wee1 catalytic domain were produced to investigate the interaction with CK2 subunits. Immunoprecipitation experiments revealed that Wee1 binds CK2β via two domains of Wee1 (comprising amino acids 59-71 and 232-332) and two regions of CK2β (comprising residues 1-5 and 155-170). Although the interaction does not affect Wee1 activity, it up-regulates CDK1 by reversing the Wee1-mediated inhibitory effect on CDK1. These findings reinforce the notion that CK2β can serve other protein kinases. It may be a universal regulatory subunit that can act independently of the CK2 holoenzyme (Olsen et al., 2010).

3. Conclusion

Even 58 years after its first description (Burnett & Kennedy, 1954), the story of protein kinase CK2 has not been fully clarified. This enzyme catalyzes phosphorylation of over 300 substrates. They are characterized by having multiple acidic residues surrounding the phospho-acceptor amino acid. Consequently, CK2 plays a key role in several physiological and pathological processes (Guerra & Issinger, 2008). After all those years of research, we are still asking the question: how is it possible that one kinase can be involved in so many

different biochemical processes in the cell? Using different biochemical and genetic methods, we have solved several problems connected with the structure and mechanism of the catalytic action of this enigmatic protein kinase. The application of mutagenesis methods in many cases has helped us and will continue to help us get answers to many problems connected with CK2 activity. Among them are the following:

- The interaction between subunits
- Catalytic specificity and efficiency
- Substrate recognition
- Regulation by the β-subunit
- Stability of the subunits
- Interactions with modulators and substrates
- The effect of phosphorylation on catalytic activity
- Constitutive CK2 activity.

A protein kinase, such as CK2, is difficult to explore with respect to its physiological functions. CK2 has been shown to be involved in numerous aspects of cell proliferation and survival, including cell cycle progression and apoptosis control (Ahmad et al., 2008; Ahmed et al., 2002; Batistuta, 2009; Gyenis & Litchfield, 2008; Meggio & Pinna, 2003; Litchfield, 2003). Alterations in the levels or activity of CK2 have been implicated in a variety of human diseases, including cancers (Guerra & Issinger, 2008). All these observations raise important questions regarding the mechanisms that control CK2 activity and specificity. These questions have a special value, since defects in regulation of these processes could contribute to tumorigenesis.

In this context, the application of mutagenesis methods, together with other techniques (*e.g.*, molecular modeling), may be very useful in designing highly effective and specific inhibitors that are promising for CK2-based target therapy.

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