Part I
Molecular and Structural Aspects

1 Structural Bases of Protein Kinase CK2 Function and Inhibition

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INTRODUCTION

"Protein Kinase CK2: A Challenge to Canons"

Protein kinase CK2—more precisely its catalytic subunit CK2α—is one of 518 protein kinases of the human kinome (Manning et al., 2002). Like all protein kinases, it catalyzes the transfer of the terminal phospho group of a nucleotide to a substrate protein (Figure 1.1).

CK2 is not an "atypical" protein kinase (APK), meaning CK2α is one of those 478 human protein kinases related by significant sequence homology and is a member of the eukaryotic protein kinase (EPK) superfamily (Hanks and Hunter, 1995). Nevertheless, CK2 is "a challenge to canons" according to a commentary by Pinna (2002) in which the author emphasized some features of CK2 non-canonical within this EPK superfamily.

In fact, since its first mentioning in the literature nearly 60 years ago (Burnett and Kennedy, 1954), the particular enzymological profile of CK2 emerged in continuous comparison to the increasing list of EPKs, and during this process, a number of exceptional properties stood out. For some of them, the unconventional character

Protein Kinase CK2, First Edition. Edited by Lorenzo A. Pinna.

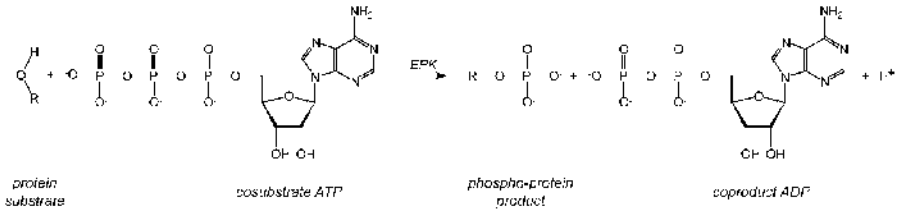

Figure 1.1. Scheme of the reaction catalyzed by a eukaryotic protein kinase (EPK). The reaction is essentially irreversible under physiological conditions. The hydroxy group of the protein substrate belongs to the side chain of serine, threonine, or tyrosine. In the case of CK2, the cosubstrate can be GTP as well as ATP.

was relativized with increasing knowledge about EPKs while for others it was intensified, but as a whole, they define the unorthodox nature of CK2.

Acidophilic and Pleiotropic Features

From the beginning, acidic phosphoproteins like casein or phosvitin (Rodnight and Levin, 1964) served as artificial and eponymous substrates, whereas other early EPKs like glycogen phosphorylase kinase (Krebs and Fischer, 1956) or cAMP-dependent protein kinase (CAPK) (Walsh et al., 1968) were basophilic with histones as typical *in vitro* substrates.

Consistently, negatively charged substrate residues around the phosphorylatable serine or threonine side chain were found to be crucial for substrate recognition by CK2 in the 1980s (Pinna et al., 1984).

In 1988, the minimal consensus sequence defining a CK2 substrate was published to be S/T-X-X-D/E (Marchiori et al., 1988). Such a small sequence motif occurs quite frequently in proteins so that the exponential growth of the number of CK2 substrate proteins to more than 300 in the last census (Meggio and Pinna, 2003) was not fully surprising. Consistently, consensus sequence analyses of the human Phospho.ELM database (Diella et al., 2004) suggested that CK2 is responsible for the generation of a substantial proportion of the human phospho-proteome (Salvi et al., 2009). Due to this broad substrate spectrum CK2 belongs to EC class 2.7.11.1 (Scheer et al., 2011) (i.e., to the non-specific serine/threonine protein phosphotransferases). A statistical analysis of the sequence regions around the phosphorylation (P + 0) site (Meggio and Pinna, 2003) confirmed the significance of the P + 3 position but additionally emphasized the P + 1 site that, if negatively charged, strongly favors CK2-catalyzed protein phosphorylation.

Dual-Cosubstrate Specificity

Although ATP is the typical cosubstrate of an EPK, Rodnight and Lavin (1964) reported already in 1964 that CK2 (which they called “phosvitin kinase”) can alternatively utilize GTP. This ability indicated structural peculiarities in the cosubstrate binding site, and in the late 1960s (Pinna et al., 1969), it was the basis for the distinction between two acidophilic “phosvitin kinases,” one of them being ATP-specific (later called “casein kinase 1” since it elutes earlier from a DEAE-anion

exchange column [Hathaway and Traugh, 1979]), while the other, that is, CK2, accepts either ATP or GTP.

Quaternary and Higher-Order Oligomeric Structure

When the knowledge to separate CK1 from CK2 and to distinguish them enzymatically was established, it turned out very soon that the former is a monomer, and CK2, when prepared from natural animal tissues, exists as a heterotetrameric holoenzyme with $\alpha_2\beta_2$-stoichiometry (Hathaway and Traugh, 1979; Thornburg and Lindell, 1977). Moreover, this quaternary structure is the prerequisite for the CK2's strongly salt-dependent ability to form higher-order aggregates. These aggregates were observed directly for the first time by Glover (1986) and intensively studied in their correlation to catalytic activity by Valero et al. (1995), but they are also responsible for a paradoxical phenomenon noticed earlier by Hathaway and Traugh (1979): CK2 binds so strongly to phosphocellulose columns that 700 mM NaCl is required for elution (leading to the name "PC0.7" [DePaoli-Roach et al., 1981]), but binding to the column does not occur below 250 mM NaCl.

For vertebrate CK2, a heterotetrameric architecture was consistently reported several times in the 1970s; however, for plant CK2, a different picture emerged in the early literature. In 1982, CK2 activity was found to be associated with monomers in wheat germs (Yan and Tao, 1982) and with homodimers in tobacco (Erdmann et al., 1982), and only 10 years later, a typical $\alpha_2\beta_2$-holoenzyme of CK2 was discovered in a plant tissue (Li and Roux, 1992). At almost the same period, evidence for CK2β-free CK2α was provided for the first time for animal (insect) cells (Stigare et al., 1993).

Polyanionic Inhibitors, Polycationic Activators

The 1970s were the golden decade of classical CK2 biochemistry and led, consistent to the acidophilic substrate preference of CK2, to the discovery of anionic substrate-competitive CK2 inhibitors like 2,3-diphospho glycerate (Kumar and Tao, 1975) or heparin (Mäenpää, 1977]. The latter advanced to a standard test substance to probe biochemically whether a new protein kinase activity is due to CK2 or not. At the same time, polyamines such as putrescine, spermidine, and spermine (Mäenpää, 1977) followed by polylysine (Criss et al., 1978) were reported to be stimulatory effectors of CK2. Later these stimulatory effects of polycations were specifically associated with the addiction of CK2 to form higher-order and filamentous aggregates (Mamrack, 1989).

ATP-Competitive Inhibitors

The first effective ATP-competitive inhibitors of CK2 described in the literature were 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and quercetin (Zandomeni and Weinmann, 1984). The possibilities to probe the selectivity of these inhibitors were rudimentary at that time, but later DRB advanced as a mother substance for synthetic CK2 inhibitors (Meggio et al., 1990).

Constitutive Activity

The discovery of glycogen phosphorylase kinase (Krebs and Fischer, 1956) and of CAPK (Walsh et al., 1968) was guided by the integration of these enzymes into a

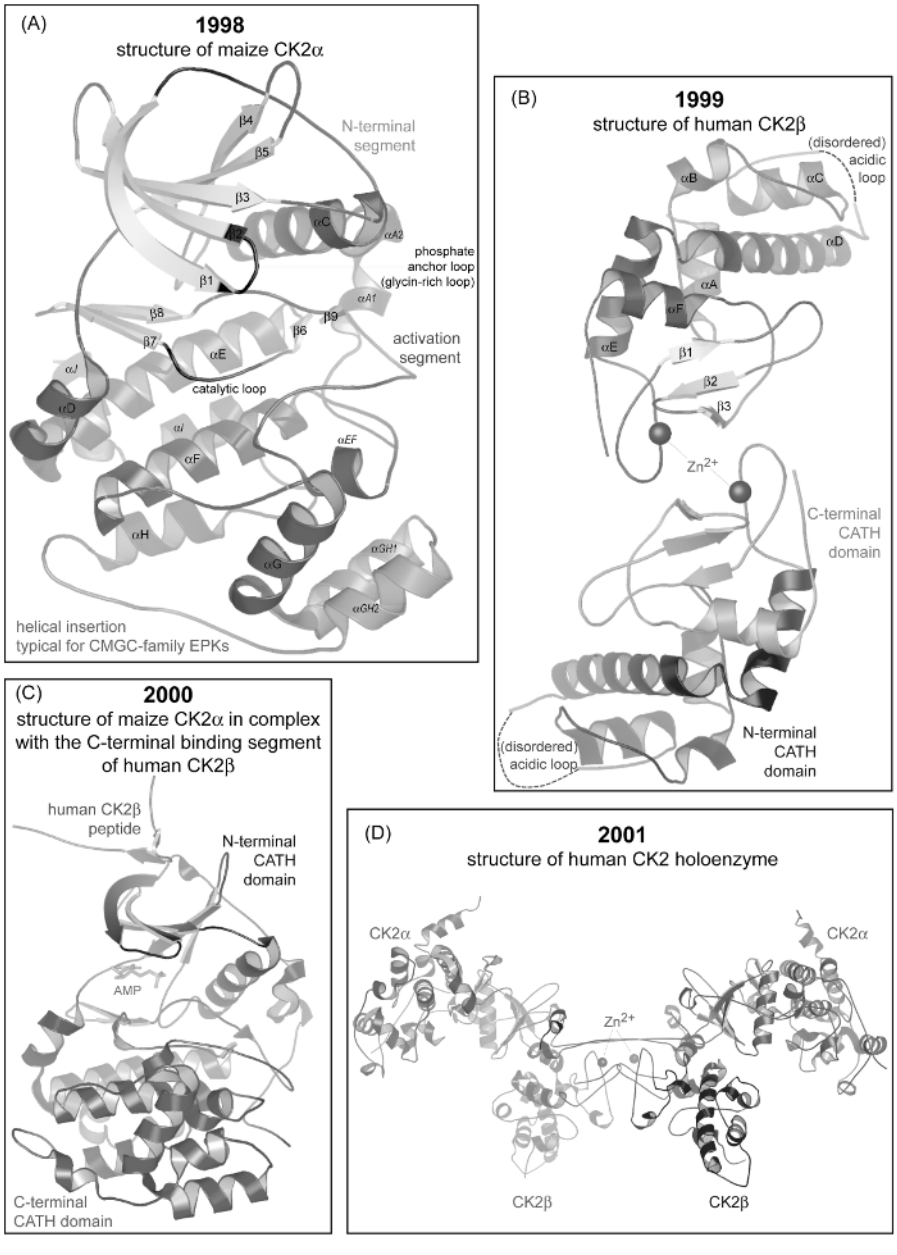

Figure 1.2. Highlights of the initial phase of CK2 structural biology. (A) Structure of CK2α from *Zea mays* (Niefind et al., 1998; PDB 1A6O, later superseded by 1LR4). The most remarkable detail of the structure was the close attachment of the N-terminal segment (blue) with the activation segment (magenta) and the helix αC, a feature that was fully consistent with the constitutive activity of the enzyme and that was confirmed in all subsequent CK2α until now. Only major secondary structure elements (no 3_{10}-helices) are drawn. In the canonical catalytic core, they are designated in analogy to CAPK (Figure 1.4A). The names of noncanonical secondary structure elements are printed in italics. (B) Homodimeric structure of human CK2β^{1-182} (Chantalat et al., 1999; PDB 1QF8). The structure revealed the existence of a zinc-binding motif next to the dimerization interface. In CATH (Cuff et al., 2011), each CK2β subunit is divided into two domains as indicated in the lower monomer. (C) Structure

regulatory pathway, i.e., both enzymes are regulators (by catalyzing phosphorylation) and are simultaneously subject to regulation (by phosphorylation, second messenger binding, and/or disassembly). Therefore, it became common practice to characterize further protein kinase activities with respect to their regulatory behavior *in vitro* (and putatively *in vivo*). However, in the case of CK2, it turned out that this kinase is "constitutively active," i.e., its activity does not depend on a phosphorylation event or on the binding of Ca^{2+}, cAMP, or other second messenger molecules.

Even the important discovery that the catalytic α-subunit is enzymatically active alone and can be stimulated *in vitro* by the non-catalytic β-subunit (Cochet and Chambaz, 1983) did not resolve the puzzle of CK2 regulation since strongly denaturing conditions were required to separate the subunits so that a dissociation under physiological conditions was regarded as impossible.

Structural Biology of CK2

In summary, when the structure biology of CK2 started in the 1990s, a number of important and non-canonical properties of the enzyme were well characterized and awaited structure-based rationalization. CK2 crystallography could satisfy many of these expectations, in particular in the first phase producing the crystal structures of maize CK2α (Niefind et al., 1998), of human CK2β (Chantalat et al., 1999), of maize CK2α in complex with a human CK2β peptide (Battistutta et al., 2000a) and of the human CK2 holoenzyme (Niefind et al., 2001) (Fig. 1.2).

Moreover, the unique enzymological profile of CK2 suggested the possibility to create highly effective and selective inhibitors that could serve as tools in chemical biology approaches as well as drugs in pharmacology. To design such compounds assisted by structural data was suggested shortly after the first CK2α structure had been published (Guerra et al., 1999). This phase of "applied CK2 crystallography" started with the structure of the complex between CK2α and emodin (Battistutta et al., 2000b) and became very soon the main field of innovation in CK2 structural biology and a driving force of CK2 research in general.

Figure 1.2. cont'd of maize CK2α in complex with human $CK2\beta^{181–203}$, i.e., with a peptide from the C-terminal region of human CK2β known to be critical for CK2α binding (Battistutta et al., 2000a; PDB 1DS5). With this structure, the CK2β-binding interface of CK2α was identified. The two CATH domains of CK2α are indicated in the figure. Noteworthy, the N-terminal part of the N-terminal segment belongs to the C-terminal CATH domain. (D) Structure of a heterotetrameric human CK2 holoenzyme complex (Niefind et al., 2001; PDB 1JWH) merging the structural information from panel A to C and disclosing the fact that the CK2α/CK2β interface is formed by three protomers, namely one CK2α subunit and both CK2β chains. The extension of the structure compared to the sequences of the wildtype proteins is indicated in Figure 1.4. For color detail, see color plate.

Aim of This Chapter

We outlined these developments in the structural biology of CK2 in two recent articles in the context of a multi-author review about the enzyme (Niefind et al., 2009; Battistutta, 2009). Here, we intend to supplement and update these contributions, giving our reasoned view of the state-of-art of the matter. Regarding the inhibitors section, here we want to focus the attention specifically on structural aspects of CK2 inhibition. Our purpose is not to survey the large and always increasing ensemble of CK2 inhibiting compounds that have been discovered. For many of these classes of inhibitors, clear structural information is lacking, and their proper mode of action is still unclear. Therefore, they will be not covered in this context, and the interested reader is referred to the original publications.

BASIC STRUCTURE/FUNCTION RELATIONSHIPS OF CK2

Domains and Databases

PFAM

Eukaryotic protein kinases possess a conserved catalytic core of about 260 residues defined for the first time in 1988 (Hanks et al., 1988) on the basis of a multiple sequence alignment including 65 enzymes, among them *Drosophila* CK2α. This core constitutes a "functional domain" and thus a "family" in the sequence-homology based PFAM database (Punta et al., 2012) (Table 1.1). It is divided into 12 particularly conserved subdomains (Hanks et al., 1988; Hanks and Hunter, 1995). With currently more than 76,000 sequences, the "Pkinase" family is among the 15 largest PFAM entries and by far the biggest among a superordinate "clan" (Table 1.1) that comprises further and more distantly related kinases like atypical protein kinases (APK) and EPK-like kinases (ELK). The last ones are certain ATP-dependent phosphotransferases with smaller substrates like choline or aminoglycosides.

In contrast, CK2β is eponymous for its own PFAM family with currently less than 600 sequences and without any relatives on the clan level (Table 1.1). This unique character of CK2β in sequence space was already noted when the first CK2β sequence was published (Saxena et al., 1987); remarkably, it did not change in the age of high-throughput sequencing.

CATH

The 3D-pendants to PFAM are CATH (Cuff et al., 2011) and SCOP (Andreeva et al., 2008), hierarchical databases that classify proteins of known 3D-structure deposited in the Worldwide Protein Data Base (wwPDB; www.wwpdb.org) (Berman et al., 2007). More precisely, in CATH the principle classification unit is the "structural domain" defined as a semi-autonomous folding unit within which the majority of non-covalent interactions are satisfied and which owns a hydrophobic core.

The first step in the CATH classification is the partitioning of a protein 3D-structure into such structural domains, irrespective of—and this is the main difference from SCOP (Andreeva et al., 2008)—evolutionary and functional similarity. Each of these CATH domains is then assigned according to structural criteria:

Table 1.1. CK2α and CK2β, the subunits of protein kinase CK2, in established sequence- and structure-based hierarchical classification databases.

	PFAM (pfam.sanger.ac.uk)	**CATH (www.cathdb.info)**		**SCOP (scop.berkeley.edu)**
		N-terminal CATH domain	**C-terminal CATH domain**	
CK2α	**clan:** CL0016; Pkinase	**class:** 3; alpha beta **architecture:** 3.30; 2-layer sandwich	**class:** 1; mainly alpha **architecture:** 1.10; orthogonal bundle	**class:** d; (α + β) proteins **fold:** d.144; protein kinase-like
	family: PF00069; Pkinase; protein kinase domain	**topology:** 3.30.200; phosphorylase kinase, domain 1 **homologous superfamily:** 3.30.200.20; phosphorylase kinase, domain 1	**topology:** 1.10.510; transferase (phospho-transferases) domain 1 **homologous superfamily:** 1.10.510.10; transferase (phosphotransferases) domain 1	**superfamily:** d.144.1; protein kinase-like (PK-like) **family:** d.144.1.7; protein kinases, catalytic subunit
CK2β	**clan:** *[not assigned]*	**class:** 1; mainly alpha **architecture:** 1.10; orthogonal bundle	**class:** 2; mainly beta **architecture:** 2.20; single sheet	**class:** g; small proteins **fold:** g.41; rubredoxin-like
	family: PF01214; CK_II_beta; Casein kinase II regulatory subunit	**topology:** 1.10.1820; protein kinase CK2 holoenzyme, chain C, domain 1 **homologous superfamily:** 1.10.1820.10 protein kinase CK2 holoenzyme, chain C, domain 1	**topology:** 2.20.25; N-terminal domain of TFIIb **homologous superfamily:** 2.20.25.20 *[no name assigned]*	**superfamily:** g.41.4; casein kinase II β subunit **family:** g.41.4.1; casein kinase II β subunit

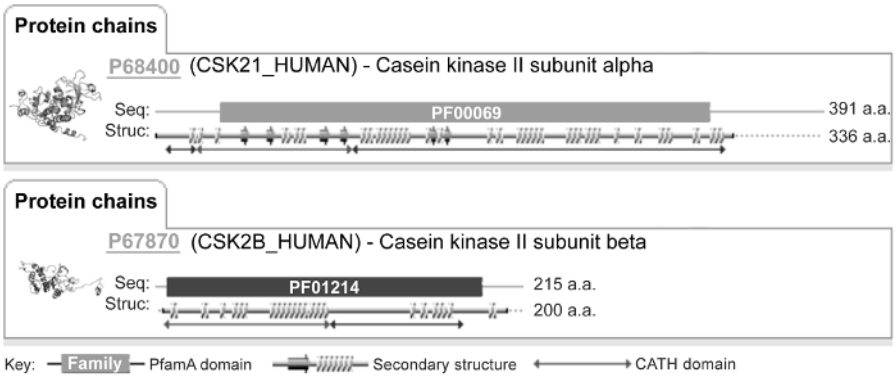

Figure 1.3. Part of the PDBSUM page of PDB entry 1JWH (human CK2 holoenzyme). PDBSUM (Laskowski, 2009; www.ebi.ac.uk/pdbsum/) offers an overview of a structure and its underlying sequence and is linked with a number of useful structural and functional databases, in particular to PFAM (Punta et al., 2012) and CATH (Cuff et al., 2011).

first to a Class, then to an Architecture, a Topology, and finally to a Homologous Superfamily (Table 1.1). Below this level, clustering proceeds according to sequence identities. Only from the H-level, an evolutionary relatedness is predicted, i.e., all domains belonging to the same Homologous Superfamily are assumed to have descended from a common ancestor.

In CATH, both CK2α and CK2β are divided into two structural domains (Table 1.1). This domain organization, along with the PFAM annotation and an overview of the extent of the protein structures compared to the underlying sequences, is nicely illustrated in PDBsum (Laskowski, 2009) curated by the European Bioinformatics Institute (www.ebi.ac.uk/pdbsum) (Figure 1.3).

For CK2α, the CATH domain architecture became apparent in 1991 with the crystal structure of the CAPK catalytic subunit (Knighton et al., 1991) (Figure 1.4A). The outstanding nature of this structure as a representative for the whole enzyme family was clear from the beginning and documented in a figure in which the positions and lengths of insertions were indicated by arrows (Figure 1.4A). Further, this figure illustrates the two N-terminal (blue) and C-terminal (pink) structural domains (often called "lobes" like in Figure 1.4B). Noteworthy, the aforementioned division into 12 sequence-conserved subdomains (Hanks et al., 1988; Hanks and Hunter, 1995) does not fully coincide with the concept of structural domains; in particular, subdomain V comprises elements of the two lobes (N-lobe: strand β5; C-lobe: helix αD) as well as the interconnecting hinge region.

In the case of CK2β, the two-domain architecture could not be inferred by homology but was revealed directly by the first crystal structure of a CK2β construct (Chantalat et al., 1999) (Figure 1.2B).

SCOP

SCOP (Andreeva et al., 2008) is the oldest database for the "Structural Classification of Proteins." Its origins go back to the definition of basic secondary structure patterns introduced nearly 4 decades ago (Levitt and Cothia, 1976). In contrast to

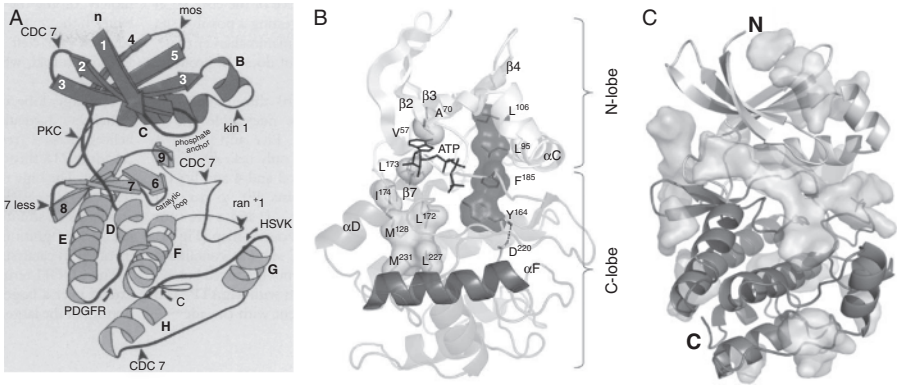

Figure 1.4. Structural illustrations of cAMP-dependent protein kinase (CAPK) reflecting 20 years of development in the comprehension of the enzyme and of eukaryotic protein kinases in general. (A) The catalytic core composed of two separated folding domains. Reprinted from Science, Vol. 253, Knighton, D.R. et al. "Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase," pp. 407–414, 1991, with kind permission from the American Association for the Advancement of Science (AAAS). (B) Two stacks of hydrophobic residues ("spines") crossing the domain border as a necessary condition for full functionality. The regulatory spine (R-spine) is drawn in red and the catalytic spine (C-spine) in yellow. Both spines are based on the hydrophobic helix αC located in the center of the C-lobe. Reprinted from Trends Biochem. Sci., Vol. 36, Taylor, S.S. and Kornev, A.P., "Protein kinases: evolution of dynamic regulatory proteins," pp. 65–77, 2011, with kind permission from Elsevier. (C) Conserved clefts and pockets on the surface of EPKs illustrated with the CAPK catalytic core. The pockets were discovered bioinformatically by comparing 10 fully active EPK structures (six Ser/Thr- and four Tyr-kinase structures) (Thompson et al., 2009). The surface cavities are either directly functionally important or can be addressed to stabilize the spines. Reprinted from Protein Science, Vol. 18, Thompson, E.E. et al. "Comparative surface geometry of the protein kinase family," pp. 2016–2026, 2009, with kind permission from Wiley-Blackwell. For color detail, see color plate.

CATH, the SCOP classification works from the bottom, i.e., from the sequence level. It inherits the sequence-based functional domain annotation of PFAM so that in SCOP, a single domain is assigned both for CK2α (and other EPKs) and for CK2β (Table 1.1).

SCOP domains clustered on the family level are reliably evolutionary related like PFAM families. However, the ambition of both PFAM and SCOP is to detect more distant homologies; the former by sophisticated sequence comparison tools like Hidden-Markov-Models (Finn et al., 2011) and the latter by inclusion of structural information.

SCOP superfamilies are insofar comparable in their significance to PFAM clans, and in fact the "protein kinase-like" superfamily of SCOP comprises APKs and ELKs in addition to EPKs, like the Pkinase clan of PFAM.

The Spine Concept of Eukaryotic Protein Kinases

The SCOP domain of EPKs reflects the development of EPK structural biology from the clear distinction of two structural domains in the first CAPK structure (Figure 1.4A) to the concept of "spines," i.e., two stacks of hydrophobic residues that form functionally essential connections across the structural domain boundaries (Kornev et al., 2008b; Kornev and Taylor, 2010; Taylor and Kornev, 2011) (Figure 1.4B). This development toward a holistic view of EPKs as complex systems, integrating catalytic functionality with sophisticated modes of regulation and of substrate recognition, was enabled by hundreds of EPK structures determined in the last 2 decades representing various functional and binding states.

The view on the SCOP superfamily level, that is the inclusion of structures of ELKs that share with EPKs the catalytic function but lack the strict controllability of the latter, was just as important. Kannan and Neuwald (2005) were the first to show that systematic sequence and structure comparisons between EPKs and ELKs can provide hypotheses about the evolution of regulatory mechanisms at the structural level.

The spine concept (Figure 1.4B) is a consequent advancement of this approach technically based on the novel "local spatial pattern" (LSP) alignment procedure (Kornev et al., 2008a). The first application of LSP alignment with EPKs (Kornev et al., 2006) took only residues with solvent access into account and led to the detection of a "hydrophobic spine" that was later re-named into "regulatory spine" or simply "R-spine" (Kornev et al., 2008b) (red stack in Figure 1.4B). The R-spine consists of four hydrophobic residues, two from either lobe, in CAPK: Leu106, Leu95, Phe185, and Tyr164. That means that the R-spine forms a non-covalent connection between the two lobes that supplement the covalent connection by the hinge region. Catalytic activity of an EPK is only possible if the R-spine is fully established.

When the LSP approach was extended to all residues (Kornev et al., 2008b), a second hydrophobic spine—referred to as "catalytic spine" or "C-spine"—became visible. The C-spine bridges the domain boundary next to the hinge region, the covalent connection of the two lobes (yellow stack in Figure 1.4B). For full establishment, the C-spine requires the adenine moiety of ATP, meaning that the C-spine is regularly opened and closed in the course of the catalytic cycle. Finally, it was recognized that the two spines experience an internal stabilization if they are connected by a large so-called "gatekeeper" residue located at the beginning of the hinge region (Kornev and Taylor, 2010).

A further extension of the spine concept was achieved when sophisticated bioinformatic tools were applied to systematically compare the surfaces of EPKs and to search for cavities that can be used for stabilization of the spines and of the entire hydrophobic core (Thompson et al., 2009). Some of the detected pockets are conserved (Figure 1.4C), but they are used in a kinase-specific manner.

The Catalytic Subunit CK2α

The structure-based sequence alignment of the human catalytic subunits of CK2α and PKA (considered the "reference" protein kinase domain) is shown in Figure 1.5.

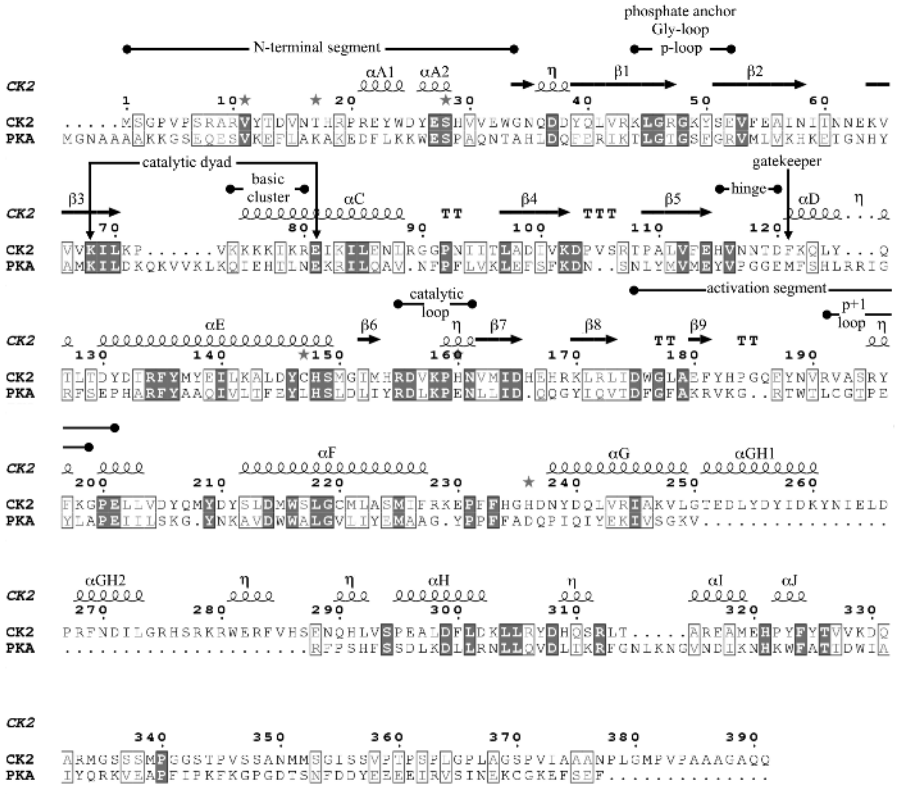

Figure 1.5. Structure-based sequence alignment (by 3D-Coffee) of human CK2α, PDB 3NSZ, and human PKA, PDB 3AGM (enhanced by ESPript Web-server). Elements of the CK2α secondary structure are indicated (α = α-helix; β = β-strand; η = 3_{10}-helix; T = Turn), as well as relevant residues and regions discussed in the text. Nomenclature of helices and strands according to Figure 1.2A. Conserved and homologues residues are indicated in white on a black background and in contoured boxes, respectively. Numbering refers to the human CK2α sequence. The secondary structure of the C-terminal tail of CK2α (from residue 333) is unknown.

Elements of the CK2α secondary structure, as well as relevant residues and regions discussed in the text, are indicated. Nomenclature of helices and strands is according to Figure 1.2A.

The N-terminal CATH Domain (N-Lobe)

The N-terminal domains of all CK2α structures in the PDB form an "S35 family" in CATH (3.30.200.20.3), i.e. the corresponding sequences are at least 35% identical. Beyond this level, three S60 families are present comprising the N-terminal domains of either human/rat CK2α (3.30.200.20.3.1), maize CK2α (3.30.200.20.3.2), and human CK2α′ (3.30.200.20.3.3), indicating the only species from which CK2α structures were solved so far.

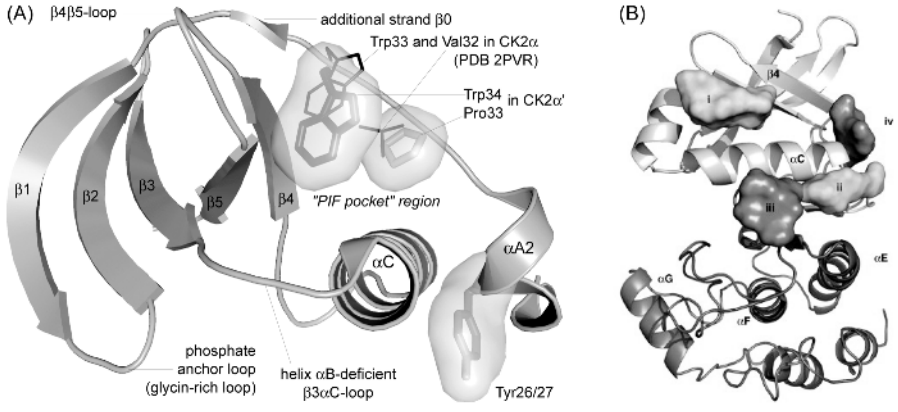

Figure 1.6. The N-terminal CATH domain of CK2α and other EPKs and its stabilization by addressing surface pockets. (A) N-lobe of CK2α in the case of strong intramolecular stabilization by tight anchoring by the N-terminal segment as found in structures of human CK2α′ (Nakaniwa et al., 2009; Bischoff et al., 2011a) and one of the existing human CK2α structures (PDB 3NSZ; Ferguson et al., 2011). The stabilization can be recognized from an extension of the β-sheet by a short strand β0 and from the occupation the space between the helix αC and the strand β5 by a conserved Trp side chain. This space is known as "PIF pocket" in AGC kinases where it serves as an inter- or intramolecular docking site (Biondi et al., 2000). (B) N-lobal surface pockets around the key regulatory helix αC. These pockets can be addressed in a kinase-specific way to stabilize the helix αC and thus the R-spine. While some EPKs use them as docking sites, CK2α fills two of them (i and iii) by side chains from the N-terminal segment (panel A). Reprinted as gray scale version from Trends Biochem. Sci., Vol. 36, Taylor, S.S. and Kornev, A.P., "Protein kinases: evolution of dynamic regulatory proteins," pp. 65–77, 2011, with kind permission from Elsevier.

The domain comprises residues Asp25 to His115 and is classified as "2-layer sandwich" (Table 1.1). Layer 1 is a five-stranded, strongly twisted, and antiparallel β-sheet (Figure 1.6A), the outside of which forms the interface to CK2β (Figure 1.2C). Layer 2 is essentially the long helix αC. The linker region between the strand β3 and the helix αC is very different in length among EPKs (Hanks and Hunter, 1995): in CAPK—representing the canonical catalytic core (Figure 1.4A)—a small helix αB is formed here, but in CK2α, only a short loop without any helical element connects β3 and αC (Figure 1.6A). On the other hand, a part of the N-terminal segment including the small helix αA2 with contacts to helix αC is part of the domain.

In human CK2α′ (Nakaniwa et al., 2009; Bischoff et al., 2011a) and in the highest-resolution human CK2α structure 3NSZ (Ferguson et al., 2011), the five-stranded β-sheet is enlarged by a short strand β0 (Figure 1.6A) formed by the region from Trp33 to Gln36. This particular feature is correlated with the occupation state of a hydrophobic surface cavity by the side chain of Trp33. In this state, the N-terminal segment looks like a belt that is tightened around the β4β5-loop (Figure 1.6A).

In contrast, in the majority of CK2α structures, the belt is somewhat released, i.e., no additional strand β0 is formed, and the Trp33 side chain remains at the entrance of the hydrophobic cavity without direct contact to helix αC (Figure 1.6A). A functional role of a putative switch between a tight conformational state of the N-lobe and a relaxed one is not apparent so far.

Special Features of the Phosphate Anchor Loop of CK2α

Functionally, the most important part of the N-terminal domain is the glycine-rich loop connecting the strands β1 and β2 (Figure 1.2A, Figure 1.6A). All three elements together—β1, β2 and the glycine-rich loop—form the subdomain I in the Hanks/Hunter nomenclature (Hanks et al., 1988; Hanks and Hunter, 1995).

The glycine-rich loop was referred to as a "phosphate anchor" in the first EPK structure (Figure 1.4A) because it serves to coordinate the triphospho group of ATP/GTP (Figure 1.7A). In many EPK-related publications, it is called the "P-loop," indicating a functional similarity to the Walker-A motif (Ramakrishnan et al., 2002) found in many GTPases and other nucleotide binding proteins for which the term "P-loop" was originally introduced (Saraste et al., 1990). However, the Walker-A P-loop not only has a consensus sequence different from the phosphate-anchor of EPKs, but it also differs significantly at the structural level as extensively discussed in the first EPK structure report (Knighton et al., 1991).

In CK2α sequences, the consensus of the phosphate-anchor region is G-R-G-K-Y/F-S, i.e., compared to most other EPKs, the final glycine residue is replaced by a serine (Ser52; Figure 1.7A). A consequence of this change on a structural level is illustrated in Figure 1.7A. In CAPK (representing the normal case of an EPK), the spatially undemanding glycine residues of the phosphate-anchor loop enable a close approximation between the β- and the γ-phospho moieties and the loop backbone so that direct hydrogen bonds to peptide groups are formed (thin dotted lines in Figure 1.7A); in binary complexes of ATP- and GTP-analogs with CK2α (Niefind et al., 1999), however, the triphospho (analogous) group is not directly anchored by the glycine peptide groups but by the side chain of Ser52 and by two water-mediated hydrogen bonds (thick dotted lines in Figure 1.7A). This, together with the absence of CAPK helix αB, generates more space at the region of phospho transfer, which may be important for the occasionally reported ability of CK2 to catalyze protein and peptide phosphorylation at tyrosine residues (Vilk et al., 2008).

The Interface to CK2β Is Formed Exclusively by the N-Lobe

A second ligand-binding region of the CK2α N-lobe is the concave outer surface of the antiparallel β-sheet, which was identified as interface to CK2β by a CK2α structure in complex with a peptide from the C-terminal tail region of CK2β (Battistutta et al., 2000a) (Figure 1.2C). The region is known as "N-lobe Cap" from other EPKs (Thompson et al., 2009), and in some cases, (like in CAPK) it functions as docking cavity for C- or N-terminal peptide segments.

In CK2α, particularly important "hot spot" residues for the interaction with CK2β are Phe54 and Leu41 (Raaf et al., 2011). The flanking β4β5 loop can exist in a stretched conformation (like in Figure 1.2A/C or in Figure 1.6A) or in a closed one, where it is bent down to the surface of the β-sheet. Binding of CK2β is incompatible with the closed conformation (Ermakova et al., 2003) and requires opening

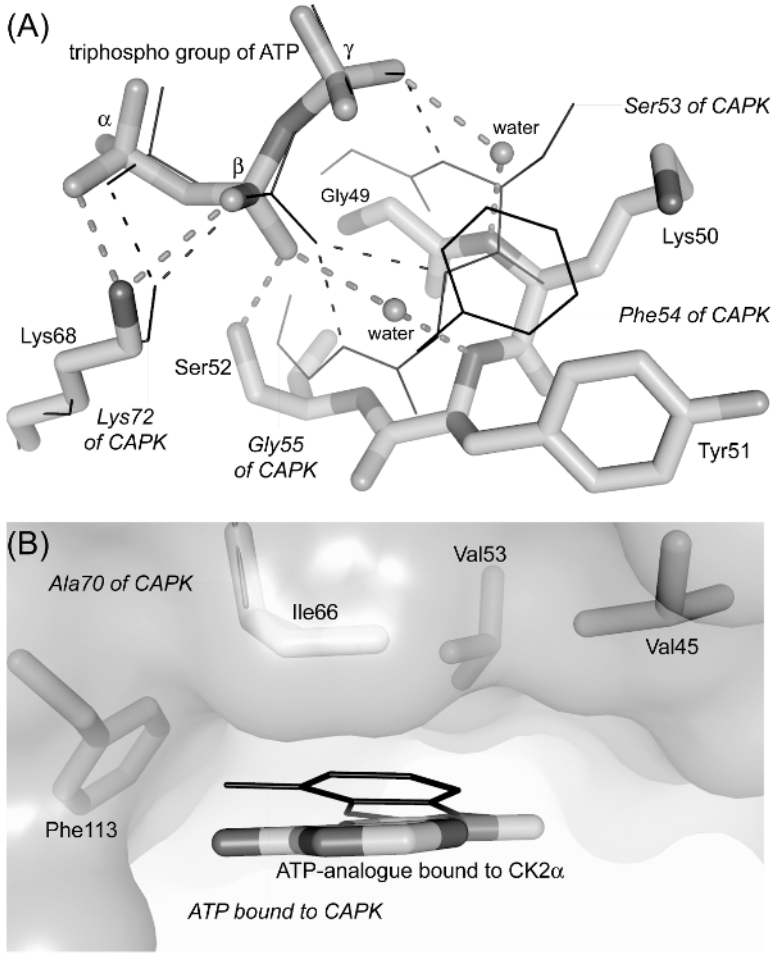

Figure 1.7. Cosubstrate binding by elements of the N-terminal CATH domain. (A) Coordination of the cosubstrate triphospho group by the phosphate-anchor loop and a conserved lysine residue of strand β3. The thick bonds refer to a binary complex of maize CK2α and an ATP-analog (Niefind et al., 1999) and the thin ones to the ternary complex of CAPK, ATP, and a substrate inhibitor (Zheng et al., 1993). Unlike CAPK in CK2α no phosphate-backbone hydrogen bonds between ATP and the phosphate-anchor loop are formed. (B) Shallow mold formed by hydrophobic side chains from the inner surface of the N-lobe β-sheet. The cavity serves to harbor the ATP/GTP purine base. In the case of CAPK and the majority of EPKs, it is deeper than in CK2α due to a replacement of Ile/Val66 (Ile66 in maize CK2α; Val66 in human CK2α) by a small alanine residue (Ala70 in CAPK). As a consequence, the adenine moiety (thin black bonds) is inclined toward the N-lobe.

of the β4β5 loop. The hypothesis that the opening motion itself may be functionally important as a driving force for CK2β binding (Raaf et al., 2008b) could never be substantiated. Such a role is improbable after a recent analysis showing that both conformations can occur without a clear correlation to the occupation state of N-lobe Cap (Papinutto et al., 2012).

The Inner Surface of the N-Lobe β-Sheet

The opposite surface of the β-sheet is a part of the nucleotide-binding site. A highly conserved residue is Lys68 from strand β3, which anchors the α- and β-phospho groups of ATP (Figure 1.7A) and is itself fixed by Glu81 from helix αC. The importance of these two residues and of their electrostatic interaction in the catalytic event is underlined by the fact that they are among the five residues fully conserved in all EPKs. In CK2α, Lys68 is particularly important also as an anchoring point for ATP-competitive inhibitors carrying an acidic function, as illustrated below.

The next inner surface residue of strand β3 is Ile66 (in maize CK2α; Val66 in human CK2α/CK2α′), which is a part of the catalytic spine (illustrated in Figure 1.4B for CAPK). Together with Val45 (strand β1), Val53 (strand β2), and Phe113 (strand β5), Ile66 forms a shallow hydrophobic well to harbor the aromatic nucleobase of ATP or GTP (Figure 1.7B). Ile/Val66 is insofar a special feature of CK2α as the equivalent C-spine residue in CAPK and many other EPKs is a small alanine (Ala70; Figure 1.4B, Figure 1.7B). As a consequence, the adenine moiety of the ATP bound to CK2α is inclined in a direction away from the N-lobe compared to CAPK (Figure 1.7B). Consistently, the mutation of Val66 to alanine led to an ATP-binding mode, which is very similar to CAPK (Yde et al., 2005).

The C-Terminal CATH Domain (C-Lobe)

The C-lobe of CK2α (CATH S35 family 1.10.510.10.31) belongs to the "mainly alpha" class and among them to the by far most frequent architecture "orthogonal bundle." Orthogonal bundle structure domains have a globular shape and contain at least some helices packed by approximately 90 degrees to each other.

In CK2α (and other EPKs), this architecture is not very pronounced; rather, most of the helices—in particular the two dominating long helices αE and αF—are largely parallel (Figure 1.2A, Figure 1.8), and only the orientation of helix αG is approximately orthogonal to the two predominating ones.

The center of the domain is formed by the large helix αF (Figure 1.8). This helix received particular attention in the last years by a systematic structural comparison among EPKs, ELKs, and APKs (Kannan and Neuwald, 2005) and by the emergence of the spine concept (Kornev et al., 2008; Taylor and Kornev, 2011). Effectively helix αF is the basis for the two hydrophobic spines that link the two lobes (Figure 1.4B).

In the course of the aforementioned efforts to understand EPKs as complex systems that combine catalytic activity and regulatory modes, another helical region of the C-lobe attracted growing interest: it is the "GHI subdomain" comprising the canonical helices αG, αH and αI. The GHI subdomain is coupled via a highly conserved Arg/Glu salt bridge (R312/E201 in CK2α) to the so-called APE-sequence motif (Figure 1.9) being a part of the small helix αEF at the C-terminal region of the activation segment (Figure 1.2A). A recent mutational study with CAPK has

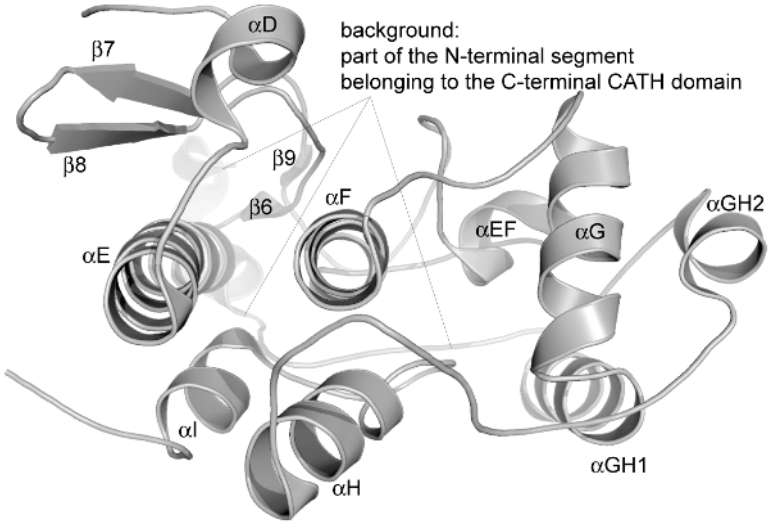

Figure 1.8. Overview of the C-terminal CATH domain of CK2α in a view along the axis of the central helix αF. Due to intensive contacts to the C-lobe, the tail of the N-terminal segment (Figure 1.2A) of the molecule (drawn foggily in the background) belongs to the C-terminal CATH domain.

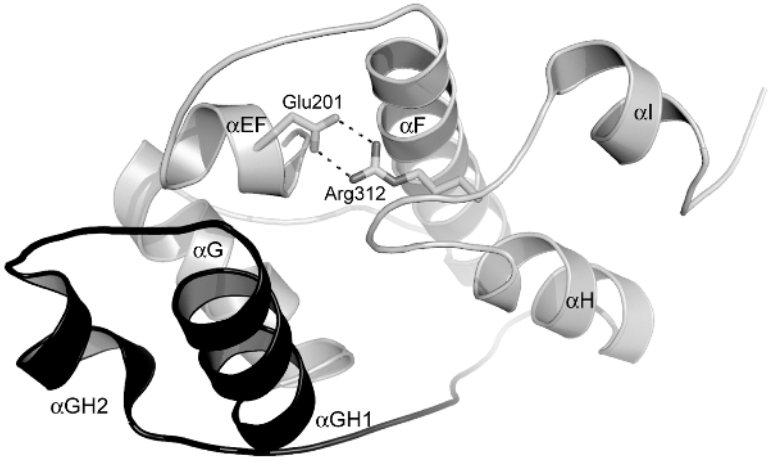

Figure 1.9. Coupling of the GHI-subdomain to the αEF-helix via a conserved ion pair. The αEF-helix is the C-terminal part of the activation segment (Figure 1.10). As typical for EPKs from the CMGC subfamily, the GHI-subdomain of CK2α contains a large predominantly helical insertion (drawn in black).

confirmed the importance of this junction (Yang et al., 2012), which is well defined in CK2α (Figure 1.9). It is noteworthy that the APE-motif is modified to GPE in CK2α, a mutation that is unique within the human kinome. Nevertheless, a look at the structure does not reveal an obvious reason for it.

In Figures 1.2A and 1.9, a non-canonical predominantly helical insertion between helices αG and αH (and thus remarkably within the GHI subdomain) is indicated. It is present in CK2α as well as in all other kinases of the "CMGC family," among which the cyclin-dependent kinases (CDK) and the MAP kinases are the most prominent. The CMGC insert of CK2α comprises two surface helices (here called αGH1 and αGH2) and is a region of increased conformational variability (Niefind et al., 2009). The homology to MAP kinases, close CK2α-relatives within the CMGC-kinases that have a so-called FXFP-binding site in this area addressed by transcription factor substrates and phosphatases (Akella et al., 2008), led to the speculation that the CMGC insert might serve as a remote-binding region for protein substrates (Niefind et al., 2009), but this has never been proven so far.

Catalytic Loop and Catalytic Mechanism

The linker region between the helices αE and αF is structurally the most variable and functionally the most important part of the C-lobe (Figure 1.10). It flanks the

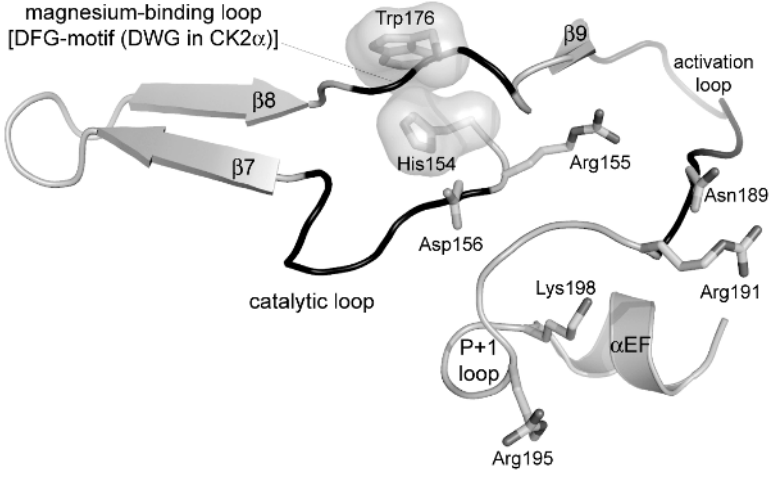

Figure 1.10. Connecting region between the helices αE and αF containing the catalytic loop, the magnesium-binding loop, and the activation loop (indicated by black). The whole of magnesium binding loop, strand β9, activation loop, P + 1 loop and helix αEF is referred to as "activation segment" (Nolen et al., 2004). The P + 1 loop is a conserved recognition site for substrates in EPKs. Its selectivity is determined by flanking site chains (Arg191, Arg195, and Lys198 in CK2α). Asn189 of CK2α is equivalent to a conserved threonine residue used as phosphorylation site in CAPK, MAP kinases, and many other "RD kinases" (Johnson et al., 1996) in which the "catalytic aspartate" (Asp156 in CK2α) is preceded by an arginine residue (Arg155 in CK2α). With His154 and Trp176, the two C-lobal members of the regulatory spine (Figure 1.4B) are drawn.

inter-domain cleft and contains two small antiparallel β-sheets as well as the so-called "catalytic loop" (Figure 1.2A; Figure 1.10) that harbors several highly conserved residues (Knighton et al., 1991) crucial for the catalytic mechanism (Figure 1.11). Most details of this mechanism were worked out with CAPK (Adams, 2001), which in this respect is also a prototype for other EPKs including CK2. It was shown with CAPK that the phospho transfer proceeds without any phospho-enzyme intermediate but directly via an in-line mechanism involving the inversion of the configuration at the γ-phosphorus atom (Ho et al., 1988). The absence of an intermediate is consistent with a sequential mechanism in which both reactants (Figure 1.1) have to be bound to the catalyst before the interconversion can occur.

Both ordered and random sequential mechanisms occur with EPKs (Schwartz and Murray, 2011). It was never experimentally investigated which one is used by CK2; nevertheless, the following structural observations favor a random sequential mechanism:

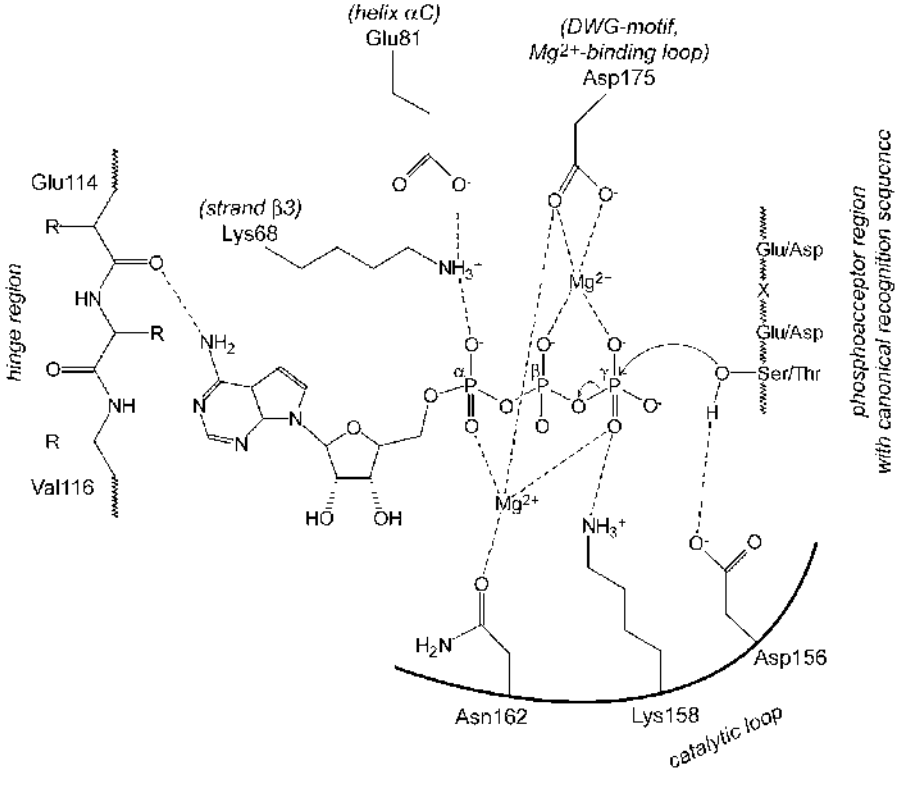

Figure 1.11. Schematic drawing of the coordination of ATP within the active site and of the catalytic key residues. Important hydrogen bonds and coordinative bonds with the two magnesium ions are indicated by dashed lines. Water molecules completing the Mg^{2+} coordination shells are left out. The sequence numbers refer to CK2α. The phosphoacceptor region shows the minimal recognition sequence of CK2α substrates.

1. In random schemes, the enzyme can bind either of the substrates productively; in the case of CK2α, productive binary complexes were at least shown for ATP and GTP (Niefind et al., 1999) albeit not for any peptide substrate.
2. Apo-structures exist for maize CK2α (Battistutta et al., 2001), human (Pechkova et al., 2003; Papinutto et al., 2012), and rat CK2α (Zhou et al., 2009), and all of them show that no significant conformational changes occur in the putative substrate-binding region after coordination of ATP. In other words, ATP binding does not seem to be a prerequisite for peptide substrate binding.

If the two substrates are productively bound to the kinase, the phospho transfer proceeds according to a concerted mechanism in which no intermediate is formed and bond formation occurs parallel to bond breakage (Schwartz and Murray, 2011). Within this scenario, associative and dissociative mechanisms are distinguished that differ in the bond distances and charge distributions of the transition states. Typically, EPKs use a dissociative mechanism with a metaphosphate-like planar transition state that is stabilized by a conserved lysine from the catalytic loop (Lys158 in CK2α; Figure 1.11). No particularly strong nucleophilic attack is required, so the role of a likewise conserved aspartate residue (Asp156 in CK2α) is probably not to act as a catalytic base but to bind and orient the reactive hydroxy group of the phospho acceptor (Figure 1.11) (Valiev et al., 2002).

Further components of the catalytic process are two magnesium ions that balance the strong negative charge of the triphospho moiety, and their coordinating side chains, which are Asn161 and Asp175 in CK2α. The latter is the first residue of the so-called "DFG-motif," which is highly conserved in EPKs but mutated to "DWG" in the case of CK2α (Figure 1.11).

Activation Segment and Constitutive Activity

Structurally, the DFG/DWF-motif is known as the "magnesium-binding loop" and forms the N-terminal part of the "activation segment" (Nolen et al., 2004), a regulatory key element that distinguishes EPKs from ELKs (Taylor and Kornev, 2011). As mentioned before, ELKs and EPKs belong to the same PFAM clan and to the same SCOP superfamily since they share common structural and catalytic features, but for full functionality in the cellular context, the latter must be strictly controllable.

In general, the activation segment plays a fundamental role in this context (Nolen et al., 2004). Apart from the magnesium-binding loop, it is composed of the strand β9, the "activation loop," and the P + 1 loop (Figure 1.10) nominated for its role in substrate recognition. In many EPKs, the activation loop can be phosphorylated at one or more sites, which often triggers large conformational rearrangements of the whole activation segment, required to obtain full catalytic competence (Nolen et al., 2004). In particular, "RD-kinases" (Johnson et al., 1996) are typically regulated by phosphorylation at a conserved threonine residue within the activation loop; the introduced phospho group then forms an ion pair with the arginine side chain preceding the catalytic aspartate.

Although CK2α is such an RD-kinase (see Arg155 and Asp156 in Figure 1.10), no phosphorylation site is present in the activation loop and no conformational variation was observed within the more than 60 CK2α structures published to date

(Niefind et al., 2009). Rather, detailed structural comparisons revealed that CK2α's activation segment is conformationally very similar to the full activation state of its nearest EPK relatives, namely the CDKs and the MAP kinases (Niefind et al., 2007).

These facts are fully consistent with the term "constitutive activity"; they could be rationalized with the first CK2α structure (Niefind et al., 1998) that revealed an intensive contact of the N-terminal segment not only with the N-lobe helix αC (Figure 1.6A) but also with the activation loop leading to the surprising assignment of the N-terminal residues to the C-terminal CATH domain (Figure 1.2C, Figure 1.8).

The whole cluster of helix αC, activation segment, and N-terminal segment is free from plasticity within the cluster of CK2α structures (Niefind et al., 2009). The importance of this arrangement was illustrated in a mutational study with human CK2α: the gradual deletion of the N-terminal segment finally led to complete inactivation of the enzyme (Sarno et al., 2002).

"DWG" Instead of "DFG"—Absolute Conservation of the Regulatory Spine in CK2α

The importance of the DFG-motif in EPKs is not restricted to the role of the aspartate in magnesium binding. Rather, the discovery of a "DFG-out" conformation in a p38 MAP kinase/inhibitor complex (Pargellis et al., 2002) pointed to the central phenylalanine whose conformational variation can be correlated with inactivation. This DFG-out conformation is often exploited by ATP-competitive inhibitors targeting EPKs in inactive states. Later, a rigorous analysis of active EPK structures emphasized the significance of this phenylalanine residue and assigned it as a member of the regulatory spine (Kornev et al., 2008; Taylor and Kornev, 2011). For full activity, the DFG-phenylalanine requires full formation of the R-spine, which is only possible if the central phenylalanine adopts the so-called "DFG-in" conformation.

CK2α and CK2α′ are the only members of the human kinome in which the DFG-motif is mutated to "DWG." A consequence of this mutation is not only an additional hydrogen bond from the tryptophane side chain to a neighboring peptide group as noticed in the first CK2α structure description (Niefind et al., 1998) but furthermore a much stronger incorporation of the more bulky side moiety into the hydrophobic core of the enzyme than found for the canonical phenylalanine. The side chain of Trp176 in CK2α makes 37 non-covalent contacts below 4 Å to atoms in its environment (Figure 1.12A) compared to only 5 in the case of Phe185 and CAPK (Figure 1.12B). Remarkably, Trp176 is in much more intense contact to its neighboring R-spine partners His154 (from the C-lobe) and Leu85 (from the N-lobe) than observed for Phe185 of CAPK and its flanking R-spine partners Tyr164 and Leu95.

Thus, there are profound structural reasons why a "DWG"-out conformation comparable to the DFG-out state of many inactive EPKs was never observed so far in CK2α. For the DFG-out conformation, it was speculated that it might be not merely an unusual feature induced by particular inhibitors, but an essential state along the catalytic cycle that might facilitate the release of ADP (Kannan and Neuwald, 2005). Recent molecular dynamics simulations with Abl kinase support

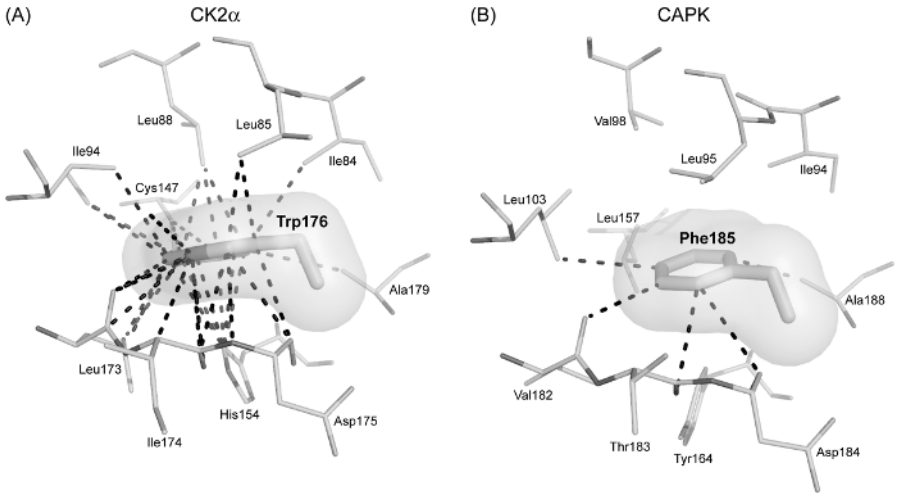

Figure 1.12. The central side chain of the DFG-motif—a member of the regulatory spine (Figure 1.4B)—and its atomic contacts to neighboring residues. The atomic contacts are drawn by dashed lines with a cutoff of 4 Å. (A) CK2α (from maize) with the unique mutation of the DFG-motif to $DW^{176}G$. More than 30 atomic contacts are indicated, among them a 3Å-hydrogen bond from the Trp176 side chain to the peptide carbonyl oxygen of Leu173. (B) PKA with a canonical DFG-motif in the "DFG-in" conformation. A comparison of the two panels reveals the intensive embedding of the Trp176 side chain into its hydrophobic environment.

such a scenario (Shan et al., 2009); however, a CK2α mutant Trp176Phe did not show a significant alteration of the v_{max} value compared to the wild type (Jakobi and Traugh, 1995), which is not consistent with a role of this position in the catalytic turnover.

Substrate Recognition: Structural Basis of the Acidophily and Correlation to Constitutive Activity

A region near the C-terminal end of the activation segment is the so-called P + 1 loop, composed by residues 191–199 (Figure 1.10) and characterized by three positively charged side chains (Arg191, Arg195, and Lys198; Figure 1.13). The name indicates that this zone harbors and recognizes the first residue after the phosphorylation site. Depending on the character of the residues constituting the P + 1 loop, the P + 1 residue of the substrate can be hydrophobic (like in CAPK) or charged. Further, in a ternary complex of CAPK, a hydrogen bond of 2.8 Å length between a peptide group of the P + 1 loop and the peptide group connecting the P and the P + 1 residue of the substrate was detected (Zheng et al., 1993). The enforced absence of this hydrogen bond is the structural basis of the preference of cyclin-dependent kinases and MAP kinases for a proline at the P + 1 position (Brown et al., 1999).

CK2 favors negatively charged side chains around the phosphorylation site, preferentially at the P + 3 and the P + 1 position but in addition at further positions around the phosphorylation site (Meggio and Pinna, 2003). The corresponding basic

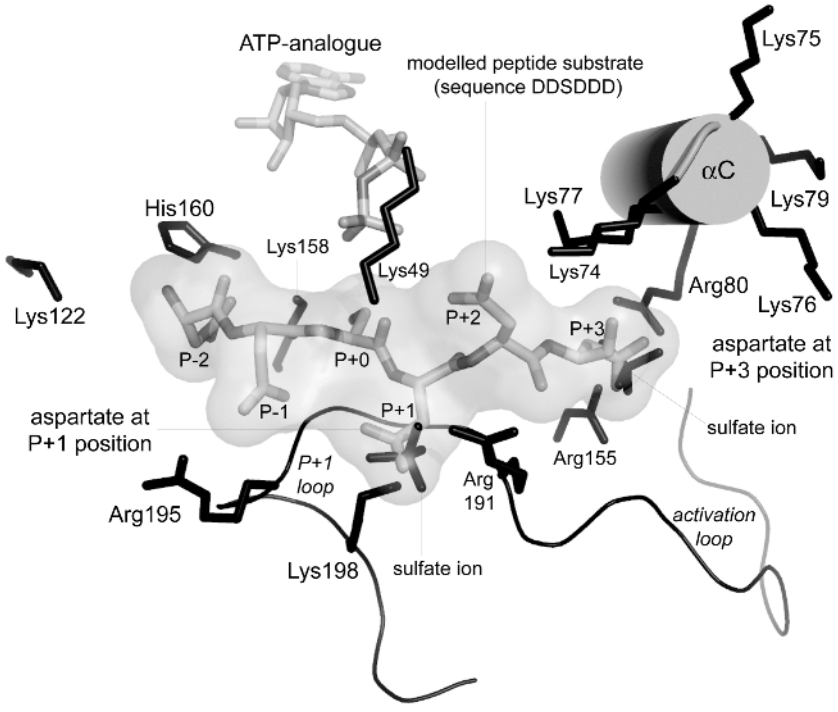

Figure 1.13. Basic substrate recognition by CK2α illustrated with a ternary complex model of CK2α, an ATP-analogue and the peptide substrate DDSDDD. The model is based on a human CK2α structure with two sulfate ions located equivalently to the two phospho groups of dually phosphorylated (fully active) MAP kinases (Niefind et al., 2007). The P + 1- and the P + 3-side chain of the substrate—the main determinants of a CK2 substrate—substitute the two sulfate ions. Basic side chains that (can) contribute to substrate recognition are drawn in black.

residues of the enzyme responsible for charge balance were determined by extensive mutational and kinetic studies (Sarno et al., 1995, 1996, 1997, 1999) and are indicated in Figure 1.13. One region of the N-lobe (the "polybasic stretch" ranging from Lys74 to Arg80 and located at the beginning of helix αC) and one of the C-lobe (the very P + 1 loop with Arg191, Arg195, and Lys198 as central determinants) were identified as particularly important, but in addition, further positively charged groups play a role.

A full structure-based rationalization of these results was never given so far since, unlike other EPKs (Zheng et al., 1993; Brown et al., 1999), no enzyme/substrate complex could be crystallized in the case of CK2. However, a number of 3D-models of such complexes were published either with CK2α alone (Sarno et al., 1997, 1999; Rekha and Srinivasan, 2003; Niefind et al., 2007) or with the CK2 holoenzyme (Poletto et al., 2008). The latest of these models based on CK2α alone is visible in Figure 1.13 and depends on a ternary complex structure of CDK2 (Brown et al., 1999) superimposed on the structure of human CK2α in complex with two sulfate ions. After 3D-fit of the two enzyme matrices, the P + 1– and the P + 3 side chain

of the CDK2 peptide substrate coincided casually with the two sulfate ions suggesting that they mark the binding sites of the negatively charged P + 1 and P + 3 side chains of the CK2 substrate protein (Niefind et al., 2007).

Moreover, it was evident that the sulfate ions were located equivalently to the two phospho groups of activated MAP kinases after phosphorylation of the T-X-Y dual phosphorylation motif, namely at the P + 1 loop and at the activation loop (Figure 1.13). This fact suggested that MAP kinases and CK2α preserved equivalent binding sites for negatively charged groups at their activation segments, but they use them differently: MAP kinases for regulation and CK2α for substrate recognition while in further CMGC family kinases like CDKs or glycogen-synthase kinase 3, a mixture of both functions can be observed (Niefind et al., 2007).

These observations are supplemented by a recent phylogenetic analysis revealing that protein phosphorylation sites can evolve from Asp/Glu residues integrated in salt bridges and vice versa (Pearlman et al., 2011). According to this study, the primordial CMGC kinase was probably MAP-kinase similar, namely disposed with a T-X-Y dual phosphorylation motif at the activation segment. In the course of evolution toward CDKs, this motif degenerated partially. CK2α was not examined by Pearlman et al. (2011), but nevertheless, the abundance of examples the authors present to show how phosphorylation sites can occur and disappear during evolution are relevant for this enzyme. They support the notion that CK2α received its pronounced acidophily at the expense of regulatability.

Hinge/Helix αD Region ("Subdomain V") and ATP/GTP-Binding

Although substrate recognition by CK2 awaits a final structure-based rationalization, the unique dual–cosubstrate specificity of CK2 was clarified (Niefind et al., 1999). The hinge connecting the two main lobes and being the central part of subdomain V plays a major role in this context by providing with its peptide backbone hydrogen-bonding partners for the purine bases of ATP and GTP (Figure 1.14A). In this context, a "hydrogen-bonding frame shift" was observed that allows an adaption of the different hydrogen-bonding potentials of adenine and guanine to the protein environment (Niefind et al., 1999).

This frame shift requires space in the binding plane of the purine bases and in the ribose subsite. Three candidate features (and their interplay) to provide this space were discussed and partially tested by site-directed mutagenesis:

1. Compared to CAPK and many other EPKs, CK2α misses a ribose anchor, i.e., a negatively charged side chain that hydrogen bonds the ribose hydroxy groups (Niefind et al., 1998).
2. The absence of this ribose anchor is correlated with an unconventional "open" conformation in the hinge region and the subsequent helix αD (Figure 1.14B). In this conformation, a relatively large space is present in a region of the nucleotide-binding site referred to as "hydrophobic region II" in a popular protein kinase pharmacophore model (see below, in particular Figure 1.21 in the Type I and Type II ATP-Competitive Inhibitors section) (Traxler and Furet, 1999).
3. Finally, the ATP/GTP site is relatively narrow and limited by large side chains flanking the ATP/GTP plane (Niefind et al., 1998; Battistutta et al., 2001). This was already mentioned above in the context of the N-lobe (Figure 1.7B), but it

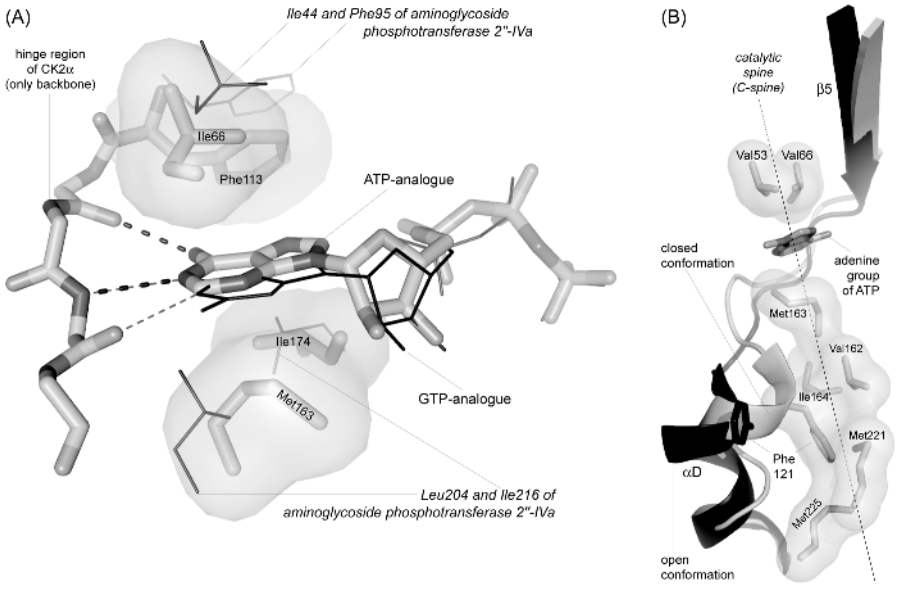

Figure 1.14. The hinge/helix αD region—function and flexibility. (A) Maize CK2α in complex with either an ATP- or a GTP-analogue (Niefind et al., 1999). Hydrogen bonds between the purine bases and the hinge region important for binding and recognition are indicated by dashed lines. Bulky side chains that determine the "purine-base binding plane" (Yde et al., 2005) are drawn embedded in a molecular surface. For comparison the equivalent side chains of aminoglycoside phosphotransferase 2"-IV, an enzyme with a similar dual-cosubstrate specificity like CK2 (Toth et al., 2010) were drawn with small black bonds. (B) The hinge/helix αD region of human CK2α with an open (black) and with a closed conformation (gray). The catalytic spine residues of human CK2α are covered by a molecular surface. Phe121 is equivalent to the CAPK-C-spine member Met128 (Figure 1.4B) and can adopt the EPK-canonical position only in the context of the closed conformation of the hinge/helix αD region.

is also obvious on the C-lobe side where, in particular, Met163 and Ile174 restrict the space (Figure 1.14B).

In summary, it appeared as if the combination of freedom within the nucleotide-binding plane and space restriction orthogonal to it is the structural prerequisite of the "hydrogen bond frame shift" and thus for the dual-cosubstrate specificity. This notion was consistent with results of a kinetic and structural analysis of the human CK2α double mutant Val66Ala/Met163Leu (Yde et al., 2005). Recently, it was further supported by the structure of an EPK-like aminoglycoside phosphotransferase with distinct dual-cosubstrate specificity (Toth et al., 2010). In this enzyme, the residues equivalent to Val/Ile66, Phe113, and Ile174 are the same as in CK2α (Figure 1.14A), and no potential ribose anchor side chain is present in the hinge/helix αD region. Recent structures of this enzyme with adenosine and guanosin confirm its potential to support the necessary "hydrogen-bonding frame shift" (Shi and Berghuis, 2012) in complete coincidence to CK2α.

Plasticity of the Hinge/Helix αD Region

The unusual "open" conformation of the hinge/helix αD region stood out already in the first CK2α structure (Niefind et al., 1998), where it was conspicuously dissimilar to all other EPK structures known at that time. Later, this unconventional conformation received particular attention in the light of the spine concept (Figure 1.14B). It was shown (Bischoff et al., 2011b; Battistutta and Lolli, 2011) that in all structures of maize CK2α and human CK2α′ and in many structures of human CK2α, the EPK-typical catalytic spine is not correctly established in spite of a completely canonical regulatory spine. The most conspicuous CK2α residue is Phe121, which is equivalent to the C-spine member Met128 of CAPK but which is, unlike the latter, not buried in a hydrophobic cavity provided by the other C-spine residues belonging to the C-lobe (black drawn conformation in Figure 1.14B).

But the hinge/αD region of CK2α is not only unorthodox because of its preference for a non-canonical "open" conformation. Rather, in the case of human CK2α, an EPK-untypical structural plasticity in this area was detected beginning with conspicuously high crystallographic temperature factors in the first human CK2α structures (Ermakova et al., 2003; Pechkova et al., 2003) and leading to the discovery of a particular "closed" conformation (Yde et al., 2005; Raaf et al., 2008a) in which the C-spine is correctly established in an EPK-canonical way (Bischoff et al., 2011b; Battistutta and Lolli, 2011) (Figure 1.14B).

From the crystallographic knowledge of human CK2α, one can safely conclude that in solution there is a conformational equilibrium with two main conformations of the peptide backbone and a variety of subpopulations concerning the side chains. The puzzling situation raises a number of questions, some of them being already addressed in the literature:

- Which internal constraints are responsible for the fact that CK2α tends either strongly (maize CK2α, human CK2α′) or at least partially (human CK2α) to a noncanonical "open" hinge/helix αD conformation?
- Why are these internal constraints favoring the "open" hinge/helix αD conformation stronger in maize CK2α (and possibly in human CK2α′) than in human CK2α? Which differences on the sequence level are the most important in this context (Battistutta and Lolli, 2011)?
- How strongly and in which way do external factors like ligands, crystallization conditions, or the partially constrained environment of the crystal lattice influence the conformational state of the hinge/helix αD region?
- In particular, what is the impact of the natural binding partners ATP and CK2β (and of protein substrates)? The limited set of existing structures of human CK2α with these ligands indicates the open hinge/helix αD conformation as the most probable (Bischoff et al., 2011b), but how substantiated is this state of knowledge?
- Is one of the two conformations the active one and the other inactive? Or are both of them passed through along the catalytic cycle, for instance for the purpose of ATP/ADP binding and release?

Currently, none of these questions has been addressed by mutational studies, by computational methods, or conformational analyses in solution. A recent

crystallographic analysis has revealed that there is no correlation between the conformational states of the hinge/helix αD region of human CK2α and of other flexible surface regions like the β4β5 loop or the phosphate-anchor loop (Papinutto et al., 2012).

Surface Pockets

The extension of the spine concept by a comparative surface analysis (Thompson et al., 2009) revealed a number of spine-stabilizing conserved surface pockets (Figure 1.4C). A typical EPK fills some of these pockets either intramolecularly (by parts of their N- or C-terminal extensions) or intermolecularly by docking of protein interaction partners.

CK2α uses both strategies: it coordinates CK2β via the N-lobe Cap, and it fills two of four cavities surrounding helix αC (Figure 1.6B) with hydrophobic side chains of the N-terminal segment (Figure 1.6A). One of them is Tyr26, which is absolutely conserved in CK2α and which occupies the equivalent space as Phe267 of cyclin A in complex with cyclin-dependent kinase 2 (CDK2), namely cavity iii of Figure 1.6B.

The second pocket filled in CK2α is equivalent to the PIF-pocket of AGC kinases (Biondi et al., 2000) (cavity i of Figure 1.6B). It is located between helix αC and the β4β5 loop and was mentioned already above in the chapter about the N-terminal CATH domain. In human CK2α, the hydrophobic couple Val31/Trp33 occupies this space, substituted both in CAPK and in the CDK2/cyclin A complex by a phenylalanine side chain, in one case (CAPK) from the C-terminus (Phe350), in the other (CDK2) from the cyclin interaction partner (Phe340). Trp33 is similarly well conserved in CK2α sequences as Tyr26; for Val31 conservative mutations to Leu, Pro or Ile occur.

The C-Terminal Segments of Human CK2α and CK2α′: Intrinsically Disordered Protein Regions

Although maize CK2α does not significantly exceed the EPK catalytic core at its C-terminal end, both human CK2α paralogs dispose of C-terminal extensions. These extensions are different in length and completely unrelated in sequence so that they were assumed to be the source of the functional differentiation between human CK2α and CK2α′. In fact the C-terminal segment of human CK2α contains four phosphorylation sites for cyclin-dependent kinase 1 (Bosc et al., 1995) and a glycosylation site (Tarrant et al., 2012) in contrast to human CK2α′.

None of the structure determinations of human CK2α and CK2α′ revealed structural information about the C-terminal segments because they were either lost by spontaneous degradation (Niefind et al., 2001), were a priori deleted by mutagenesis (Ermakova et al., 2003; Nakaniwa et al., 2009) or were disordered due to internal flexibility (Bischoff et al., 2011a; Pechkova et al., 2003). Consistently, using the DISOPREP2 server (bioinf.cs.ucl.ac.uk/disopred; Ward et al., 2004), the C-terminal regions are predicted with high confidence to be "intrinsically disordered proteins" (IDP) that lack 3D-structure when alone in solution (Babu et al., 2011).

IDPs attract growing interest due to their frequency (Ward et al., 2004) and functional importance, e.g., for protein degradation and as flexible interaction mediators (Babu et al., 2011). For the latter function, human CK2α provides a nice

example since the only structural information about the C-terminal region was provided indirectly, namely by the structure determination of a ternary complex of O-linked β-N-acetylglucosamine transferase (O-GIcNAc transferase or OGT). OGT catalyzes the transfer of an N-acetylglucosamine group from uridine disphosphate (UDP) N-acetylglucosamine to serine side chains of protein and peptide substrates, among them to Ser347 from the C-terminal segment of human CK2α (Tarrant et al., 2012). OGT was crystallized together with the coproduct UDP and with a peptide containing the sequence G^{341}GSTPVSSANMM^{352} and thus a part of the C-terminal region of human CK2α (Lazarus et al., 2011). In complex with OGT, this peptide is well ordered and adopts a slightly bent conformation (Figure 1.15).

This structure supports the notion that the C-terminal segment of human CK2α plays a fundamental role by changing in a synergistic way its constitution via phosphorylation and glycosylation, and its conformation via docking to protein interactions partners. Recent data suggest that the resulting adaptability may be particularly relevant for the modification of the enzyme's substrate specificity (Tarrant et al., 2012).

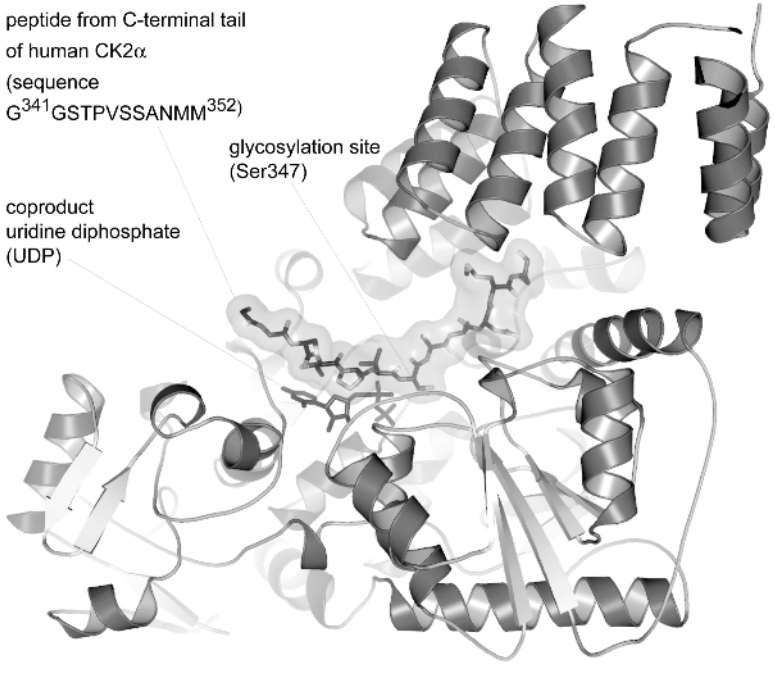

Figure 1.15. Ternary complex structure of human O-GIcNAc transferase with uridine disphosphate (UDP) and a peptide substrate derived from the (otherwise disordered) C-terminal tail of human CK2α (Lazarus et al., 2011). UDP is the coproduct of the OGT-reaction after transfer of the acetylglucosamine moiety to a serine side chain of a substrate.

The Regulatory Subunit CK2β

Overview

For CK2α, we have shown that the structural domains underlying the CATH annotation are—irrespective of their heuristic value—limited in their significance: the CATH domains of CK2α (and other EPKs) are neither evolutionary nor functional units, and even structurally they are not really separated but connected by the two hydrophobic spines (Figure 1.4B) and share thus a common hydrophobic core.

With CK2β, this diagnosis is even more pronounced: a CK2β subunit consists of two CATH domains (Figure 1.2B, Figure 1.3), but these are less independent from each other than the two CK2α CATH domains, since they are so closely attached that no interdomain cleft is directly visible. And even regarded as a single entity, the two CK2β CATH domains do not form a structural and functional unit for two reasons: first, CK2β necessarily requires dimerization for structural stability and functional competence (Canton et al., 2001), and second, even a dimer of the two CK2β CATH domains lacks the C-terminal regions of the molecules that are necessary for CK2β's main role, namely to bind CK2α.

In spite of these limitations, we proceed here with a description of the CK2β CATH domains due to their didactical value in order to receive a comprehension of the structure.

The N-Terminal Domain of CK2β

Like the CK2α C-lobe, the N-terminal CATH domain of CK2β belongs to the "Mainly Alpha" class and to the "Orthogonal Bundle" architecture. In this case, however, this designation is rather suitable: the helical elements in the N-terminal part of the domain are arranged like a rectangle, and in the C-terminal part of the domain, there is a helical "knee" composed of the long helix αD that is followed directly and essentially without an interconnecting loop by the shorter helix αE (Figure 1.16). This topology is unique among protein CATH domains so that already on the T-level of CATH the N-terminal domain of CK2β has no relatives.

A further conspicuous feature of the domain emphasized already in its first structural description (Chantalat et al., 1999) is the clustering of acidic and rather conserved residues, in particular in a region designated as "acidic groove." This acidic groove is about 35 Å long, 7 Å wide, and 4.5 Å deep (Chantalat et al., 1999) and had been demonstrated to be the main binding site of polyamines such as spermidine or putrescine (Leroy et al., 1995), an observation that did not directly explain the stimulatory effect of these compounds on CK2 but that was at least consistent with the fact that CK2β had to be present to observe this type of CK2 stimulation (Filhol et al., 1991).

The acidic groove is formed exclusively by elements of the N-terminal domain, among them the large helix αD and an "acidic loop," which is the connecting region between the helices αC and αD (Figure 1.2B, Figure 1.16). The frequency of acidic residues in this loop region and its possible role as an interaction site for polybasic substances had been noted early in the CK2 literature (Pinna, 1990) and motivated mutational studies showing that a reduction of the negative charge by site-directed mutagenesis had an upregulating effect on CK2 activity similar to the balancing with polycationic molecules (Boldyreff et al., 1993; Meggio et al., 1994).

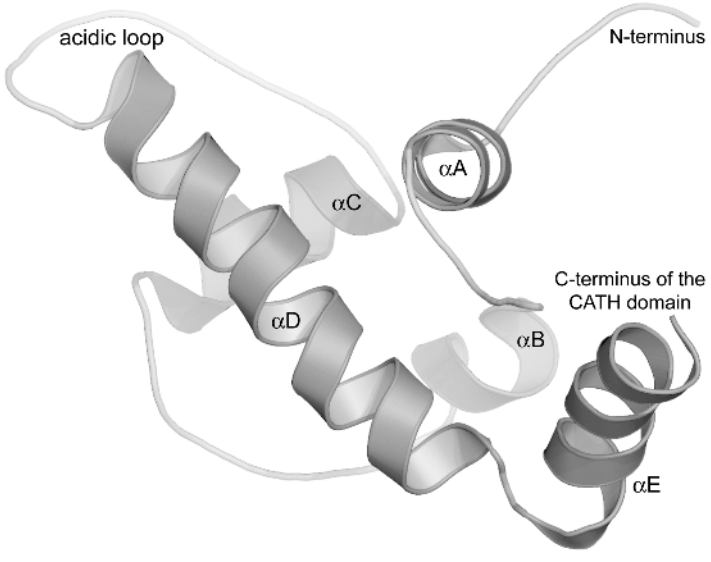

Figure 1.16. Overview of the N-terminal CATH domain of human CK2β.

It is noteworthy to mention that the stimulating effect of a polycation like polylysine is no longer detectable if a substrate itself contains a polybasic region in suitable distance from the phosphorylation site. This was demonstrated by Poletto et al. (2008) for eIF2β1–22, i.e., a 22-meric peptide from the N-terminal tail of the eukaryotic translation initiation factor 2β containing a stretch of eight lysine residues in its C-terminal part. This substrate stimulates its phosphorylation by itself, but it requires for this task CK2β with an intact acidic loop, an effect that could be plausibly rationalized on a structural level by an eIF2β1–22/CK2 holoenzyme model (Poletto et al., 2008).

The experimental structural information about the acidic loop is poor since, due to its high negative charge density, it is highly flexible and not visible in the majority of the 16 CK2β molecules currently in the Protein Data Bank archive. Only in the case of favorable stabilizing crystal contacts rough tracing of the peptide backbone was possible (Niefind et al., 2001; Bertrand et al., 2004).

The C-Terminal Domain of CK2β

The CATH assignment of the C-terminal CK2β domain to the class "Mainly Beta" and the architecture "single sheet" is somewhat surprising since the domain contains the medium-size helix αF that is known for its contribution in CK2α binding (Niefind et al., 2001) (Figure 1.17). The following facts form the rationale of the CATH annotation:

- The center of the domain is a three-stranded β-sheet (Figure 1.2B; Figure 1.17), which is antiparallel and therefore not necessarily accompanied by helices.
- In contrast to the N-terminal domain, the C-terminal domain of CK2β has several CATH relatives at the topology level, and the majority of them are indeed free from larger helical elements.

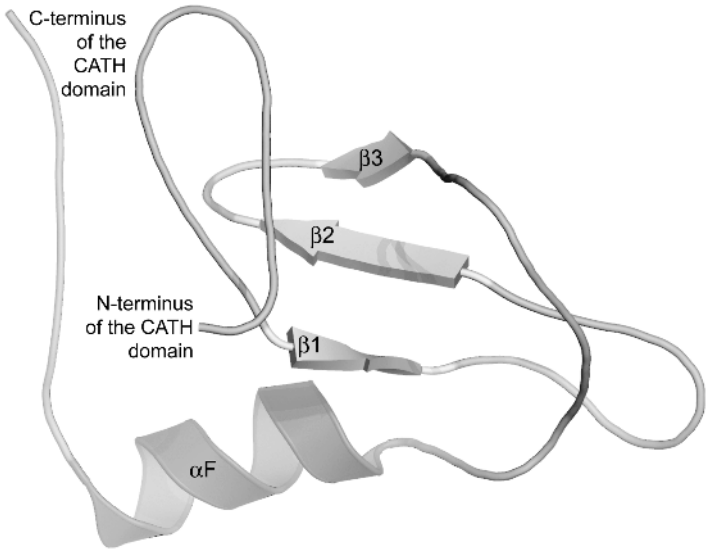

Figure 1.17. Overview of the C-terminal CATH domain of human CK2β.

- Essential common elements of CK2β and some of its CATH relatives are two loop connections that establish a zinc-binding site formed by four cysteine residues (Figure 1.18). This motif, known as "zinc ribbon," is surprisingly similar to the zinc-binding sites of the transcriptional elongation factor TFIIS and of the RNA polymerase II subunit 9 (RPB9), but in contrast to typical zinc-finger motifs no helix is required here (Chantalat et al., 1999).

Stability of Zn^{2+}–Coordination and Dimerization

Remarkably, when Cochet and Chambaz (1983) isolated CK2β for the first time, they applied a procedure involving denaturation and refolding. No metal ion was identified at that time as a necessary cofactor; obviously the zinc-binding site is either rather stable or can be easily re-established even after the impact of drastic conditions. Accordingly, the first hint in the CK2 literature that CK2β might dispose of a zinc-binding motif resulted from bioinformatics, namely after the sequence similarity of the cysteine-rich region to typical metal-binding sites had been noticed (Gatica et al., 1993). More detailed discussions of this feature were published shortly afterward (Reed et al., 1994; Allende and Allende, 1995), but the experimental confirmation was delivered only with the first CK2β crystal structure (Figure 1.2B) by Chantalat et al. (1999).

Simultaneously, this structure clearly suggested the key role of the zinc-binding motif, namely to support CK2β dimerization (Figure 1.18). Remarkably, dimerization readily occurs in spite of a relatively small interface area of 540 $Å^2$ (Chantalat et al., 1999), far below the average of 1685 $Å^2$ for stable homodimers (Jones and Thornton, 1996). Chantalat et al. (1999) explained this with the accumulation of hydrophobic residues at the interface and by a significant number of hydrogen bonds. Two of these H-bonds serve to extend the three-stranded β-sheet into the other

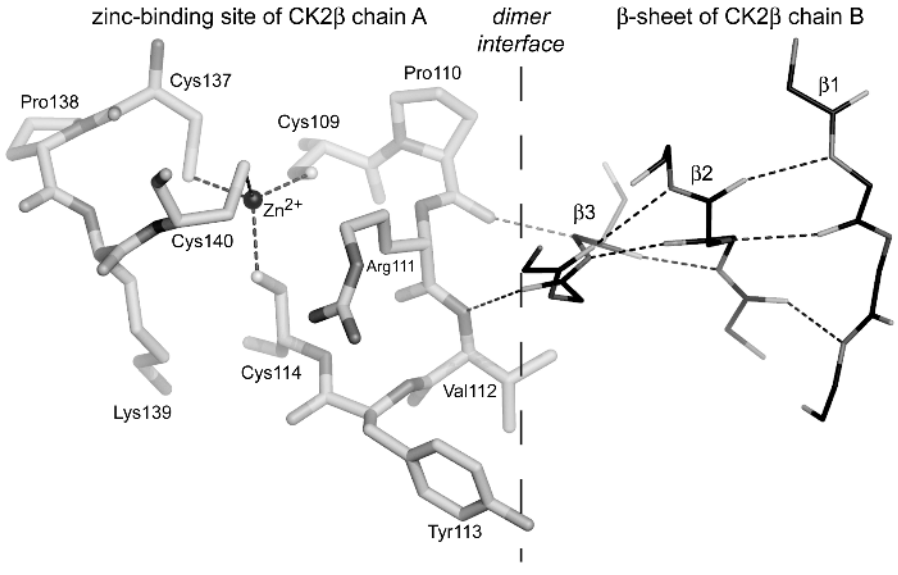

Figure 1.18. The zinc-binding site of CK2β and its role for homodimerization. The sophisticated geometry of the Zn^{2+}–coordinating protein environment enable an extension of the three-stranded antiparallel β-sheet of the neighboring subunit. Hydrogen bonds and coordinative bonds of the Zn^{2+}–ion are indicated by dashed lines.

monomer (Figure 1.18), namely to Pro110 and Val112. These two residues are part of the interconnection between the first two Zn-motif cysteines (Cys109 and Cys114), and Pro110 is as well conserved as these cysteines, indicating that the whole arrangement is sterically extremely challenging.

The chemical basis of CK2β's zinc-binding site stability is the ideal tetrahedral Zn^{2+} coordination known from inorganic zinc sulfide minerals (zinc blende and wurtzite) and established by four "soft" sulfur ligands each of which forms a coordinative bond with the central Zn^{2+} ion, which thus receives the saturated electron configuration of krypton. Destabilization of this arrangement could be achieved by the exchange of two sulfur against oxygen atoms via site-directed mutagenesis: the double mutant human $CK2\beta^{Cys109Ser/Cys114Ser}$ is no longer able to dimerize and to form a tetrameric CK2 holoenzyme *in vitro* and *in vivo* (Canton et al., 2001). In summary, the structural work of Chantalat et al. (1999) in combination with the mutagenesis study of Canton et al. (2001) clearly elucidated the intimate connection between zinc ion binding and dimerization of CK2β as a prerequisite of CK2 holoenzyme formation.

CK2β Body—CK2β Tail

To avoid aggregation typically associated with full-length CK2β, Chantalat et al. (1999) and later Bertrand et al. (2004) used CK2β constructs for crystallization lacking the C-terminal segment of about 35 amino acids (out of 215 in human CK2β), and thus a zone containing the key region for CK2α binding according to a mutational study (Boldyreff et al., 1993). The critical role of this region for holoenzyme formation was emphasized by the discovery that a human CK2β peptide

(CK2β$^{181–203}$) can bind specifically to maize CK2α (Figure 1.2C) (Battistutta et al., 2000a).

Thus, originally protein chemical problems combined with functional assignments suggested to distinguish between a "CK2β body" responsible for dimerization and a "CK2β tail" required for CK2α binding. Interestingly, this distinction did not become obsolete with later and more complete CK2β structures (Niefind et al., 2001; Raaf et al., 2008b; Zhou et al., 2009) but is preserved in the actual CATH annotation of CK2β comprising only the first 180 residues (Figure 1.3).

The CK2β tail is left out from CATH because it does not make a contact to its own body. Rather, it penetrates the dimerization plane, proceeds in touch to the CK2β body of the second monomer up to Tyr188 where it folds back to form a small two-stranded antiparallel β-sheet (strands β4 and β5, Figure 1.19). This structural motif is important for CK2α binding, but its formation does not require the presence of CK2α (Raaf et al., 2008b). After Pro194, an intrinsically disordered protein region (Babu et al., 2011) begins as shown by the only CK2α-unbound full-length CK2β protein that was crystallized so far (PDB 2R6M) (Zhou et al., 2009) in accordance with a prediction of the DISOPREP2 server (bioinf.cs.ucl.ac.uk/disopred; Ward et al., 2004). In contact to CK2α, however, the small helix αG is formed (Figure 1.19).

The CK2-Holoenzyme

Overview

The CK2 holoenzyme structure of 2001 (Figure 1.2D) (Niefind et al., 2001) merged the knowledge about the interaction of CK2α and CK2β existing at that time:

- The constituting element of the heterotetrameric complex is the CK2β dimer at its center (Graham and Litchfield, 2000).
- Two CK2α subunits (or CK2α′ or a mixture of both paralogs) are attached to this dimer, but they do not touch each other as shown before by yeast-two-hybrid studies (Gietz et al., 1995; Boldyreff et al., 1996).
- The C-terminal tail of CK2β (Figure 1.19) forms the main part of the interface to CK2α (Boldyreff et al., 1993).
- On the CK2α side, the interface is located at the outer surface of the N-lobe β-sheet like in the structure of maize CK2α with the peptide human CK2β$^{181–203}$ (Battistutta et al., 2000b).

The holoenzyme structure (Figure 1.2D) did not reveal significant conformational differences between CK2β-bound and -unbound CK2α. This was coarsely consistent with the constitutive activity of the enzyme although it left open the problem of how CK2β manages to modulate the activity of CK2α. In fact, this problem remains unsolved today.

The CK2α/CK2β Interface

The aforementioned yeast-2-hybrid studies (Gietz et al., 1995; Boldyreff et al., 1996) had left open whether within the heterotetrameric complex a CK2α subunit

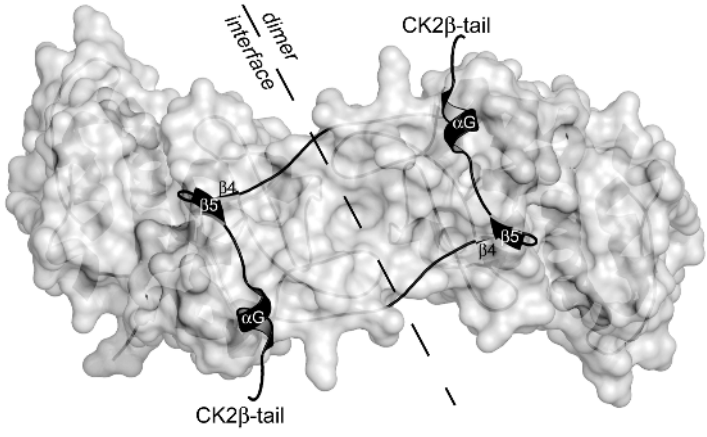

Figure 1.19. The CK2β tail (black) penetrating the dimer interface and being attached to the CK2β body of the neighboring subunit. The CK2β body dimer is covered with a molecular surface.

touches either CK2β chains or only one of them. The CK2 holoenzyme structure unambiguously clarified this question and revealed that the two CK2β monomers cooperatively form the interface to CK2α (Figure 1.10), which requires the particular extended conformation of the C-terminal CK2β tail described above.

Thus, the CK2α/CK2β contact within the CK2 holoenzyme is heterotrimeric (Niefind et al., 2001). The tail of one CK2β subunit provides 490 $Å^2$ of the interface to CK2α supplemented by about 340 $Å^2$ from the CK2β body of the second CK2β subunit stemming mainly from the helix αF and to lower parts from the helices αD and the helical interconnection between the helices αB and αC (Figure 1.20).

Only by this cooperativity can the CK2β dimer generate sufficient contact surface to form a stable heterocomplex. With 830 $Å^2$, the overall contact area is relatively small and in a size range typical for non-permanent protein/protein complexes (Niefind et al., 2001). This was a surprising message of the CK2 holoenzyme structure given the old report about the necessity of denaturing conditions to separate the CK2 subunits (Cochet and Chambaz, 1983). It led to the development of the first effective CK2β competitor, namely a cyclic peptide derived from the main CK2α contact region of the CK2β tail (Laudet et al., 2007), and together with *in vivo* fluorescence imaging data (Filhol et al., 2003), it supported "a new view of an old molecular complex" (Filhol et al., 2004) that displays an unexpected dynamic in its quaternary structure and in its intracellular localization.

The design of CK2β-competitive peptides by Laudet et al. (2007) revealed that Tyr188 and Phe190 are the most important interaction "hot spots" of CK2β. As mentioned above, on the CK2α side, two critical pendants are Phe54 and Leu41 (Raaf et al., 2011). The interaction is thus mainly hydrophobic and lacks hydrogen bonds as selectivity determinants. This observation fits with reports that CK2β can interact with other protein kinases like CHK1 (Guerra et al., 2003) in a CK2α-competitive manner.

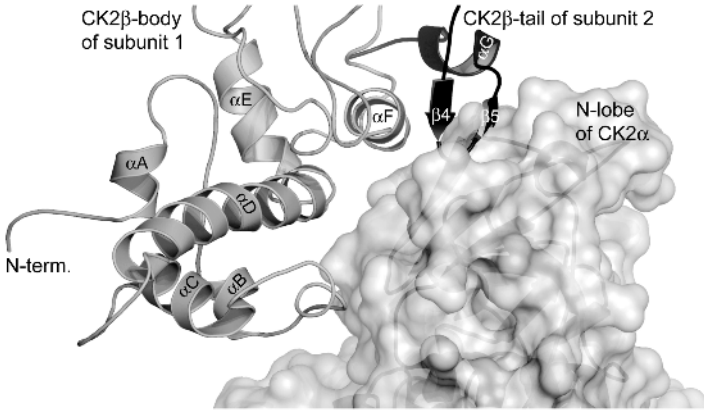

Figure 1.20. The heterotrimeric CK2α/CK2β contact region. The presence of the CK2β tail (black) that plunges its β4β5 loop into the N-lobe CAP of CK2α is a necessary condition for complex formation, but the CK2β body of the other CK2β subunit (gray) contributes significantly to its stability.

Higher-Order Aggregates and Polycationic Activation: Facets of a Constitutively Inactive Kinase?

The open architecture of the CK2 holoenzyme (Figure 1.2D), the small size of the CK2α/CK2β interface and the resulting large exposed surface of the heterotetrameric complex provide an ideal basis for interactions with substrate proteins but also for higher-order aggregation phenomena (among them autophosphorylation and salt-dependence of solubility, activity, and affinity to phosphocellulose columns) as they were observed since the 1970s. (See Introduction.) In fact, the "butterfly" shape of the holoenzyme (Figure 1.2D) inspired modeling efforts to rationalize these phenomena: Rekha and Srinivasan (2003) constructed a trans-model composed of two tetramers for autophosphorylation, and Poole et al. (2005) realized that such a dimer of heterotetramers is necessarily open for coordination of further tetrameric CK2 holoenzyme complexes and thus for the formation of filamentous aggregates.

Poole et al. (2005) presented an intriguing "global view of CK2 function and regulation," discussed the physiological existence and relevance of CK2 aggregates, and finally suggested that *in vivo* CK2 (at least a low-mobility pool of the enzyme excluded from nuclear import [Filhol et al., 2003]) might be a "constitutively inactive" kinase that is stimulated by alterations in the ionic status and by polycationic activators. In fact, in the model of CK2 filaments (Poole et al., 2005), the acidic loop of CK2β and thus a part of the interaction region of polyamines (Leroy et al., 1995) is close to the basic zones (P + 1 loop and "basic stretch") in the active site area of a CK2α subunit within a neighboring tetramer. A disruption of this interaction by polycations should be stimulatory since it provides access to the otherwise blocked active site.

Indirect (*in vitro*) experimental support of these notions came from a detailed analysis of the crystal packing associated with the CK2 holoenzyme structure (Niefind and Issinger, 2005). It showed that within the hexagonal crystals, the CK2

tetramers are grouped into trimeric rings stabilized by the same type of inter-tetrameric ionic interactions as predicted for the linear filaments, namely between the (otherwise completely disordered) "acidic loop" of CK2β and the positively charged substrate recognition regions of CK2α.

In summary, the structure determination of the tetrameric CK2 holoenzyme (Niefind et al., 2001) had paradoxical consequences, namely regulatory concepts that challenge its relevance or even its existence under physiological conditions: the small CK2α/CK2β interface supported ideas about its dynamics (Filhol et al., 2004) and of "promiscuous subunit interactions" (Allende and Allende, 1998), and the butterfly shape of the tetramer inspired a revival of the "regulation by aggregation"-hypothesis (Poole et al., 2005).

The question remains whether the CK2 heterotetramer itself (Figure 1.2D) is more than a "transition state" between higher-order aggregates and the isolated subunits CK2α and CK2β.

CK2 INHIBITORS

Protein Kinases as Therapeutic Targets

The exploitation of protein kinases as potential therapeutic targets originated some 25 years ago in Basel, when Novartis started the ambitious project to identify small molecules able to inhibit members of this class of enzymes (Bozulic et al., 2007). Since then, the interest in the development of protein kinase inhibitors has grown enormously, not only in the pharmaceutical industry but also in basic, academic research. In fact, these small molecules can be essential tools for studying many key cellular functions such as signal transduction, cell cycle regulation, development, proliferation, apoptosis and others (Cohen, 2002; Cowan-Jacob et al., 2009; Hemmings et al., 2009). Commonly, protein kinases exist in an inactive state, and the full catalytic activity is triggered by specific, tightly controlled stimuli. When this regulation fails, kinases can display an uncontrolled activity that is often associated with some kind of disease, in particular with cancer. The recent history of protein kinases research has demonstrated that these enzymes are attractive and successful drug targets, particularly in the treatment of cancer.

Although constituting less than 2% of the human genome (Manning et al., 2002), protein kinases are currently the second most important drug targets after G-protein-coupled receptors, with kinase inhibitors as objectives of around 20–30% of drug discovery projects in pharmaceutical companies (Bozulic et al., 2007; Cohen, 2002; Levitzki, 2003). In this context, there has been a huge impact on the field of structural biology, which is able to provide an extensive knowledge on the structure-activity relationships of protein kinases and kinase inhibitors, to such an extent that structure-guided drug design is an integral part of the modern drug discovery and lead optimization procedures (Cowan-Jacob et al., 2009; Fabian et al., 2005). These processes rely on the accumulation of accurate and detailed structural information, essentially by means of X-ray macromolecular crystallography, that can guide new chemical synthesis, greatly reducing the demands of chemistry resources. Usually in this structure-based approach, one tries to promote favorable enthalpic

interactions (typically hydrogen bonds) between lead compounds and the macromolecular target of interest while minimizing the negative effects of the decreased entropy of the system.

Although kinases are commonly associated with cancer, they are clearly involved in other pathologies as well, expanding their potential therapeutic spectrum. For instance, targeting protein kinases looks interesting for the development of anti-infectious drugs, in particular for facing the challenge of multi-drug resistant pathogens to current antimicrobials (Schreiber et al., 2009). Protein kinase inhibitors can also be regarded as novel potential anti-inflammatory drugs (Cohen, 2009), as well as compounds useful for the treatment of autoimmune diseases (Plas and Thomas, 2009).

CK2 as Potential Drug Target

CK2 is involved in many cellular processes (Ahmad et al., 2005; Ahmad et al., 2008; Ahmed, 2008; Ahmed et al., 2002; Homma and Homma, 2008) and has been demonstrated essential for cell viability (Buchou et al., 2003; Fraser et al., 2000; Padmanabha et al., 1990). Anomalously high levels of the enzyme activity detected in several types of cancer have suggested the involvement of CK2 in tumorigenesis phenomena (Gyenis and Litchfield, 2008; Piazza et al., 2012; Sarno and Pinna, 2008; Tawfic et al., 2001), and indeed CK2 has been demonstrated to increase the cell oncogenic potential (Channavajhala and Seldin, 2002). In principle, CK2 can be considered to be a potential drug target for cancer therapy on the basis of several pieces of evidence: at protein level, CK2 activity is elevated in various cancers; it is a potent suppressor of apoptosis and strongly promotes the survival of the cell; it reinforces the multi-drug resistant phenotype (Di Maira et al., 2007). These are interesting and promising pre-clinical results that suggest that CK2 is able to establish global favorable conditions for tumorigenesis (Sarno and Pinna, 2008), supporting the rationale of inhibiting CK2 as a potential approach for cancer therapy.

CK2 is not related only to tumorous diseases (Guerra and Issinger, 2008). CK2 inhibitors also have an anti-viral and anti-infectious potential interest, since it was seen that viruses are able to exploit cellular CK2 to phosphorylate proteins essential for their life cycle (Meggio and Pinna, 2003). Moreover, new roles for CK2 have been recently discovered in the molecular pathology of different neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis, for which new effective drug candidates are urged due to the lack of treatments. Indeed CK2 can be considered to be a potential new target for the treatment of neurodegenerative diseases (Perez et al., 2011), as well as other pathologies such as inflammation and cardiovascular diseases (Cozza et al., 2011).

Protein Kinases ATP-Competitive Inhibitors and Selectivity

Because of the conservation of the structural features within and near the ATP-binding cleft and the presence of different ATP-binding proteins, selectivity is one of the major issues in the development of ATP-competitive protein kinase inhibitors. Another problem to cope with is the high intracellular concentration of ATP,

approximately 1–10 millimolar (mM), that competes with inhibitors in the binding to the target kinase. However, many ATP-competitive protein kinase inhibitors (more than 130) are now in clinical trials, and an increasing number (more than a dozen approved for sale in the United States) are available on the market as anti-tumor drugs (Cohen, 2002; Fabbro et al., 2012; Melnikova and Golden, 2004). Many structural studies have indicated that small but crucial differences among diverse protein kinases are present, providing a rationale for selective drug design. This variability is significant enough to account for substrate selectivity, even if not complete.

In some cases, the selectivity of inhibition relies on flexible elements present in the catalytic domain of protein kinases, such as the highly conserved DFG motif, which can either enhance or interfere with the binding of small-molecule inhibitors. As illustrated above, this tripeptide Asp-Phe-Gly, located at the beginning of the activation segment, is the pivotal point of the loop movement between active ("DFG-in") and inactive ("DFG-out") conformations of protein kinases that can both be targeted by inhibitors. The DFG motif is highly conserved in kinases but not in CK2, where the phenylalanine is substituted by a tryptophan (Trp176; Fig. 1.12), with the consequence that the "DWG-out" conformation is highly disfavored and indeed never observed, and therefore cannot be targeted by small-molecule inhibitors. Upon kinases activation, other different conformational changes are generally possible, modifying exposed and buried residues that may be involved in inhibitor binding, but this is not the case for the structurally stable and intrinsically active CK2 where large conformational changes have never been observed.

An absolute selectivity against a specific target does not seem to be a mandatory requirement for kinase inhibitors that aim to become a useful drug. A study on a panel of 113 kinases revealed that none of the ATP-competitive inhibitors, which were in clinical trials or clinical use at that time, was totally selective (Fabian et al., 2005). These results were confirmed by a more recent analysis on 38 kinase inhibitors tested against an enlarged panel of 317 distinctive human kinases, comprising around 55% of the human kinome (Karaman et al., 2008). In particular, it showed that even the most selective inhibitor, lapatinib (Tykerb, an EGFR/ERBB2 inhibitor) binds to three distinct kinases with a $K_d < 3\,\mu M$, while staurosporine, as expected being the most promiscuous kinase inhibitor, binds to as many as 253 kinases with a $K_d < 3\,\mu M$. The lack of an absolute selectivity holds also for imatinib mesylate (Gleevec® or STI571), the first and probably the best known kinase inhibitor in the market, approved by the FDA on May 2001. Originally, imatinib was approved for the treatment of the chronic myelogenous leukemia (CML), targeting the intrinsically active Tyr-kinase BCR-ABL. Nowadays, this drug is used also in the treatment of other cancers of the blood cells, in gastrointestinal stromal tumors (GIST) and in dermatofibrosarcoma protuberans (Druker, 2004a; Druker, 2004b).

Signaling pathways exploited by cancer cells to promote proliferation and survival are regulated by different protein kinases, comprising CK2, and therefore drugs targeting simultaneously different kinases can be an effective strategy and looks to be a desirable methodology to treat cancers (Fabbro et al., 2012). The concept of "one drug, many targets," i.e., that nonspecific drugs are sometimes useful and even desirable, has recently attracted increasing attention and has been

properly recognized not only for kinase inhibitors but also for other classes of drugs (Imming et al., 2006). However, it remains possible, of course, that the lack of specificity could represent a serious drawback for the possibility to give rise to unwanted side effects due to "off-target" activities, and, anyway, can be a problem if inhibitors are used to analyze the biological function of a given kinase. As a matter of fact, the biological consequences of multi-kinase activity are still poorly defined (Karaman et al., 2008). Pertinent to this is the concept of "anti-target kinases," i.e., kinases that should not be targeted because of severe side effects. Currently, there is not a general consensus on which kinases of the human kinome are "off limits," and whether or not it is appropriate to include CK2 in this "black list."

Type I and Type II ATP-Competitive Inhibitors

According to the pharmacophore model (Traxler and Furet, 1999; Traxler et al., 1999), the ATP-binding site of protein kinases can be divided into three hydrophobic (adenine region and hydrophobic regions I and II) and two hydrophilic regions (sugar pocket and phosphate binding region) (Figure 1.21). The so-called "type I" kinase inhibitors are defined as those that target the active conformation ("DFG-in" conformation in the activation loop) and bind principally to the hydrophobic adenine region. They constitute the vast majority of the currently available protein kinase ATP-competitive inhibitors (Liu and Gray, 2006; Zhang et al., 2009), and this holds also for CK2. Type I inhibitors typically form one to three hydrogen bonds with the backbone of residues of the hinge region; these hydrogen bonds mimic those normally formed by the amino group of ATP adenine. Generally, the basis of the selectivity of these type I kinase inhibitors relies on the ability to present different chemical functions to other more variable regions of different protein kinases (Gray et al., 1998; Liu et al., 1999).

Inhibitors that preferentially bind to the inactive conformation of the protein kinase ("DFG-out" conformation) but still have contacts to the hinge region are referred to as "type II" kinase inhibitors (Cowan-Jacob, 2006; Liu and Gray, 2006; Zhang et al., 2009). Examples are Tyr-kinases inhibitors imatinib, nilotinib, sorafenib, or vatalinib. These inhibitors usually show ATP-competitive behavior, similar to type I inhibitors, as they exploit an additional hydrophobic pocket adjacent to the ATP-binding site that becomes exposed as a consequence of the movement of the activation loop from the DFG-in to the DFG-out conformation. These inhibitors share a similar pharmacophore where the conserved glutamate from helix αC (Glu81 in CK2) and the backbone amide of the aspartate from the DGF motif are involved in hydrogen bonds with the inhibitor. The energetic preference of a specific kinase to adopt this particular DFG-out conformation can be used to gain selectivity in the design of inhibitors, such as in the cases of TIE2 (Hodous et al., 2007) or MET (Schroeder et al., 2008). The structural properties at the basis of the capacity of a particular protein kinase to adopt or not the DFG-out inactive conformation remain to be established. However, as outlined in Figure 1.12, on the basis of the many available crystal structures of CK2 and CK2 complexes, it seems hardly possible that this kinase can adopt such DFG-out (in this case it would be "DWG-out") conformation and that it can be targeted by type II inhibitors.

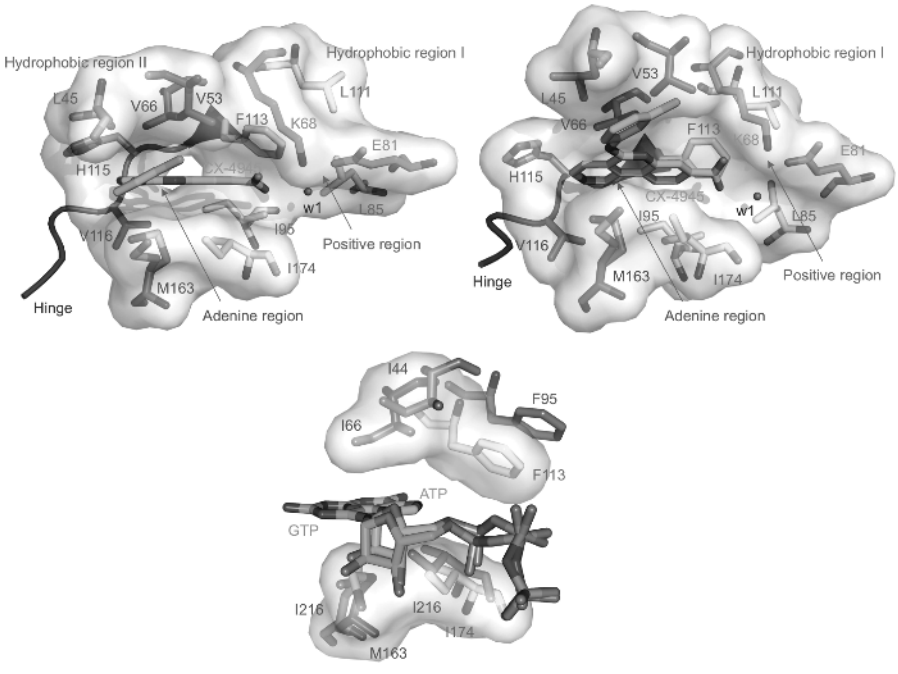

Figure 1.21. Main structural features of the CK2α ATP-binding site shown in two different orientations (upper panels). The active site is occupied by the CX-4945 inhibitor (cyan). Residues of the three hydrophobic regions common to the "kinase pharmacophore" are shown in yellow (hydrophobic region I, in the deepest part of the cavity), in orange (hydrophobic region II, at the entrance of the cavity), and in magenta (adenine region). Other important elements of the CK2α pharmacophore are the hinge region (dark red) and the area with a positive electrostatic potential near the salt bridge between Lys68 and Glu81, where the fully conserved water molecules w1 is located. These two regions are the main polar anchoring points for CK2 inhibitors. Lower panel: similarity between the active site of CK2α (colors as in upper panels) and of bacterial aminoglycoside phosphotransferase-2″-IVa from Enterococcus casseliflavus (gray). ATP and GTP as they bind to maize CK2α are shown. This enzyme is an EPK-like kinase (ELK). ELKs are members of the Pkinase clan of PFAM (Table 1.1) like EPKs. They share the catalytic properties of EPKs but lack their regulatability (Taylor and Kornev, 2011). That CK2α is concerning cosubstrate specificity and activity control more similar to an ELKs than to its CMGC relatives fits to its position next to the root of the CMGC family branch of the kinome tree (Manning et al., 2002). For color detail, see color plate.

Structural Aspects of CK2 Inhibition by Type I ATP-Competitive Inhibitors

The catalytic subunit of CK2, CK2α, bears most of the sequence and structural features common to all protein kinases. Regarding the sequence, two notable exceptions remarked also above, which are relevant for inhibition strategies, follow: (a) in the phosphate-anchor loop (residues 46–51), the third glycine in the general consensus sequence GXGXøG is missing (ø is usually a tyrosine, as in CK2, or a phenylalanine, as in PIM kinases, discussed below); and (b) the presence of a tryptophan (W176) instead of a phenylalanine in the otherwise conserved DFG triplet

at the beginning of the activation segment (Fig. 1.12). Another distinctive feature of CK2α is the presence of the basic cluster at the beginning of the helix αC (residues 74–80), where six out of seven consecutive amino acids are basic.

An important peculiarity of CK2 is the constitutive activity, with the consequence that only the active (DFG-in) conformation can be targeted and only type I inhibitors are accessible. The strategy to exploit specific flexible elements for selectivity, for instance to induce and stabilize an inactive DFG-out conformation by type II inhibitors, does not seem feasible for CK2. On the other hand, the inability of CK2α to adopt an inactive conformation has the advantage to avoid the problem of drug resistance, in tyrosine kinases often related to point mutations at the level of the gatekeeper residue, typically a threonine (Cools et al., 2004; Fabbro et al., 2012; Kobayashi et al., 2005; Sawyers, 2004). The side chain of the gatekeeper residue contributes to control the size of a hydrophobic back pocket, the hydrophobic region I of the protein kinase pharmacophore (Traxler et al., 1999). This pocket accommodates moieties of ATP-competitive inhibitors, such as Imatinib and some potent CK2 inhibitors (as shown below), but no functional groups of ATP itself. The type of drug resistance connected to the presence of a small gatekeeper residue (i.e., threonine) can hardly occur in protein kinases that possess a larger, hydrophobic gatekeeper residue, such as methionine, leucine, or phenylalanine (about 75% of all protein kinases). This is the case of CK2α that at the gatekeeper position carries a phenylalanine (Phe113).

Although for CK2, it is not possible to exploit the selectivity of specific inactive conformation with type II inhibitors, its catalytic site displays some unique properties that can be utilized in the design of inhibitors with a high degree of specificity. This is indicated by the rare ability to utilize both ATP and GTP and by the unusual low sensitivity to staurosporine inhibition (IC_{50} of 19.5 μM versus values in the low nanomolar range for other kinases [Meggio et al., 1995]). Actually, as described below, fairly specific, potent, and cell-permeable inhibitors of CK2 have been successfully developed in the last years (Battistutta, 2009; Bortolato et al., 2008; Cozza et al., 2010; Cozza et al., 2011; Mazzorana et al., 2008; Sarno et al., 2011; Sarno et al., 2005b; Sekigushi et al., 2009). Structural formulas of CK2 inhibitors discussed in this chapter have been reported in Figure 1.22.

Many CK2 inhibitors have been studied in complex with the maize enzyme that, as was described above, shows some structural differences in comparison with the human enzyme, particularly in the conformation of the hinge/αD region (Figure 1.23).

A recent analysis of the flexible regions of CK2 suggests that ligands that do not use the hinge/αD region to anchor to the protein active site would bind in a similar way to the maize and to the human enzyme, while ligands that interact also with this region can bind differently, at least in principle (Papinutto et al., 2012). Most of CK2 inhibitors for which structural studies have been performed belong to the first class of ligands. They establish direct polar interactions only with a limited, conformationally rigid portion at the N-terminal part of the hinge/αD region (i.e., with backbone of residues Glu114 and Val116) and/or the deeper part of the cavity, principally with some conserved water molecules and Lys68. For instance, it has been shown that emodin (1,3,8-trihydroxy-6-methyl-antraquinone) and quinalizarin (1,2,5,8-tetrahydroxy-anthraquinone) (Figure 1.22) can bind in a similar way to both

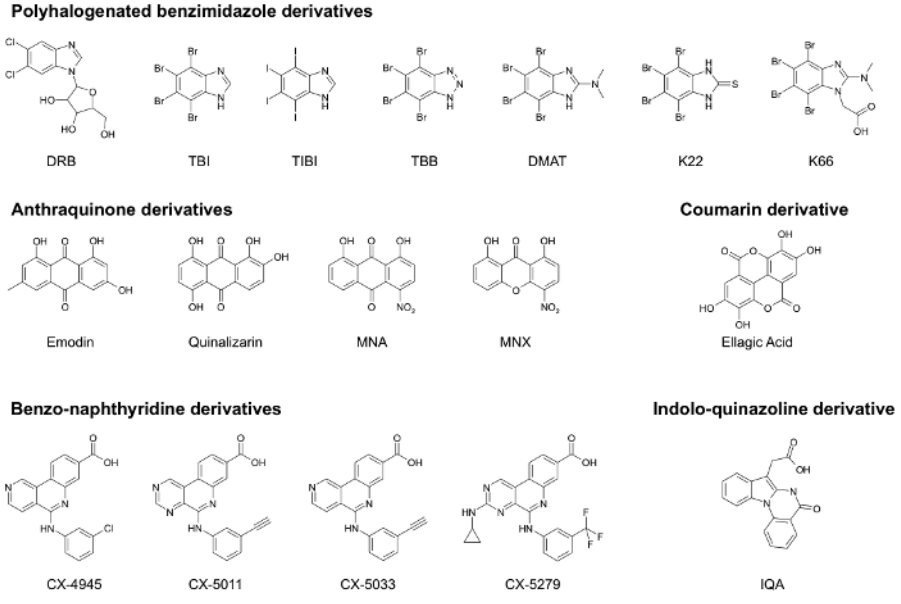

Figure 1.22. Structural formulas of the principal CK2 inhibitors discussed in this chapter, grouped by chemical classes.

the open and the closed hinge/αD conformation of the human enzyme, as well as to the open conformation of the maize one (Figure 1.23 and Figure 1.24). This suggests that, at least for inhibitors of this type, structural information obtained with the maize enzyme are comparable with those obtained with the human enzyme, despite the difference in the hinge/αD region, which indeed is not exploited for binding.

Some recent comprehensive reviews are available describing in details single families of CK2 inhibitors and the interested reader is referred to them (Battistutta, 2009; Cozza et al., 2010; Cozza et al., 2011; Prudent and Cochet, 2009; Sarno et al., 2011). Here we illustrate general principles of CK2 inhibition as emerged from all those data.

Common Structural Properties of Type I ATP-Competitive Inhibitors Targeting CK2

The majority of kinase inhibitors, also those crystallized in complex with CK2α, are type I ATP-competitive and, therefore, bind to the active (DFG-in) conformation of the catalytic site of the enzyme, in the position normally occupied by an ATP (or GTP) molecule. In general, the overall structure of the protein is only marginally affected by the binding of these small molecules. The C-terminal lobe is poorly influenced by the formation of the complex, and the N-terminal lobe shows a general higher degree of flexibility, in particular, but not exclusively, in the phosphate-anchor loop. Other areas showing plasticity are the side chain of His160, the hinge/αD region, and the stretch from residue 102 to residue 108, comprising the external

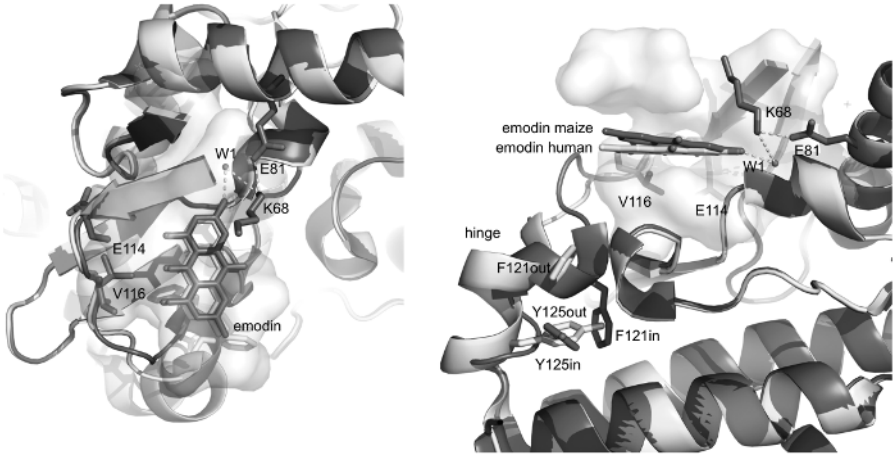

Figure 1.23. Inhibitor emodin bound to the maize (hinge/αD open, yellow) and the human (hinge/αD closed, marine blue) enzyme. Two residues that undergo major movements are Phe121 and Tyr125, shown in the two conformations open (out) and closed (in). Irrespective of the hinge/αD conformation, emodin establishes very similar interactions with the two enzymes. The major difference is a tilt in the plane accessible to emodin (and, generally, to active site ligands), shown in the right panel. For color detail, see color plate.

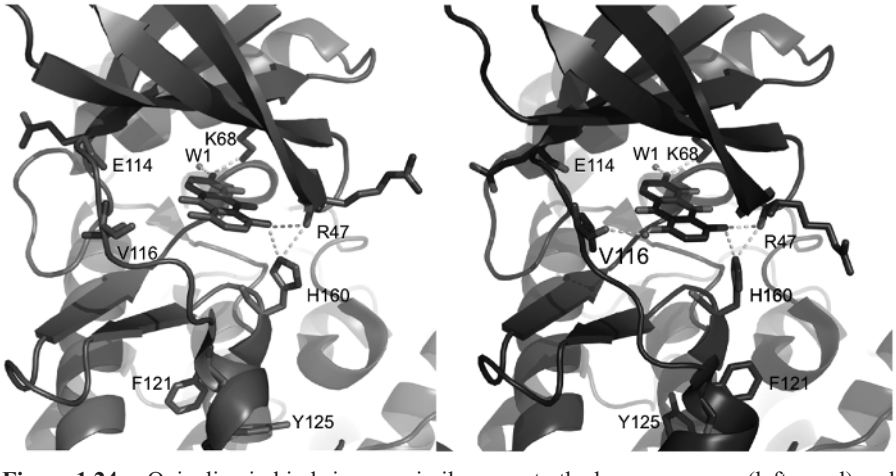

Figure 1.24. Quinalizarin binds in very similar ways to the human enzyme (left panel) and the maize enzyme (right panel), regardless of the conformation of the hinge/αD region, closed in the former and open in the latter (note the different orientations of Phe121 and Tyr125). Polar interactions of quinalizarin with Lys68 and water molecule W1 on one side, and with Arg47 carbonyl (from the phosphate-anchor loop) and His160 side chain on the other side are identical. The direct interaction between the phosphate-anchor loop and His160 mediated by quinalizarin is a unique feature among CK2α/inhibitor complexes.

loop between β strands β4 and β5 (β4β5 loop) (Papinutto et al., 2012). One of the major perturbations in the CK2α protein matrix was observed at the level of the phosphate-anchor loop and of His160 side-chain in the complexes of quinalizarin with the maize and human enzymes (Figure 1.24) (Cozza et al., 2009; Papinutto et al., 2012). However, the "active state" conformation of the helix αC and of the activation loop, main hallmarks of a protein kinase active state, are particularly well conserved in all CK2 structures, including the apo-enzyme.

Role of Apolar Interactions and Small Dimension of the CK2α ATP-Binding Site in Binding and Selectivity

In general, for type I CK2 inhibitors, an important energetic contribution for binding is due to apolar forces, namely hydrophobic interactions and van der Waals contacts, involving the hydrophobic surface of the CK2α binding site formed by residues Leu85, Val95, Leu111, Phe113, and Ile174 (hydrophobic region I); Val53, Ile66, Val116 and Met163 (adenine region); and Val45 and Tyr115 (hydrophobic region II) (see Figure 1.21). For tetrabromobenzo derivatives, a linear correlation between the log(Ki) and the variation in the accessible surface area (ΔASA) upon binding was identified, indicating that the apolar interactions are ultimately responsible for their rank in potency, as confirmed by a Linear Interaction Energy analysis (LIE model) (Battistutta et al., 2007; Battistutta et al., 2005). The structure-activity analysis of more than 60 different coumarins and the derived LIE model showed that apolar interactions give the largest contribution to the free energy of binding also for this class of compounds (Chilin et al., 2008). For the pyrazolo-triazine derivatives, the SAR analysis confirmed the important role played by apolar forces involving interactions between the extended hydrophobic portions of the inhibitors and the hydrophobic region I (with the alkyl linker of inhibitors), the adenine region (with the pyrazolo-triazine ring system), and the hydrophobic region II (with the cyclopropyl group).

The analysis of the active site of different protein kinases revealed that it is smaller in size in CK2α because of some bulky side chains, which reduce the space available to cofactors and inhibitors. As outlined above, the most important of these residues are Ile66 (maize) or Val66 (human) and Ile174, that in many protein kinases are replaced by less bulky amino acids, namely alanine versus Ile/Val66, alanine, threonine or leucine versus Ile174. For instance, the active site of CDK2 (belonging to the closely related CMGC group of protein kinases) is larger than in CK2 for the presence of Ala31 instead of Ile66 and of Ala144 instead of Ile174. As a consequence, the inhibitor TBB (4,5,6,7-tetrabromo-1-benzotriazole) binds in different ways to the two proteins, showing a remarkable selectivity for CK2 (De Moliner et al., 2003) (Figure 1.25). Inhibition data on CK2α mutants confirmed the importance of Ile66 and Ile174; for the single mutants Ile174Ala and Val66Ala and for the double mutant Ile174Ala/Val66Ala, the TBB IC_{50} increases from 0.50 to 1.74, 13.0 and 12.5 μM, respectively (Sarno et al., 2005b). The reduced inhibitory sensitivity of these mutants is a common characteristic of many CK2 inhibitors, with some notable exceptions described below. The smaller size of the CK2α active site can also account for the unusually modest sensitivity to the large molecular-sized staurosporine, a promiscuous protein kinase inhibitor (Meggio et al., 1995).

In tetra-halogenobenzo derivatives, the bulkiness of the four halogen atoms is essential for the potency of the inhibitors that increases upon replacement of chlorine with bromine and, even more, with iodine, and decreases when the two unique bulky side chains of CK2 (Val66 and Ile174) are mutated to alanines. For instance, the tetraiodo derivative TIBI (4,5,6,7-tetraiodo-benzimidazole) is almost 10-fold more potent than the analogue tetrabromo-benzimidazole compound TBI, although its selectivity is similar (Sarno et al., 2011). The higher potency is correlated to the increased hydrophobicity of the halogen atoms but also to their different polarizability, which allows the formation of stronger halogen bonds for iodine compared to bromines and chlorines.

Role of the Positive Region

From the analysis of the known maize and human CK2α cocrystal structures, it was noted that if a negatively charged moiety is present in a ligand (inhibitor or cosubstrate), it tends to cluster in a well-specified zone of the ATP-binding cleft, near the conserved salt bridge between Lys68 and Glu81. This holds for the acidic hydroxyl groups of MNA (1,8-dihydroxy-4-nitro-anthraquinone) and MNX (1,8-dihydroxy-4-nitro-xanthen-9-one) as well as for the carboxylic functions of IQA ((5-oxo-5,6-dihydro-indolo[1,2-a]quinazolin-7-yl) acetic acid) and CX compounds (CX-4945, 5-[(3-chlorophenyl)amino]-Benzo[c]-2,6-naphthyridine-8-carboxylic acid, and the derivatives CX-5011, CX-5033 and CX-5279), the acidic triazole ring of TBB, the chloride anions present in the DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole), and DRB (5,6-dichloro-ribofuranosyl-benzimidazole) (see Figure 1.22 for structural formulas), as well as for an oxygen of the α-phosphate in ATP and GTP (Battistutta et al., 2007). A quantitative analysis of the electrostatic potential in the CK2α active site revealed the presence of a positively charged region located in the deeply buried area of the cavity, between the hydrophobic region I and the salt bridge between Lys68 and Glu81 (Figure 1.21), with a mean positive electrostatic potential of 1.5–2.0 kcal/mol. As seen by the systematic analysis of the binding of different classes of CK2 inhibitors, the electrostatic interaction with this area greatly contributes to the different orientation of the ligands in the active site of CK2α. A striking example of this effect is seen for the different binding modes of the two closely related tetrabromobenzo derivatives TBB and TBI. TBB, with a pKa of approximately 5, binds with the acidic triazole ring inside the positive area, while TBI, with a pKa of approximately 9, is shifted toward the hinge region and forms two halogen bonds with Glu114 and Val116 (Figure 1.25), like all other tetrabromobenzo-imidazole derivatives analyzed. In CX-4945, while the tricyclic scaffold occupies the adenine region, the carboxylic function is able to penetrate into the deepest part of the active site, simultaneously interacting with the conserved water W1 and Lys68. As highlighted by SAR data, suppression or replacement of the carboxylate resulted in more than 400-fold loss of activity (Pierre et al., 2011), confirming the fundamental role of the negative charge of the carboxylic function in the binding. Indeed, the starting point for the development of the CX-4945 binding model was the crystal structure of the complex between the maize enzyme and the inhibitor IQA, the first structure of an inhibitor with a carboxylic function bound to CK2α (Sarno et al., 2003).

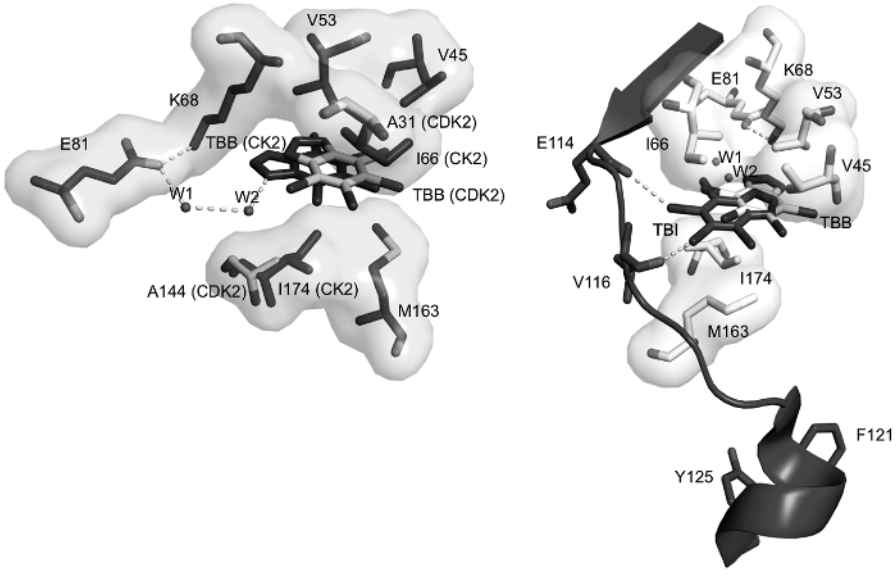

Figure 1.25. Left panel: TBB binds to CK2α (light gray) and CDK2 (dark gray) with different poses. Bulky CK2α residues Ile66 and Ile174 are substituted with two alanine (31 and 144) in CDK2, whose active site results larger. The different shapes of the active sites determine the two diverse binding modes of TBB to the kinases. Right panel: TBB (light gray) and TBI (dark gray) bind to CK2α with different orientations, despite their very similar structural formulas (see Figure 1.22). Unlike TBI (pKa ≈ 9), because of the acidic pKa (≈5) TBB is able to interact with the positive area near Lys68 and W1. TBI is shifted toward the hinge region, making halogen bonds with Glu114 and Val116 backbones (see also Figure 1.26).

Conserved Water Molecules

In the apo form of CK2α, the positive electrostatic area described in the previous section is occupied by three water molecules (Figure 1.26). The one in the deepest part of the cavity, called water molecule 1 (W1), is highly conserved in all the known human and maize CK2α crystal structures (Battistutta et al., 2007). It makes hydrogen bonds with the amidic NH of Trp176, with one carboxylic oxygen of Glu81 and with another water molecule (W2), that is present in many structures. When W2 is absent, its position is invariably occupied by a portion of a ligand, as in the case of MNA, MNX, Emodin, IQA, benzamidine or CX compounds, and this suggests that it is directly expelled by the ligand itself, and that this water should be considered to be a sort of competitor for that position. The third water in the positive area of apoCK2α, W3, is present in only two other structures, namely in the complexes with TBI and the related K22 (4,5,6,7-tetrabromo-1H,3H-benzimidazol-2-thione); in the complexes with DMAT and DRB, a chloride ion was found in that position. In the other cases, W3 is usually replaced by atoms of the bound ligand and, most importantly, by functional groups that can carry a negative charge. In other words, ligands carrying an acidic function have a propensity to cluster in a position corresponding to that of waters W2 and W3, in the region with the positive electrostatic potential, at a distance ≤3.5 Å from Lys68. This mode of interaction is uncommon for type I

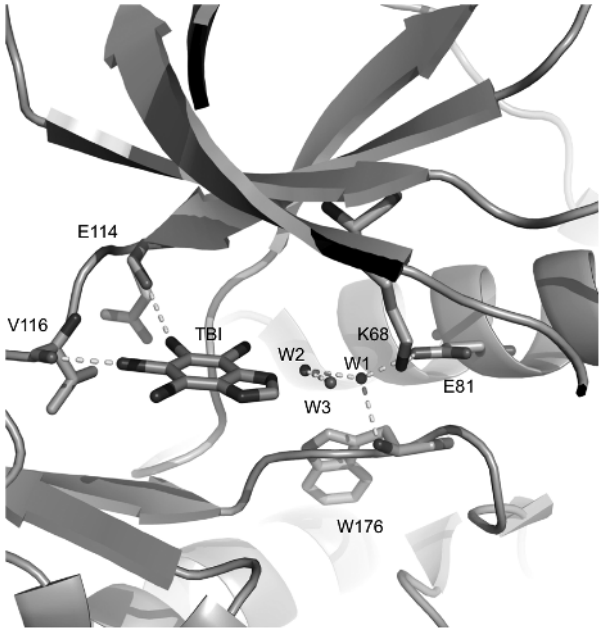

Figure 1.26. Three important water molecules reside in the positive area near Lys68. W1 is strongly anchored to the Lys68 side chain and to the Trp176 backbone amide nitrogen, and it is conserved in all CK2α structures, so it can be considered a "constitutive" component of the kinase. W2 is present in many structures but is displaced by inhibitors strongly interacting with W1 and Lys68, typically harboring a carboxylic function. W3 is present in the apo form of the enzyme and only in few complexes with small inhibitors such as TBI shown in the figure. Note the two halogen bonds (dashed lines) between TBI bromines and carbonyls of Glu114 and Val116 from the hinge region.

ATP-competitive inhibitors of protein kinases, which usually are more shifted toward the hinge region, making polar interactions with it. For CK2, this happens for ligands without acidic functions, which prefer to interact with the hinge region, in particular with the backbone carbonyls of Glu114 and Val116. The scaffolds of the macrocyclic pyrazolo-triazines compounds (Nie et al., 2007; Nie et al., 2008) and of the tricyclic CX compounds are so extended that occupy almost entirely the CK2α binding pocket, and indeed these are among the most potent CK2 inhibitors, with IC_{50} in the very low nanomolar range.

Role of Other Water Molecules in Mediating Inhibitor Binding

Besides the three conserved water molecules cited in the previous section, there are other waters that participate in the binding of several inhibitors (CX compounds, quinalizarin, emodin, etc.), mediating interactions with the protein matrix. Their analysis can be useful for the design of ligands with tighter binding properties. These waters are visible only in high-resolution crystal structures, and generally they are not predicted by docking programs whose poses for specific compounds can therefore be misleading. For instance, in the theoretical model of CX-4945 binding to

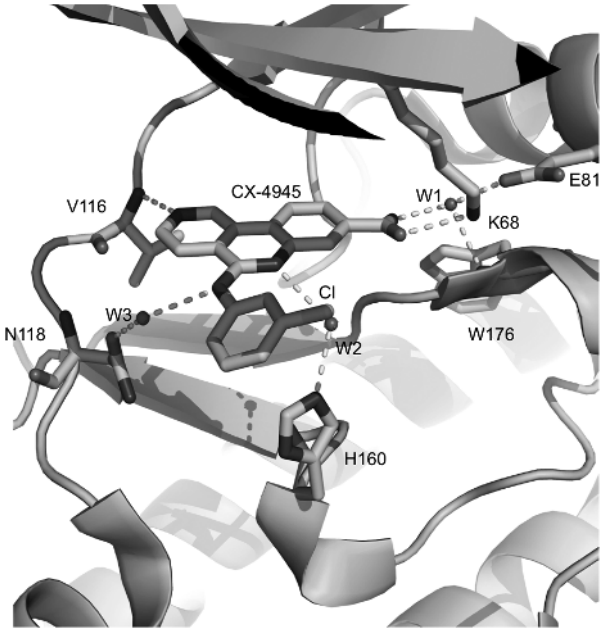

Figure 1.27. Inhibitor CX-4945 (see Figure 1.22 for structural formula) bound to the CK2α active site. Besides the conserved W1 near Lys68, note the other two water molecules W2 and W3 mediating the interactions of the inhibitor with the protein matrix. CX-4945 is able to establish polar interactions with the two possible anchoring sites present in the CK2α ATP-binding pocket, i.e., the N-terminal portion of the hinge region (Val116 backbone) and the positive area in the deepest part of the cavity (W1 and Lys68). The orientation of the 3-chlorophenil moiety toward the N-terminal lobe is determined by the unusual "up" conformation of His160 that interact with W2.

CK2α, the 3-chlorophenyl moiety points toward the C-terminal lobe, while in the two crystal structures it is oriented toward Gly46 of the phosphate-anchor loop of the N-terminal lobe (Figure 1.27).

The reason for this 3-chlorophenyl orientation was not completely clear on the basis of the first crystal structure at 2.71 Å resolution (Ferguson et al., 2011), while it could be nicely explained on the basis of that at 1.60 Å (Battistutta et al., 2011). The nitrogen in ortho to the 3-chlorophenylamino substituent forms a hydrogen bond with a water molecule that in turn is bound to the N^{ε} atom of His160, which adopts an unusual up conformation that hinders the downward rotation of the chlorophenylamino group (Figure 1.27). In the same structure, as well as in that of the related CX-5011 compound, another water molecule (W3 in Figure 1.27) mediates the interaction between the ligands and the side chain of Asn118 from the hinge region. This interaction does not cause an increase in potency, as indicated by SAR analysis (Pierre et al., 2011), but can be exploited for structure-based designed of improved inhibitors. Furthermore, a computational analysis indicates that the enhanced interaction with water W3 is responsible for the unique insensitivity of CX-5011 to double mutations V66A and I174A (Battistutta et al., 2011).

Potency in CK2 Inhibition

A preliminary note on the matter of potency is that caution must be used in the case of inhibitory values (K_i or IC_{50}) in the very low nanomolar or subnanomolar range. In these cases, to get reliable data on the potency of compounds, assays should be performed at comparably low concentration of the kinase (Shen et al., 2005), which is not always possible or not always taken into consideration. Moreover, good practice rules indicate that nonlinear regression analysis of the enzyme activity versus inhibitor concentration should be preferred to the graphical interpolation of linearized, mostly outdated, equations, which lead to the distortion of the experimental error.

The analysis of several low-molecular weight inhibitors crystallized in complex with CK2α indicate that the following fundamental features are characteristic of a type I ATP-competitive inhibitor: appropriate hydrophobicity, to benefit from the hydrophobic effect and be readily transferred from the aqueous phase to the hydrophobic active site; excellent shape complementarity with the small active site of CK2α (to maximize van der Waals contacts and avoid steric clashes), carrying characteristic residues such as Tyr50, Val66, Phe113, His160, Met163, and Ile174, whose combination makes the CK2α active site quite unique among protein kinases; and, most importantly, the capability to simultaneously establish electrostatic interaction with the positive area near the conserved water W1 and Lys68, and hydrogen bonds with three possible anchoring points at the beginning of the hinge region: Val116 backbone amide (NH) and C=O and Glu114 backbone carbonyl (C=O). As seen above, these anchoring functions are used also by cosubstrates ATP, that interacts with Glu114 C=O and Val116 NH, and GTP, that interacts with Val116 NH and Val116 C=O (Niefind et al., 1999).

One common peculiar characteristic of many CK2 inhibitors is that hydrogen bonds with the hinge region are not essential for binding, differently from other protein kinases, highlighting the importance of the apolar interactions in targeting CK2. However, crystal structures of very potent compounds indicate that to reach potency in the very low nanomolar range, inhibitors must target both the hinge region and the positive area with polar interactions.

The best-characterized inhibitor carrying all of these features is CX-4945, for which also thermodynamic binding data are known (Ferguson et al., 2011). ITC analysis confirms a very strong binding of CX-4945 to the human enzyme, with a K_d <10 nM. The binding is dominated by a strong enthalpic contribution ($\Delta H° = -56.8$ kJ/mol), with a slightly negative (unfavorable) $T\Delta S°$ term of −9.6 kJ/mol, implying that, in this case, hydrophobic interactions, generally characterized by a strong favorable entropy, are not the main determinant for the binding. Given the extended hydrophobic nature of the inhibitor and of the binding site, the negative $T\Delta S°$ suggests that other unfavorable entropic contributions must be taken into account and that the latter are able to overcome the gain in entropy expected from the large hydrophobic contribution. The unfavorable entropic term was attributed to the reduction in the degrees of freedom both of the ligand and of the protein upon binding (Battistutta et al., 2011). At the level of the inhibitor (see Figure 1.22), the two torsional angles free to rotate in solution around the disubstituted amino function are frozen in the complex, and in

CK2α, the intrinsic flexibility of the phosphate-anchor loop and His160 is strongly reduced upon ligand interaction (probably via a "conformational selection" rather than an "induced fit" mechanism). An unfavorable entropic contribution can also derive from the loss of degrees of freedom of the crystallographic waters surrounding the inhibitor. The strong enthalpic contribution, essential for the CX-4945 binding, is indicative of strong noncovalent interatomic interactions. The crystal structure reveals that there are two major polar interactions: one hydrogen bound to Val116 NH in the hinge region and the salt-bridge with Lys68 (Figure 1.27). The removal of one of these interactions causes a dramatic decrease in the inhibitor affinity (Pierre et al., 2011), showing that they play a fundamental role for the favorable enthalpic term. The substitution of the carboxylic function with an amide group, still able to form hydrogen bonds with the protein matrix, increases the IC_{50} from 1 to 417 nM, underlining the importance of the electrostatic nature of the interaction with Lys68. From this detailed analysis, it can be derived that a possible improvement in this class of inhibitors could be the removal of the rotational degrees of freedom around the amine function.

Thermodynamic data coupled to crystal structures are available also for two members of another class of CK2 inhibitors, 2,6-disubstituted pyrazines derivatives, with potency in the nanomolar range (Kinoshita et al., 2011; Suzuki et al., 2008). Like CX-4945, these inhibitors carry a carboxylic function, and ITC data show that also for this class of compounds the binding free energy is dominated by the enthalpic term ($\Delta H° = -46.3$ and -57.4 kJ/mol for compound 1 and 2, respectively), with smaller entropic contributions ($T\Delta S° = 2.2$ and -6.9 kJ/mol, respectively). Therefore, also for these inhibitors, strong polar interactions and a good shape complementarity, with good van der Waals contacts, are essential for binding to overcome the only slightly favorable or even unfavorable entropic term.

The importance of a good shape complementarity between the ligand and the CK2α active site is also illustrated by the fact that the insertion of a methyl group in ortho of the carboxylic function of CX-4945 is not tolerated because this position is placed very close to Phe113 (the so-called "gatekeeper" residue) and Ile95, both in the hydrophobic region I. In this case, there is a dramatic loss of potency of those analogues (Pierre et al., 2011), while, conversely, the other ortho position has enough space to host a methyl function without compromising the affinity.

Selectivity in CK2 Inhibition

Recently, the Gini coefficient was proposed as a quantitative parameter to express the selectivity of kinase inhibitors against a certain panel of kinases. This parameter is derived from the plot of the cumulative fraction of total inhibition against the cumulative fraction of tested kinases (Graczyk, 2007). The relative selectivity of inhibitors determined with the Gini coefficient does not depend on the ATP concentration, although depends on the size and composition of the used kinase panel, ideally becoming an invariant parameter only when it is calculated using the whole kinome (518 protein kinases). Nonselective inhibitors are characterized by Gini values close to zero, for instance staurosporine showing a Gini value of 0.150 on a panel of 85 kinases, while highly selective compounds show Gini values close to 1,

with the very selective MAP kinase kinase 1 (MEK1) and MEK2 inhibitor PD184352 (also known as CI-1040) exhibiting a particularly high Gini value of 0.905. Interestingly, the PD184352 selectivity is a result of its non ATP-competitive mode of binding (Ohren et al., 2004).

The selectivity of many CK2 inhibitors were characterized also with another parameter, the "promiscuity score" (PS), that is the average inhibition (in percentage) of all of the kinases of a defined panel, tested at an inhibitor concentration that decrease the activity of the kinase under consideration by around 90% (Pagano et al., 2008b). PS and selectivity are therefore inversely proportional, i.e., low PS values indicate high selectivity. To be a reliable indication of selectivity, the PS for different inhibitors must be calculated on the same and sufficiently large panel of kinases, at least 60 or more, representative of all of the subfamilies of the kinome.

Whatever the score used to evaluate the selectivity of an inhibitor, it is clear that the extent and the diversity in the members of the panel of tested protein kinases are crucial to get reliable information. For instance, TBB was the first CK2 inhibitor whose specificity was analyzed toward a "large" (for that time, 2001) panel of more than 30 protein kinases, representative of the main branches of the kinome (Sarno et al., 2001), and was considered to be a very specific inhibitor of CK2, the only kinase drastically inhibited at 10 μM TBB concentration. However, later, when the panel was enlarged to include 70 protein kinases, it turned out that other kinases are strongly inhibited, such as DYRK1A, DYRK3 and all the members of the PIM family, HIPK2, ERK8, and PKD (Pagano et al., 2008b), with a Gini coefficient for TBB of 0.375, considerably poorer than those for quinalizarin (0.612) and CX-4945 (0.615), among the most selective CK2 inhibitors known today.

About the proper extent of the panel of kinases to test, it is interesting to note that for the two highly potent inhibitors, CX-4945 and CX-5011, the Gini coefficients derived from a panel of 102 kinases were very similar to the values on a larger panel of 235 kinases (almost 50% of the known kinome) (Battistutta et al., 2011). It seems that a panel of around 100 kinases adequately distributed among the kinome appears to be sufficient to provide reliable Gini coefficients to compare the selectivity of different compounds.

Data are available on the selectivity of many interesting CK2 inhibitors, including some commercially accessible, using relatively large panels of 60 or more, distantly related, protein kinases (Sarno et al., 2011). These data revealed that kinases targeted by CK2 inhibitors are not randomly distributed throughout the kinome, but tend to fall into the same subset of enzymes (the same indicated above for TBB), irrespective of the chemical class of the compounds. This suggests significant analogies in the ATP-binding sites of these kinases, but also the possibility to exploit the available repertoire of CK2 inhibitors as a source for the development of inhibitors selective for those other kinases. In general, it was shown that CK2 inhibitors often target also PIM kinases with similar efficacies, with the remarkable exception of quinalizarin and the tetrabromobenzo derivative K66 (1-carboxymethyl-2-dimethylamino-4,5,6,7-tetrabromo-benzimidazole) (Sarno et al., 2011).

For some inhibitors, on the basis of the crystal structure in complex with CK2α, it was possible to derive indications about the basis of their selectivity. For instance, for the CX compounds, the comparison between the Gini coefficients of CX-4945

(0.615), CX-5011 (0.735), CX-5279 (0.755), and CX-5033 (0.570) suggests that the higher general selectivity of CX-5011 and CX-5279 results from the replacement of the pyridine with a pyrimidine ring, and not from the structural changes on the aniline (Ar-NR2R3) moiety (Battistutta et al., 2011).

On the other hand, for the specific case of PIM1, the selectivity seems correlated with the bulkiness of the substituent in position R2, not to the presence of the pyrimidine instead of the pyridine. The phosphate-anchor loop of CK2α has an intrinsic flexibility and is able to accommodate the three different substituents equally well, as shown by the corresponding crystal structures. This does not seem possible in the case of PIM1. As deduced by the crystal structures of PIM1 complexes with inhibitors (1XWS and 3BGQ [Pierce et al., 2008]), the phenylalanine of the glycine-rich loop is pointing toward the active site, in a position that superposes with that occupied by the substituted phenyl of the inhibitors in the CK2α complexes, in accordance with the lower potency of these compounds against PIM1.

From the comparison between the crystal structures of the CK2α complexes and the models obtained from the molecular docking of emodin and quinalizarin into PIM1, it was possible to derive the structural features responsible for the higher sensitivity of PIM1 to emodin, and for its reduced sensitivity to quinalizarin, a much more selective CK2 inhibitor (Cozza et al., 2009). Three PIM1 residues are responsible for this difference, namely Phe49 (corresponding to Tyr50 in CK2α), Leu174 (Met163 in CK2α), and Glu171 (His160 in CK2α). It is interesting to note that only 21% of the protein kinases included in the selectivity panel used for quinalizarin carry a tyrosine residue at the position homologous to CK2α Tyr50, and only 10% have a methionine residue homologous to CK2α Met163, almost all of which have a phenylalanine instead of Tyr50. Moreover, within the selectivity panel, CK2α is the only kinase bearing the His160 residue. It can be derived that at least for quinalizarin (but perhaps also for other inhibitors), the remarkable selectivity for CK2 mainly relies on the unique combination of the three residues Tyr50 from the phosphate-anchor loop and His160 from the catalytic loop and the adjacent Met163.

The comparison of the CK2α active site with those of other 71 different human protein kinases indicates that the peculiarity of the CK2α ATP-binding cleft is not only due to the small size and the hydrophobic character, but also to its electrostatic properties (Cozza et al., 2010). Indeed, only Polo Like Kinase 1 shows an electrostatic distribution similar to CK2α, and, interestingly, PIM1 and PIM2, often targeted by CK2 inhibitors, are quite different in this respect, suggesting a way to improve selectivity.

Non ATP-Competitive Protein Kinase Inhibitors

In parallel to the development of ATP-competitive inhibitors, there is a growing interest in compounds that do not target the active site but are still able to down-regulate the activity of protein kinases. The non-ATP competitive or allosteric kinase inhibitors, which display no contact to the hinge region, are usually referred to as "type III" inhibitors. The following compounds are examples of allosteric kinase inhibitors: CI-1040, which inhibits MEK by occupying a pocket adjacent to the ATP

binding site (Ohren et al., 2004); GNF2, which binds to the myristate binding site of ABL (Adrian et al., 2006); the pleckstrin homology domain-dependent AKT inhibitors (Lindsley et al., 2005); BMS-345541, which inhibits the IKK (McIntyre et al., 2003); and the rapamycin derivatives targeting mTOR (Wang and Sun, 2009). In general, non ATP-competitive inhibitors are expected to show fewer off-target side effects and a higher degree of specificity, mainly because residues and structural characteristics outside the ATP binding pocket tend to be less conserved. Furthermore, since these inhibitors do not compete with cellular ATP, they can be normally used at concentrations closer to their biochemical K_i, unlike ATP-competitive ones. Some allosteric non-ATP-competitive inhibitors have been recently proposed also for CK2.

Another class of non ATP-competitive inhibitors is composed by small molecules or, more frequently, peptide-based inhibitors, able to directly compete with the binding of protein substrates (Bogoyevitch et al., 2005; Bogoyevitch and Fairlie, 2007). The most interesting example of this type of inhibitor is the compound classified as ON01910 (and its analogue ON012380), a potent inhibitor of polo-like kinase1 (Plk1) (IC_{50} around 9 nM) implicated in cell cycle progression and over expressed in many tumor cells (Gumireddy et al., 2005). As shown below, an example of a substrate-competitive inhibitor is available also for CK2. Bisubstrate analogs, able to bind simultaneously to both the ATP-binding and substrate-binding sites, have also been proposed as potent and highly specific kinase inhibitors (Parang and Cole, 2002; Parang et al., 2001; Shen et al., 2005). At least theoretically, agents able to selectively target key protein-protein interactions would appear to serve as ideal inhibitors of cell signaling as well as potential therapeutics. In some cases, non-ATP-competitive inhibitors can be used to selectively inhibit the activity of a kinase against only a subset of its targets.

In general, peptide-based inhibitors, available also for CK2, show some inconveniences, notably the following: low affinity for the target and hence low inhibitory potency; intracellular instability and difficult uptake; and general bioavailability problems with respect to classical drug-like small organic compounds. Recent advances (delivery technologies, new strategies to transform modest binding consensus sequences into high-affinity ligands) have led to a renewed interest in the development of such compounds able to disrupt intracellular protein-protein interactions. In several of these instances, unprecedented affinity (<nM) and selectivity (>1,000-fold versus closely related protein targets) have been achieved (Pinna and Cohen, 2005). For instance, cyclization of peptides improves stability against proteases while affording conformational constraints that may enhance inhibitory potency; globally modified peptides, for example with the addition of long alkyl chain, can have enhanced active site affinity via coordination to ancillary binding pockets. Anyway, the conversion of consensus recognition sequences into small molecules with drug-like properties (peptidomimetics) is time-consuming and requires a combination of detailed structural information of the target protein and an intensive synthetic effort.

An interesting feature of the non-ATP competitive mode of inhibition is that it can provide compounds that may work well in combinations with other drugs, an approach that may achieve better clinical results, potentially with less toxic side effects.

Non-ATP-Competitive Inhibitors of CK2

The first non-ATP-competitive inhibitor of CK2 for which structural information was available is DRB. In addition to the ATP-binding site (competitive inhibition constant 29.2 μM), a second molecule of DRB was found bound to an external hydrophobic cavity ("secondary or allosteric binding site") near the β4β5 loop, the main anchoring point between CK2α and CK2β in the holoenzyme formation. This binding shows a noncompetitive inhibitory effect on CK2 activity that could be discriminated from the ATP-competitive one (noncompetitive inhibition constant 39.7 μM) (Raaf et al., 2008). Obviously, this secondary binding site is important also for the possibility to selectively interfere with the assembly of the tetrameric $\alpha_2\beta_2$ holoenzyme.

The outcome of the binding to this secondary binding site can be diverse. Indeed, a stimulatory effect on the phosphorylation of peptide substrates was observed in the case of various peptides reproducing the C-terminal end of CK2β, encompassing the region that physically interacts with the α subunit in the structure of the tetrameric holoenzyme (Marin et al., 1995; Marin et al., 1997). In particular, a 23-mer peptide corresponding to the C-terminal sequence 181-203 of the human CK2 regulatory β subunit is able to stimulate the activity of CK2α against a calmodulin-derived peptide, even if to a lesser extend compared to the full-length β subunit. The occurrence of strong interactions between CK2α and the β peptide (181–203) was confirmed by plasmon resonance experiments. The crystal structure of the complex with maize CK2α (Fig. 1.2C) revealed that the peptide binds in the same hydrophobic pocket of the second DRB molecule, in the secondary binding site (Figure 1.28), interacting with the protein through several polar and apolar interactions (Battistutta et al., 2000a).

More recently, a cyclized 11-mer peptide containing the sequence Arg186-His193 of CK2β, designed on the basis of the α/β interface of CK2, was shown to inhibit the assembly of the holoenzyme (as was previously seen also for the CK2β linear peptide [170–215]) and to affect its substrate preference (Laudet et al., 2007). Presumably, this peptide binds in the same secondary binding site as the linear β peptide (181–203) and DRB, but the exact mode of binding is not known, as well as whether it has a stimulatory or inhibitory effect on the activity of CK2α.

Another interesting peptide is the one derived from the cystic fibrosis transmembrane conductance regulator protein CFTR, corresponding to the 500–518 sequence of the NBD1 domain. Despite the fact that this peptide encompasses a region displaying the consensus for CK2 phosphorylation, and hence could behave as a substrate-competitive inhibitor, it was shown to inhibit the CK2α activity with a purely noncompetitive mechanism. The inhibitory effect of the peptide is only detectable with the isolated catalytic α-subunit, and not with the heterotetrameric CK2 holoenzyme (Pagano et al., 2008a). Inhibition data suggest that the CFTR peptide binds to an allosteric site of CK2α that is no longer accessible in the tetrameric holoenzyme, as is the case of the secondary binding site.

Recently, another non-ATP-competitive class of inhibitor was identified, comprising the podophyllotoxine indolo derivative W16, that was indicated as an allosteric inhibitor (Laudet et al., 2008). It is also able to selectively disrupt the CK2α/CK2β assembly and hence it was hypothesized to bind to the same secondary binding site targeted by DRB.

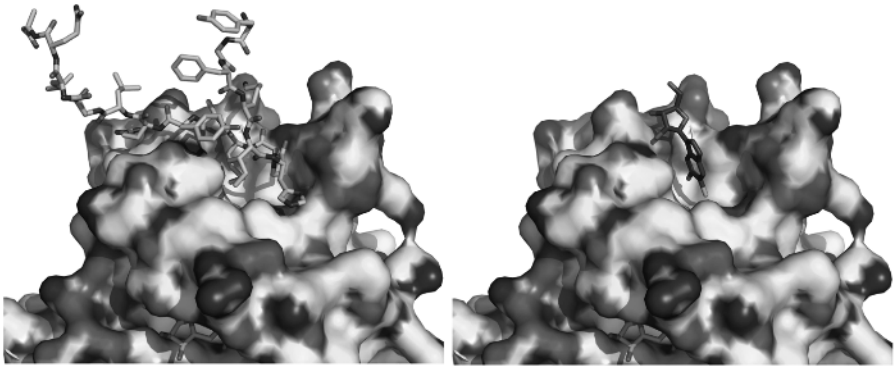

Figure 1.28. Close-up view of the secondary (allosteric) binding site occupied by the CK2β peptide (181–203) (left panel) or by inhibitor DRB (right panel), in the N-terminal lobe of the α-subunit (shown as surface). This is the site of major interaction between the CK2α and CK2β subunits in the tetrametric $\alpha_2\beta_2$ holoenzyme.

Till now, there are no clear explanations for the structural effects of the binding to the secondary "allosteric" site of CK2. Several structures are available with different molecules in this allosteric site, those of human CK2α in complex with DRB, with small ligands such as glycerol (Raaf et al., 2008), ethylenglycol or PEG (Papinutto et al., 2012), or the entire β subunit (Niefind et al., 2001), together with that of maize CK2α in complex with the CK2β peptide (181–203) (Battistutta et al., 2000a). None of these structures shows remarkable conformational changes in the active site that can be clearly correlated to a modulation (either negative or positive) of the catalytic activity of isolated CK2α. It is clear that more experimental data are needed to clarify the nature and the structural bases of the hypothesized allosteric regulation at the level of the secondary binding site and the reasons why the binding to this site can have both stimulatory and inhibitory effects, depending on the ligand. This is a quite important issue to address because of the potential relevance of the availability of this type of non-ATP-competitive inhibitors. Furthermore, compounds targeting this secondary binding site should also be able to inhibit the CK2 subunit's regular assembly and could be valuable tools for the study of the role of the β subunit *in vivo*, especially in the case of those substrates whose phosphorylation is entirely dependent on the β subunit (class III substrates, see Pinna, 2002).

Polyoxometalates (POM) are another family of interesting nonclassical inhibitory compounds. They are inorganic compounds, nanometer-sized complexes of early-transition metal ions and oxo ligands, with inhibitory activity in the low nanomolar range (IC_{50} < 10 nM for the best ones). Although not having any drug-like property, they can be interesting because they seem to behave as allosteric compounds targeting CK2α in a site different from the ATP-binding, the protein substrate-binding, or the allosteric sites (Prudent et al., 2008). No clear structural information on the POMs binding site is available yet; however, they are supposed to interfere with the interaction between the activation segment and the N-terminal region. They showed a promising selectivity but are limited to a relatively small panel of 29 protein kinases. As indicated above, this information needs to be validated on a larger panel of carefully chosen kinases.

Other important tools for studying CK2 function would be compounds able to selectively inhibit the phosphorylation activity of the enzyme by interfering with the binding of protein substrates, i.e., substrate-competitive inhibitors. In this respect, up till now there is only one peptide that has been reported to inhibit CK2 *in vivo* acting as a protein substrate competitor. This CIGB-300 peptide (formerly P15-Tat) was identified from the screening of a random cyclic peptide phage library using a synthetic CK2 phosphoacceptor site of the HPV-16 E7 oncoprotein, and fused to the cell-penetrating peptide derived from the HIV-Tat protein. This inhibitor exhibited pro-apoptotic activity and anticancer properties *in vitro*, in tumor animal models, and in patients with cervical malignancies (Perea et al., 2008a; Perea et al., 2004; Perea et al., 2008b). Whether the anti-cancer potential of this peptide is mediated by CK2 inhibition or not remains unclear.

In Vivo Versus *In Vitro* Inhibitory Activity

To be useful as biochemical tools and to be considered compounds with pharmacological potential, inhibitors must be cell permeable and active in cells. CX-4945, CX-5011, and CX-5279 all display cell permeability and high efficacy as antiproliferative agents when tested on a variety of cancer cells (Pierre et al., 2011). Interestingly, although the EC_{50} values are 3 orders of magnitude higher than the IC_{50} values for CK2 inhibition, in the case of CX-5011 and CX-5279, the EC_{50} remain below the *in vitro* IC_{50} on PIM1. These data suggest that the antiproliferative efficacy of these compounds is indeed mediated by CK2.

Polyhalogenated benzimidazole (or triazole or pyrazole) derivatives, namely DMAT, TBB, TBI (all three commercially available), and TBCA (tetrabromocinnamic acid), and condensed polyphenolic derivatives, namely MNX, MNA, and NBC (8-hydroxy-4-methyl-9-nitrobenzo[g]chromen-2-one), are all cell permeable and were among the most frequently used CK2 inhibitors for *in vivo* studies. Other interesting recently developed type I ATP-competitive inhibitors with good pharmacological profiles are those based on pyridocarbazolo and benzopyridoindole scaffolds, with *in vivo* efficacy in a mouse xenograft model of human glioblastoma (Prudent et al., 2010).

CK2 inhibitor quinalizarin is also cell permeable; it is able to inhibit endogenous CK2 in HEK293 and Jurkat cells. Such an inhibition, which is higher than that observed with TBB, correlates with a marked cytotoxic efficacy. Of particular interest is the fact that quinalizarin only marginally increases necrosis, with a much more remarkable enhancement of apoptosis. For other inhibitors, such as TBB and DMAT, the cytotoxic effect is substantially related to necrosis rather than apoptosis, raising the possibility that the cytotoxic effect is at least partially due to the inhibition of protein kinases other than CK2. All this, together with the narrower selectivity of quinalizarin, makes this inhibitor superior for *in vivo* studies.

However, there are other cases in which the practical usefulness of potent *in vitro* CK2 inhibitors is hindered by deliverability problems, limiting their pharmacological potential and the possibility to used them as biochemical tools *in vivo*. This is the case for the potent and selective CK2 inhibitor IQA, which is not sufficiently stable in water (Sarno et al., 2005a), and ellagic acid (Sekiguchi et al., 2009), which fails to cross the intestinal barrier where it is converted to urolithin (Seeram et al., 2006).

CK2 Inhibitors as Biochemical Tools

Many functional roles of CK2 have been discovered and deciphered using ATP-competitive inhibitors that therefore have been proven to be useful biochemical tools. A significant example is the demonstration of the anti-apoptotic potential of this kinase that was inferred by using different selective and potent cell permeable type I ATP-competitive CK2 inhibitors (Di Maira et al., 2005; Loizou et al., 2004; Piazza et al., 2006; Sarno et al., 2005a; Sarno et al., 2005b; Scaglioni et al., 2006).

Although kinases are commonly associated with cancer, these enzymes are also clearly involved in other pathologies (Guerra and Issinger, 2008), expanding their potential therapeutic spectrum. In this regard, it is significant to note that, recently, the use of CK2 inhibitors TBB, DMAT, and TBCA was important to demonstrate the involvement of CK2 in neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (Perez et al., 2011). This is particularly important because of the current lack of effective treatments for neurodegenerative diseases, and the consequent increased efforts dedicated to the search of new therapeutic targets that could provide new effective drug candidates. The involvement of CK2 in Alzheimer's disease was inferred on the basis of the fact that peptide oAβ42, when in soluble intracellular form, acutely inhibits synaptic transmission (a pathophysiology that characterizes AD), and that this effect is mediated by the activation of CK2, and it is prevented by the specific pharmacological CK2 inhibitors DMAT and TBCA (Moreno et al., 2009; Pigino et al., 2009). Moreover, CK2 inhibitors TBB and DMAT were shown inducing a significant reduction in heme oxygenase activity, indicating the potential protective neuronal effect of these molecules (Perez et al., 2011). TBB was also important in demonstrating that CK2 plays an important role in the induction of long-term neuronal potentiation (Perez et al., 2011).

Is CK2 Really a Druggable Target?

As outlined along this review, potentially CK2 is an appealing drug target for its involvement in several diseases, among which include cancer and neurodegenerative disorders. In recent years, several potent, selective, and cell-permeable CK2 inhibitors have been identified and characterized. These compounds constitute a valuable combination of biochemical tools but also set up the proof-of-concept that the selective inhibition of CK2 is feasible. However, the facts that CK2 is essential for cell viability and that it is involved in many physiological processes, with hundreds of potential substrates, raise the question whether this protein kinase can be in practice, not only in theory, an effective drug target for therapeutic intervention.

At the current stage of knowledge, answering this question is not an easy task because many aspects must be taken into consideration, and often only qualitative, rather than quantitative, pieces of information are available. In drug development, particularly for life-threatening diseases, it is clear that the benefit-risk balance is a key concept to cope with, and for tumors, often only chemotherapy or radiotherapy, with clear massive side effects, is currently available. The discovery of the involvement of CK2 also in neurodegenerative disorders is particularly important because the lack of effective treatments for these diseases suggests that CK2 may represent

a valuable and promising target for therapeutic intervention. So, in both cases, the severity of the diseases and the lack or hazards of alternative therapies can make more tolerable potential risks of side effects associated to a specific drug, for instance a CK2 inhibitor. Indeed, from the preliminary studies available so far, there is no reason to believe that a CK2 targeting drug could have a lower therapeutic index than other protein kinase inhibitors in clinical use.

Another important point to consider is the rising concept of the non-oncogene addiction of malignant cells to high level of CK2, whose anomalous high activity, not caused by genetic alterations but by still unknown epigenetic events, is supposed to generate a cellular environment particularly favorable to neoplasia (Ruzzene et al., 2011; Sarno and Pinna, 2008). In this view, CK2 is not a real "promoter" of malignancy but just a "driver" of it, being able to support, mainly though its anti-apoptotic role, other biological alterations truly responsible of the tumor onset (Ruzzene and Pinna, 2010). This model indicates that a general effective strategy would be to attenuate, i.e., pharmacologically downregulate, rather than completely abolish, the abnormally high activity typical of cancer cells of this protein kinase. This could be particularly effective in the context of the multitarget drug approach, i.e., the use of a combination of drugs to target a particular disease. In this case, many biological pathways utilized by the tumor to originate, grow, resist death, and spread are simultaneously hit in order to achieve an additive or synergistic effect.

Other data seem to support the notion that CK2 is a valuable drug target. In particular, in xenograft models, inhibition of CK2 activity by siRNA and antisense vectors is able to induce apoptosis in cancer cells with no or small effects on normal cells (Slaton et al., 2004). Furthermore, it was repeatedly reported that malignant cells where CK2 level is abnormally high are more susceptible to CK2 inhibitors-induced apoptosis than their normal controls, where CK2 is lower on the average (Kim et al., 2007; Piazza et al., 2006; Silva et al., 2008). Data of the Phase I clinical trial for the Cylene inhibitor CX-4945 for the treatment of different kinds of cancer are encouraging, indicating that it has favorable safety, and pharmacokinetic and pharmacodynamics characteristics. The possible positive progression of this inhibitor in further steps of clinical trials will be important to set up the concept that pharmacological inhibition of CK2 is really feasible.

Another multitarget drug discovery (MTDD) approach entails finding agents that are capable of simultaneously addressing two or more targets. Based on this idea, the rationale for new anticancer molecules is currently moving from highly specific inhibitors acting on single protein kinases to ligands binding simultaneously to multiple cancer targets. Indeed multitargeting agents have the theoretical ability to provide more efficient antitumor activity and to delay the onset of tumor resistance. With the recent approval by FDA of Sorafenib and Sunitinib, which target the vascular endothelial growth factor receptor, the platelet-derived growth factor receptor, the FMS-like tyrosine kinase 3 (FLT-3), and the stem cell factor receptor (c-Kit), this new scenario is becoming generally accepted. An interesting example of this approach applied to CK2 is the recent identification of difurandicarboxylic acid derivatives as potent dual inhibitors of this protein kinase and PIM kinases, two structurally and functionally related kinases. Crystal structures of CK2α and PIM1 complexes suggested that the basis of the selectivity of this class of compounds mainly relies on the narrower ATP-binding site of both CK2α and PIM1, and

particularly on the presence of two conserved isoleucine (Ile174 and Ile95 in CK2α, Ile185 and Ile104 in PIM1) (Lopez-Ramos et al., 2010). Other kinases usually present smaller residues in the corresponding positions.

CONCLUSIONS AND OUTLOOK

The determination of many crystal structures of maize and human CK2α catalytic subunits in complex with different families of type I ATP-competitive inhibitors has been very productive in deciphering the structural basis for targeting this enzyme with high potency, and, consequently, permitting the effective structure-based design of new potent compounds. Starting from the first inhibitors with K_i in the micromolar range (emodin ≅2 μM, DRB ≅24 μM), now potent inhibitors in the very low nanomolar range are available. Pharmacologically, the most promising one is currently CX-4945, in Phase I clinical trials for the treatment of different kinds of cancers. Some of the available inhibitors show good cell permeability and selectivity and, therefore, are very useful for *in vivo* functional studies of CK2. Tested on extended panels of protein kinases from different branches of the kinome, quinalizarin and CX derivatives appear among the most selective compounds, with Gini coefficients above 0.6. Notably, they are poor inhibitors of PIM1, a protein kinase often targeted by other CK2 inhibitors.

A peculiarity of the best CK2 inhibitors is the ability to simultaneously establish polar interactions with both the hinge region (through hydrogen bonds) and the positive area near Lys68 and Glu81 (through salt-bridges). Usually, Type I ATP-competitive inhibitors of protein kinases interact principally with the hinge region, and Type II inhibitors target the region near Glu81, which binds to inactive "DFG-out" conformations of the enzymes. This further supports the concept that the active site of CK2 is atypical among the protein kinases family, as discussed throughout this review.

In the context of the multitargets drug approach, it is of interest that the recent development of compounds like the difurandicarboxylic acid derivatives and the triazolyl-thieno quinolines derivatives, as potent dual inhibitors of PIM1 and CK2, protein kinases are often involved in the same or closely related biochemical pathways at the basis of the same pathologies.

Much less structural information is available for non-ATP-competitive CK2 inhibitors. Some interesting compounds have been discovered in the recent past but, apart from DRB, the lack of 3D structures of their complexes with CK2 is a clear obstacle for the further developments of these hits. In particular, how the binding to surface clefts different from the ATP-binding site can allosterically influence the catalytic activity of the intrinsically active CK2 currently is obscure. Much more efforts in this direction are needed to assess whether an out-of-the-box strategy, successful for some Tyr protein kinases, can be applicable also to CK2.

In this context, a possible complication is the evidence that the catalytic α-subunit is incorporated in the tetrameric $\alpha_2\beta_2$ form of the holoenzyme, and in more extended supramolecular assemblies. As outlined above, *in silico* modeling and the analysis of crystal packings provided preliminary evidence about the structural

characteristics of these assemblies, but the validity of these concepts for the solution state and in particular for the cell remains to be clarified.

In this regard, crystallography, which dominated CK2 structural biology so far but that can only provide snapshots, may come to its limits, and alternative experimental approaches like small-angle X-ray scattering (SAXS) or high-resolution imaging techniques may be more suitable. A recent SAXS study (Moucadel et al., 2011) providing evidence that the conformational space open for CK2α may be broader than inferred from crystal structures could serve as a programmatic example.

It is a general tendency in the structural biology of protein kinase no longer to understand particular conformational states (like the DFG-out conformations of MAP kinases and other EPKs) observed in complex with certain inhibitors as artificial peculiarities but as potential intermediates along catalytic and regulatory cycles (Kannan and Neuwald, 2005; Bukhtiyarova et al., 2007; Shan et al., 2009). They may be trapped by inhibitors, but most probably they are relevant for physiological conditions as well. Most hints pointing in this direction have been collected for the DFG-motif, but based on that experience, it would not come as a surprise if the intriguing plasticity of the hinge/helix αD region observed so far exclusively in human CK2α will finally turn out to be a functionally important and even more representative rather than exceptional property.

Thus, the hinge/helix αD plasticity is a nice example for a general feature of science, namely to provide with any answer a novel question. In this sense, we believe that even after 15 years, the structural biology of protein kinase CK2 is far from its end.

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