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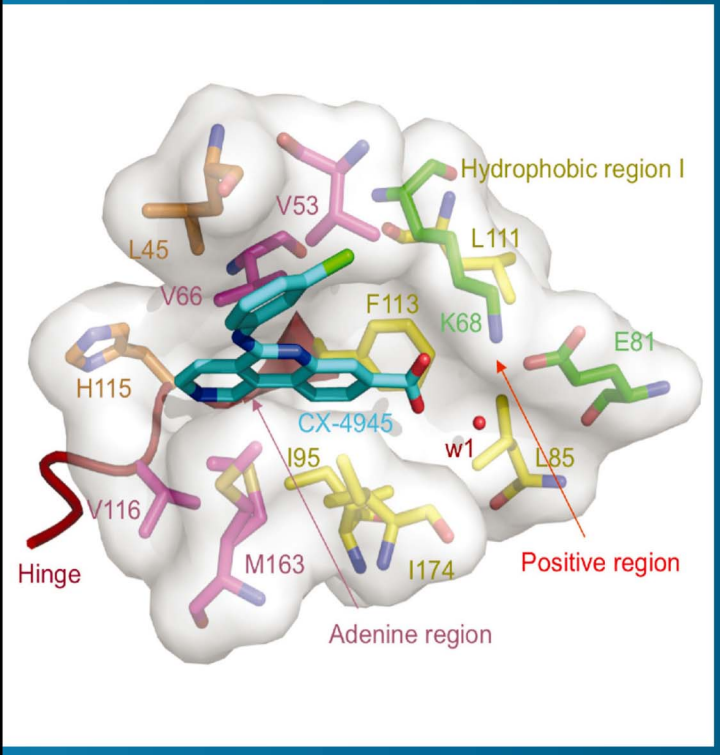
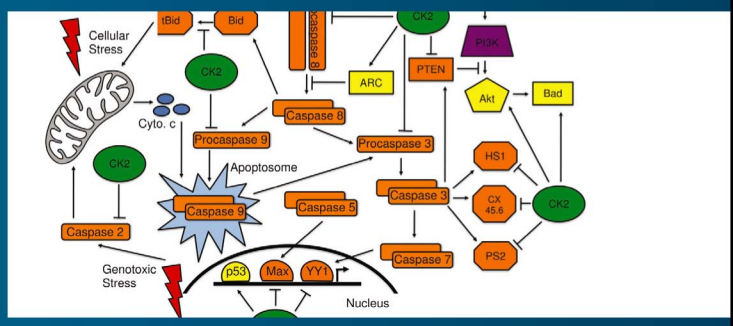
Protein Kinase CK2

Edited by **Lorenzo A. Pinna**





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Edited by

Lorenzo A. Pinna

Department of Biomedical Sciences
University of Padua
Padua, Italy

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Cover image: The cover figure provides examples of CK2 peculiar properties by showing, clockwise, starting from left upper corner: the unique butterfly shape of CK2 holoenzyme, composed of two catalytic subunits bound to a dimer of the noncatalytic subunit; the CK2 catalytic subunit pharmacophore occupied by an inhibitor now in clinical trials as an anticancer drug; the convergence of CK2 with caspase pathways; the dorsal axis duplication induced by injecting Xenopus laevis embryos with CK2 mRNAs. Figures are drawn from Chapters 1, 5, and 4, respectively.

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CONTRIBUTORS

KHALIL AHMED
Research Service, Minneapolis V.A. Health Care System
Department of Laboratory Medicine and Pathology
Masonic Cancer Center
and Department of Urology
Department of Otolaryngology
University of Minnesota
Minneapolis, MN, USA

ROBERTO BATTISTUTTA
Department of Chemical Sciences
University of Padua
Padua, Italy

LUCA CESARO
Department of Biomedical Sciences
University of Padua
Padua, Italy

CLAUDE COCHET
INSERM, UJF, CEA, DSV/iRTSV
Biology of Cancer and Infection
Grenoble, France

ALEXANDRE DESHIERE
INSERM, UJF, CEA, DSV/iRTSV
Biology of Cancer and Infection
Grenoble, France

ISABEL DOMINGUEZ
Hematology Oncology
Department of Medicine
Boston University School of Medicine
Boston, MA, USA

DENIS DRYGIN
Cylene Pharmaceuticals
San Diego, CA, USA

ODILE FILHOL
INSERM, UJF, CEA, DSV/iRTSV
Biology of Cancer and Infection
Grenoble, France

MICHELLE GABRIEL
Department of Biochemistry
Schulich School of Medicine and Dentistry
The University of Western Ontario
London, Ontario, Canada

CLAUDIA GÖTZ
Medical Biochemistry and Molecular Biology
University of the Saarland
Homburg, Germany

BARBARA GUERRA
Department of Biochemistry and Molecular Biology
University of Southern Denmark
Odense, Denmark

OLAF-GEORG ISSINGER
Department of Biochemistry and Molecular Biology
University of Southern Denmark
Odense, Denmark

BETSY T. KREN
Research Service, Minneapolis V.A. Health Care System
Masonic Cancer Center
Department of Medicine
Minneapolis, MN, USA

ESTHER LANDESMAN-BOLLAG
Section of Hematology-Oncology
Department of Medicine
Boston University School of Medicine and Boston Medical Center
Boston, MA, USA

TOMMASO LEGNAIOLI
Center for Research in Agricultural Genomics
Molecular Genetics Department
Parc de Recerca UAB
Edifici CRAG, Campus UAB
Bellaterra (Cerdanyola del Vallés)
Barcelona, Spain

DAVID W. LITCHFIELD
Departments of Biochemistry and Oncology
Schulich School of Medicine and Dentistry
The University of Western Ontario
London, Ontario, Canada

LAURA MACIAS ALVAREZ
Hematology Oncology
Department of Medicine
Boston University School of Medicine
Boston, MA, USA

DAVID W. MEEK
Division of Cancer Research
Medical Research Institute
The University of Dundee
Ninewells Hospital
Dundee, Scotland, United Kingdom

YOSHIHIKO MIYATA
Department of Cell and Developmental Biology
Graduate School of Biostudies
Kyoto University
Kyoto, Japan

MATHIAS MONTENARH
Medical Biochemistry and Molecular Biology
University of the Saarland
Homburg, Germany

KARSTEN NIEFIND
University of Cologne
Department of Chemistry
Institute of Biochemistry
Cologne, Germany

MONTSERRAT PAGÈS
Center for Research in Agricultural Genomics
Molecular Genetics Department
Parc de Recerca UAB
Edifici CRAG, Campus UAB
Bellaterra (Cerdanyola del Vallés)
Barcelona, Spain

FRANCESCO PIAZZA
Department of Medicine
Haematology and Clinical Immunology Branch
University of Padua School of Medicine
and Venetian Institute of Molecular Medicine
Haematological Malignancies Unit
Padua, Italy

LORENZO A. PINNA
Department of Biomedical Sciences
University of Padua
Padua, Italy

JESUS REVUELTA-CERVANTES
Hematology Oncology
Department of Medicine
Boston University School of Medicine
Boston, MA, USA

MARTA RIERA
Center for Research in Agricultural Genomics
Molecular Genetics Department
Parc de Recerca UAB
Edifici CRAG, Campus UAB
Bellaterra (Cerdanyola del Vallés)
Barcelona, Spain

MARIA RUZZENE
Department of Biomedical Sciences
and Venetian Institute of Molecular Medicine
University of Padua
Padua, Italy

MAURO SALVI
Department of Biomedical Sciences
University of Padua
Padua, Italy

DAVID C. SELDIN
Section of Hematology-Oncology
Department of Medicine
Boston University School of Medicine
and Boston Medical Center
Boston, MA, USA

JANEEN H. TREMBLEY
Research Service, Minneapolis V.A. Health Care System
Department of Laboratory Medicine and Pathology
Masonic Cancer Center
Minneapolis, MN, USA

GRETCHEN M. UNGER
GeneSegues
Chaska, Minnesota

ISABEL CRISTINA VÉLEZ-BERMÚDEZ
Center for Research in Agricultural Genomics
Molecular Genetics Department
Parc de Recerca UAB
Edifici CRAG, Campus UAB
Bellaterra (Cerdanyola del Vallés)
Barcelona, Spain

JING JIANG WU
Research Service, Minneapolis V.A. Health Care System
Department of Laboratory Medicine and Pathology
Minneapolis, MN, USA

PREFACE

A KINASE FOR ALL SEASONS

In the twilight of his scientific life, the Nobel laureate Edwin G. Krebs became more and more attracted by protein kinase CK2. In a 1999 paper (Mol. Cell. Biochem. 191: 3–12), tellingly entitled "CK2, a protein kinase of the next millennium," he wrote that such a title was "intended to emphasize the fact that CK2 is such a rich topic for investigation that research involving this enzyme will continue for decades to come." This statement is one of the justifications for devoting an entire book to an individual member of the human "kinome," a huge gene family including more than 500 enzymes.

Indeed the long history of CK2, from its early—and in some ways "premature"—discovery in 1954 to the present day, is unique and paradoxical in several respects. CK2 activity was the first example of an enzymatic phosphorylation reaction affecting a protein rather than a small metabolite, leading Eugene Kennedy to coin the term "protein (phospho) kinase" (J. Biol. Chem. 211:: 969–980, 1954). For decades, however, and at variance with other protein kinases discovered between 1955 and 1980, notably phophorylase kinase, PKA, PKG, and PKC, which were immediately recognized to participate in signal transduction pathways, the biological role of CK2 remained obscure. Indeed, its physiological targets remained entirely unknown for many years, its activity being measured *in vitro* with proteins that were not its physiological target, such as casein, leading to it being "misnamed" "casein kinase 2," a historical name still hinted at by its current acronym of CK2.

The first physiological targets of CK2 were discovered in the late 1970s, causing CK2 to be independently "re-discovered" by a number of researchers working in different areas, for example as a "glycogen synthase kinase 5" (GSK-5) (Cohen P et al. Eur. J. Biochem. 124: 21–35, 1982) and a "Troponin-T kinase" (Villar-Palasi C et al. J. Biol. Chem. 256:7409–7415, 1981), as has been discussed elsewhere (Pinna LA Cell. Mol. Biol. Res. 40:391–399, 1994). Later, by a remarkable "snowballing" effect, the pleiotropy of CK2 eventually came to surpass that of any other individual protein kinase, with more than 300 substrates identified by 2003 (Meggio F and Pinna LA FASEB J. 17:349368, 2003). However, even this number is a huge underestimate of the total number of CK2 substrates that undoubtedly exist, bearing in mind that recent proteomic analyses have revealed that a large proportion of naturally occurring phosphorylation sites in proteins display the unique acidic motif C-terminal to the phosphorylated residue that is recognized specifically by CK2. This suggests that more than 20% of the entire human phosphoproteome may be generated by this individual protein kinase (see Salvi and Cesaro's discussion in Chapter 3 of this book).

The pleiotropy of CK2 is now considered to be just one facet of this remarkable enzyme, its unique feature being its "constitutive" activity, an intriguing property whose structural basis is discussed by Niefind and Battistutta in the first chapter of

this book. This is in contrast to many other protein kinases, which are silent under basal conditions and only become active in response to specific stimuli. CK2 seems to always be present in cells in an active conformation, without the need of phosphorylation events to sustain its activity. In this respect, it is therefore quite different from kinases that participate in signaling "cascades." However, to exclude CK2 from participation in signaling pathways would be an incorrect inference, contradicted by the overwhelming evidence that CK2 impinges on many signaling pathways, but in a unique "lateral" fashion rather than a "vertical" linear manner (see Chapter 5 by Gabriel and Litchfield and Chapter 11 by Ruzzene in this book).

Constitutive activity also underlies another paradox of CK2: many oncogenes encode protein kinases endowed with inappropriate activity or a gain of function mutation. Although this might appear to exclude CK2 from being an oncogene, since no gain of function mutations have ever been reported, nonetheless CK2 is clearly implicated in many cell biology phenomena that are associated with cancer, and the expression and activity of this protein kinase is invariably high in malignant cells compared to untransformed cells. This issue is dealt with in several chapters of this book. An attractive explanation for this apparent contradiction seems to be that diverse neoplastic cells become "addicted" to abnormally high levels of CK2 to such an extent that pharmacological downregulation of CK2 can reverse the tumorigenic phenotype. There are two important consequences of this situation. Firstly, cells where CK2 is abnormally high are "predisposed" to malignant transformation, thus deserving the neologism "oncophilic" cells (Ruzzene et al. Mol. Cell. Biochem. 356: 5–10, 2011). Secondly, CK2 may represent a pharmacological target for the treatment of a wide range of neoplastic diseases. The structures and mode of binding of several inhibitors in complex with CK2 are described in the first chapter of this book, and a potent and selective CK2 inhibitor is now undergoing clinical trials for the treatment of different kinds of tumors as discussed in detail by Drygin in the last chapter of this book.

Another consequence of the constitutive activity of CK2 is that many viruses and other infectious agents have learned how to exploit its presence in the host cell for the phosphorylation of proteins that are essential to their life cycle. Therefore, CK2 also represents an attractive target for anti-infectious therapies although in contrast to cancer, where a partial downregulation of abnormally high CK2 activity may suffice, the suppression of host cell CK2 activity may have undesired consequences that still have to be evaluated. Other pathologies where an involvement of CK2 is suspected, mostly based on the scrutiny of its protein targets, are neurodegenerative syndromes, cardiovascular diseases, inflammation, and cystic fibrosis as reviewed by Guerra and Issinger (Curr Med Chem. 15:1870–86, 2008). In these cases, however, the roles of CK2 still need to be unravelled, and it is unclear whether any beneficial effects will come from downregulation or upregulation of CK2 activity.

The widespread and continuously increasing interest in CK2 in the scientific community is obvious from even a cursory scrutiny of the literature, the number of paper mentioning "CK2" in their title rising from 94 in 2000, to 159 in 2005, and 329 in 2011. This mainly reflects the increasing numbers of investigators who are inevitably coming across this kinase in the course of their studies. Although the "love affair" of most scientists with CK2 is transient, there remains a hard core group of "CK2 addicted" labs where this topic has been studied for decades, and

this community of CK2 investigators meets periodically to discuss their most recent findings and to try to delineate new perspectives in the field. The first conference was held in Heidelberg, Germany, in 1994, followed by other conferences in Villard de Lans, near Grenoble, France (1997), in San Esteban, Chile (2001), in London, Ontario, Canada (2004), in Padua, Italy (2007), and in Cologne, Germany (2010). These international conferences on CK2 have been sponsored and generously supported by IUBMB. It is therefore not surprising that a book of the Wiley-IUBMB series is now devoted to CK2.

The first part of this book will deal with structural aspects underlying the unique properties of CK2, its specific susceptibility to pharmacological inhibition, and its extraordinary pleiotropy. In the second part, the fundamental role of CK2 in a wide number of biological functions will be illustrated, and the third part will be devoted to the potential roles of CK2 in malignancy, which is providing new strategies and tools to treat neoplasia.

Chapter 1 by Karsten Niefind and Roberto Battistutta provides a thorough and detailed overview of present knowledge about structural features that underlie the enigmatic mode of regulation of CK2 and its susceptibility to a wide spectrum of potent, selective, and cell permeable inhibitors that are invaluable in helping to dissect the cellular functions of this kinase, as well as to counteract its oncogenic role. This theme will be exemplified throughout the book.

Chapters 2 and 3, by Mathias Montenarh and Claudia Götz and by Mauro Salvi and Luca Cesaro, respectively, deal with the pleiotropic nature of CK2 function, by presenting an updated repertoire of its interacting partners and a proteomic analysis that supports the concept that a substantial proportion of the whole human phosphoproteome is generated by this single kinase.

A global view of the biological role of CK2 from both an embryogenetic and phylogenetic standpoint is provided by Isabel Dominguez and collaborators in Chapter 4, where the phenotypes of CK2 deregulation in model organisms, with special reference to yeast, C. elegans, Drosophila, and mouse are described.

Chapter 5 by Michelle Gabriel and David Litchfield mainly focuses on the unusual mode of operation of CK2 in signaling pathways and on devices by which the apparent "lack of control" of CK2 can be overcome. In this connection, special reference is made to "substrate level regulation" mediated by hierarchical phosphorylation.

The next three chapters by David Meek, Yoshihiko Miyata, and Olaf-Georg Issinger and Barbara Guerra, respectively, deal with specific and relevant aspects of CK2 functionality, namely its potential role in the regulation of the tumor suppressor protein p53 (Chapter 6), its role in the Hsp90 chaperone machinery, which is essential for the survival of the "onco-kinome" (Chapter 7), and its involvement in cell survival (Chapter 8).

Chapter 9 by Montserrat Pagès and collaborators is entirely devoted to the distinctive properties of CK2 in plants, where unique structural features of the kinase may reflect roles in a variety of specialized functions.

Chapter 10 is an introduction to the implied involvement of CK2 in neoplasia, where David Seldin and Esther Landesman-Bollag summarize studies that have proved that CK2 has the capability to act as an oncogene. They also show that the overexpression of CK2 is associated with reduced survival and with invasiveness of cancer cells.

In a similar vein, albeit from a different angle, Maria Ruzzene provides evidence in Chapter 11 for a "vicious circle" whereby cells sporadically expressing abnormally high levels of CK2 are predisposed to malignancy if an oncogenic mutation occurs, leading to the selective increase of these cells, which in turn are more susceptible than "normal" cells to the cytotoxic efficacy of CK2 inhibitors.

The concept that malignant cells are more susceptible to loss of CK2 activity than normal cells is also dealt with by Khalil Ahmed and collaborators in Chapter 12, whose important message is that CK2 is deregulated in all cancers examined and that its downregulation results in potent induction of apoptosis. The authors also describe recent progress in targeting CK2 cancer cells in a specific manner, leading to eradication of the cancer.

An overview of the role of CK2 in normal and malignant hematopoiesis is presented by Francesco Piazza in Chapter 13, showing that CK2 is upregulated in a variety of acute and chronic lymphoid and myeloid malignancies and suggesting that this protein kinase could be a suitable therapeutic target in these cases.

The role of CK2 in the progression of breast carcinoma through its control of epithelial cell plasticity is the topic addressed by Claude Cochet, Alexandre Deshiere, and Odile Filhol in Chapter 14, where the authors describe an unbalanced expression of CK2 subunits in a subset of breast tumor samples providing a detailed explanation for the molecular events underlying this process.

In Chapter 15, Denis Drygin provides a thorough and stringent survey of arguments supporting the concept that CK2 is a "logical target" in cancer therapy, especially if its inhibition is combined with chemotherapeutic agents. In that chapter, the efficacy of CK2 inhibitors whose mode of action is detailed at the molecular level in Chapter 1, is highlighted by showing how the "first-in-class" CK2 inhibitors have entered clinical trials. This has demonstrated for the first time that CK2 can be safely and extensively inhibited in humans without unacceptable side effects.

Needless to say, I am enormously grateful to all of the authors for having participated in this editorial enterprise and for having provided such an excellent series of contributions.

I also wish to thank Professor Angelo Azzi, President of the IUBMB Executive Committee, Professor Willy Stalmans, Chairman of the IUBMB Publication Portfolio, and Professor William J. Whelan, Editor-in-Chief, IUBMB Life, for having given me the opportunity to crown my academic career by editing a book devoted entirely to my "favorite" enzyme, which has monopolized my attention for decades and I hope will continue to keep me busy scientifically in the future.

Special thanks also to Justin Jeffryes, Wiley's Executive Editor, for his encouragement and continuous support, to Anna Ehler for her invaluable help in editorial matters, and to Luca Cesaro for his help in collecting and assembling the authors' contributions and for preparing the cover figure of the book.

Lorenzo A. Pinna

THE WILEY-IUBMB SERIES ON BIOCHEMISTRY AND MOLECULAR BIOLOGY

Protein Kinase CK2
Editor: Lorenzo A. Pinna

Part I
Molecular and Structural Aspects

1 Structural Bases of Protein Kinase CK2 Function and Inhibition

KARSTEN NIEFIND[1] AND ROBERTO BATTISTUTTA[2]

[1]University of Cologne, Department of Chemistry, Institute of Biochemistry, Cologne, Germany
[2]Department of Chemical Sciences, University of Padua, Padua, Italy

CONTENTS

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

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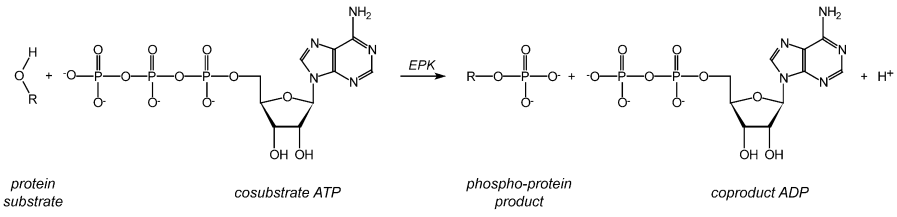

Figure 1.1. Scheme of the reaction catalyzed by a eukaryotic protein kinase (EPK). The reaction is essentially irreversible under physiological conditions. The hydroxy group of the protein substrate belongs to the side chain of serine, threonine, or tyrosine. In the case of CK2, the cosubstrate can be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

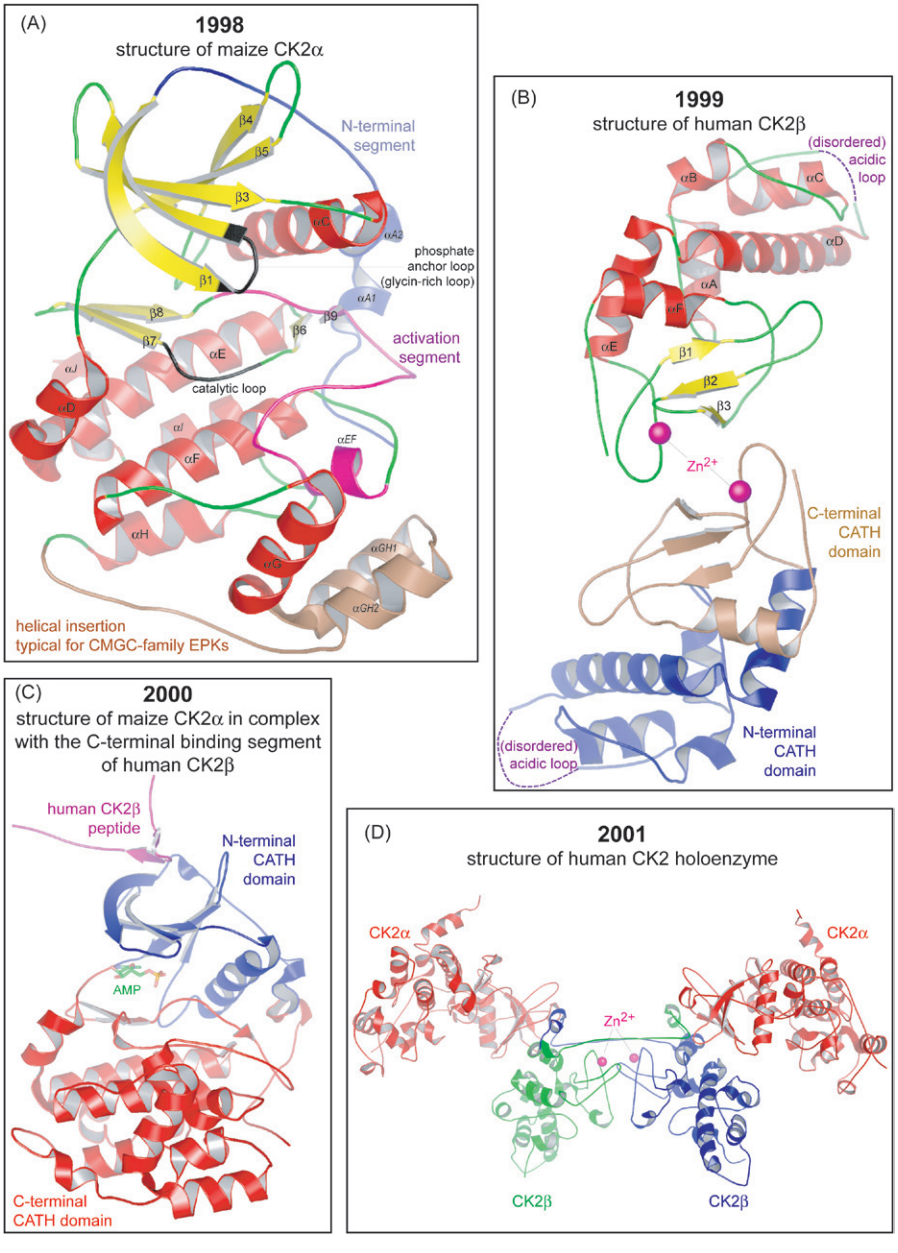

Figure 1.2. Highlights of the initial phase of CK2 structural biology. (A) Structure of CK2α from *Zea mays* (Niefind et al., 1998; PDB 1A6O, later superseded by 1LR4). The most remarkable detail of the structure was the close attachment of the N-terminal segment (blue) with the activation segment (magenta) 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.

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: