Studies on the Differential Specificity of Protein Kinases and Its Applications

BY

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ABSTRACT


Protein kinases are enzymes that catalyse the phosphoryl transfer from the γ-phosphate of ATP to acceptor amino acids in proteins. The specificity of selected model protein kinases was studied at three different levels using a) novel bi-substrate-analogue inhibitors, b) synthetic peptide substrates and c) mutated protein substrate analogues.

A new class of protein kinase bi-substrate-analogue inhibitors was designed on the basis of adenosine-5'-carboxylic acid derivatives, where a short arginine containing peptide was attached to the 5'-carbon atom of the adenosine sugar moiety via a linker chain. These compounds showed high inhibitory potential against two basophilic protein kinases, the protein kinase A (PKA) and protein kinase C (PKC), with IC50 values in the nanomolar range, but no inhibitory activity towards the acidophilic kinases CK1 and CK2. The inhibitors were efficiently applied for affinity purification of PKA using MgATP as well as L-arginine as eluting agents.

Ca\textsuperscript{2+} -dependent protein kinase (CDPK-1) was purified from maize seedlings and its substrate specificity was studied using a set of synthetic peptides. These were derived from the phosphorylatable sequence RVLSRLHS(15)VRER of maize sucrose synthase 2 (SuSy2), and a consensus sequence motif A/LXRXXSXZR (where X denotes a position with no strict amino acid requirements and Z a position strictly not tolerating arginine) was defined from a study using arrays of systematically varied peptides attached to cellulose membrane (SPOTs\textsuperscript{TM} membranes). The SuSy2 derived peptides were also found to be efficient substrates for mammalian PKC, but showed low reactivity in the case of PKA. On the basis of this peptide motif, a positionally oriented peptide library approach based on ESI-MS detection of phosphopeptides in initial velocity conditions was designed for quantitative kinetic characterization of protein kinase specificity profiles. On the basis of the obtained data an optimal peptide substrate for PKC, FRRRRSFRRR, was designed.

The specificity of protein kinase A was studied using site-directed mutagenesis in the phosphorylation site of L-type pyruvate kinase (L-PK), and comparison of the obtained data with the data from previous studies on structurally altered peptide substrates revealed that amino acid alterations in short peptide substrates cause stronger effects on the phosphorylation rate than the corresponding alterations in the protein substrate L-PK.

Key words: Protein kinases, protein kinase inhibitors, substrate specificity, peptide libraries, affinity chromatography.

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V. Loog, M., Oskolkov, N., O'Farrell, F., Järv, J., and Ek, P. Substrate specificity of protein kinase A in reaction with protein and peptide substrates. Manuscript

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<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
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<tr>
<td>CaMKII</td>
<td>calcium- and calmodulin-dependent protein kinase</td>
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<td>cAMP</td>
<td>cyclic AMP</td>
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<td>CDK</td>
<td>cyclin-dependent protein kinase</td>
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<td>CDPK</td>
<td>calcium-dependent protein kinase</td>
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<td>CK1</td>
<td>casein kinase 1</td>
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<tr>
<td>CK2</td>
<td>protein kinase CK2, formerly casein kinase II</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>ESI-MS</td>
<td>electrospray mass-spectrometry</td>
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<td>GSK</td>
<td>glycogen synthase kinase</td>
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<td>L-PK</td>
<td>L-type pyruvate kinase</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MLCK</td>
<td>myelin light-chain kinase</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
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<tr>
<td>PKA</td>
<td>protein kinase A, cAMP-dependent protein kinase</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
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<td>PKI</td>
<td>heat-stable protein kinase inhibitor</td>
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<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>RACK</td>
<td>receptor for activated C-kinase</td>
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Introduction

Protein kinases are enzymes which catalyse the phosphorylation of substrate proteins by transfer of the $\gamma$-phosphate of ATP to the acceptor amino acid. Depending on the acceptor amino acids, the protein kinases can be classified as either serine/threonine kinases or tyrosine kinases, which are the two most important and numerous subdivisions. This introduction aims to give an overview of the important aspects of the specificity of serine/threonine protein kinases, with emphasis on the examples of two thoroughly studied model enzymes: protein kinase A (PKA), and protein kinase C (PKC).

The phosphorylation modulates the activity of many proteins and protein kinases play a key role in multiple signalling and regulatory processes in cell. It has been estimated that as much as 20-50% of all the cellular proteins undergo phosphorylation \textit{in vivo} [124, 173]. The regulation of protein activities by phosphorylation is a reversible process, with the reverse direction catalysed by protein phosphatases. The extracellular signals are relayed from the plasma membrane to specific intracellular targets mostly through several phosphorylation events catalysed by different protein kinases, each of which, in order to maintain the exactness of the signalling flux, should possess individual target recognition specificity and localisation specificity. There is a great diversity of protein kinase forms involved in eukaryote signalling and according to the preliminary estimation of Celera’s human genome project, the human genome codes for 868 protein kinases [168], corresponding to 2.8% of all genes, which makes this the largest enzyme family in eukaryotic cells.

Ubiquitous involvement of protein kinases in the regulation of important processes including cell growth, differentiation and apoptosis, associates them with many diseases. Abnormal protein kinase activities have been identified as key factors in several cancers [76, 86]. These findings have validated several protein kinases as potential drug targets and the design of specific protein kinase inhibitors presents a promising field of therapeutic intervention with diseases [5, 10, 147].

The functioning of protein kinases as “molecular switches” in checkpoints of cellular signalling also makes these enzymes attractive as biomarkers. Recently it has been shown that differential protein kinase expression patterns can reflect the molecular phenotype of different disease states [14, 15, 48, 80, 135]. Determination of protein kinase activity profiles in normal and diseased cells as fingerprints of the signalling status of the cell may lead to important medical applications including molecular classification and diagnosis of disease, design of individual treatment strategy, identification of therapeutic markers and drug targets, and profiling of response to toxins and pharmaceuticals.

Despite the large amount of sequence data revealing the existence of hundreds of protein kinase forms, still only a small fraction of these enzymes have been characterised at the protein level and the substrate specificity has been investigated for fewer still. Understanding the subtle differences in specificity requirements for substrate binding regions of different representatives of this conserved family of enzymes would facilitate the design of selective inhibitors, which can be considered as potential drugs.
There is also a great demand for selective inhibitors in the field of signal transduction research, where these compounds can be used for dissecting the signalling pathways mediated by phosphorylation of particular kinases and thereby unravelling the signalling networks. Secondly, the understanding of the structural requirements for substrates of protein kinases leads to the design of selective and highly efficient peptide substrates for detection of individual protein kinase activities in cells. The latter direction is particularly interesting in the light of the promising new trends in developing protein kinase chips for high throughput simultaneous kinase activity screening [87, 173, 186].

New specific ligands are also needed for development of group specific affinity isolation methods for protein kinases. Protein kinases as regulatory catalyst molecules have low abundance in cells and the methods for their enrichment using specific affinity isolation methods are therefore in great demand in field of proteomics research. Such affinity adsorbents shorten the gap between genomics and proteomics by providing the possibilities to study kinase function at protein level.

The current thesis focuses on the questions of phosphorylation site-specificity of peptide and protein substrates, and also on the possibilities and strategies for developing highly efficient inhibitors and affinity ligands for protein kinases. Since the research for achieving these goals can be substantially accelerated by development of novel combinatorial library methods and rational ligand design strategies, proportionally more emphasis is paid to these questions.

1. The protein kinase superfamily

The classification of protein kinases and the definition of the eukaryotic protein kinase superfamily were done by Hanks, Quinn and Hunter [40]. The common motif which all protein kinases share and are related by is the homologous “kinase domain”, also known as the “catalytic domain”, which consists of 250-300 amino acid residues. The kinase domain contains 12 conserved subdomains and it folds to closely similar three-dimensional core structures in different protein kinases (reviewed in [38, 39, 153]). The classification given by Hanks et al. [40] was based one the phylogenetic trees derived from an alignment of kinase domain amino acid sequences. It was found that the sequence similarity of the kinase domains was a good indicator of other characteristics common to different kinases, revealing four main families with related substrate specificities and modes of regulation. The rest of the kinases falling outside these major groups were difficult to classify into defined subsections. The four groups are shown below together with the list of the few representatives which are more frequently referred to in subsequent chapters of this thesis:

1) The AGC group units protein kinases with mainly basic amino acid specificity determinants including the cyclic nucleotide-regulated protein kinase family with the well known representatives PKA and PKG,
diacylglycerol-activated/phospholipid-dependent protein kinase C family and the RAC family of kinases
2) The CaMK group covers the family of kinases regulated by Ca$^{2+}$/calmodulin. The plant Ca$^{2+}$-dependent protein kinases, studied in one of the papers of this thesis also belong to this group
3) The CMGC group includes the family of cyclin-dependent kinases (Cdks), the MAP kinase family and casein kinase II
4) The PTK group: the protein-tyrosine kinase group

The first 3-dimentional structure of a protein kinase was the crystal structure of the catalytic subunit of protein kinase A (cAMP-dependent protein kinase, PKA) in complex with its peptide inhibitor PKI and MgATP, solved in 1991[79]. Later additional structures of PKA in different ligand complexes were solved [24, 106-108, 126, 184, 185] and PKA has become the most thoroughly studied representative of the protein kinase superfamily serving as a model system for research on newly identified and isolated protein kinases.

Protein kinases have been identified and characterised from other animal phyla, besides the mammals, including other vertebrates, as well as plants, fungi and microorganisms, and the comparative studies have demonstrated that the basic aspects of protein phosphorylation pathways regulating the cell functions have been maintained throughout the course of eukaryotic evolution [38].

2. The catalytic domain of protein kinase A as a model for the protein kinase superfamily

PKA is the most investigated and understood of all the protein kinases of the superfamily and many general rules derived from these extensive structural and kinetic studies will certainly apply to the entire protein kinase superfamily. This protein kinase is unique due to its dissociative mechanism of activation in response to the second messenger cyclic AMP (cAMP). The inactive holoenzyme of PKA is a tetramer consisting of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits in the holoenzyme leads to its dissociation into two active catalytic subunits and a dimer of regulatory subunits [151]:

$$R_2C_2 + 4 \text{ cAMP} \rightarrow R_2(\text{cAMP})_4 + 2 \text{ C}$$

There are three known genes that code for the catalytic subunit. The Cα is the predominant form and is expressed in most tissues [162]. The C β is more tissue specific, being highly expressed in neuronal tissues [9, 140, 163] and the Cγ has been found in testis and its expression is most limited [3].

The schematic representation of the catalytic and regulatory subunit structure is given in Fig. 1. The general three-dimensional architecture of the catalytic subunit is
Figure 1. Schematic view of the catalytic subunit and regulatory subunit of PKA.

shown in form of the ribbon diagram in Fig. 2, as a ternary complex, where the recombinant catalytic subunit was co-crystallized with MgATP and a 20-residue peptide inhibitor PKI(5-24) [79]. The catalytic subunit consists of a smaller upper lobe and a larger lower lobe. The small lobe is dominated by antiparallel β-sheets and is responsible for ATP binding. The loop between the beta sheets 1 and 2 of the small lobe, encompassing the signature motif Gly50-X-Gly52-X-X-Gly55, is called the phosphate anchor since it is binding the phosphate groups of ATP via several hydrogen bonds [6, 184, 185]. The third beta strand contains a lysine residue (Lys72), which is conserved throughout the protein kinase superfamily. This lysine coordinates the α- and β-phosphates of the ATP and also forms a salt bridge with Glu91 located in the middle of helix C. The latter interaction is important for maintaining the active state in case of other studied protein kinases [56, 64]. The adenine ring of ATP binds in a confined, mostly hydrophobic pocket, located in the cleft under the β strands 1 and 2. Most of the interactions of ATP, except the contacts with the γ-phosphate and the hydrophobic interactions with the adenine ring, are associated with the small lobe.

The large lobe is predominantly α-helical and is associated with catalysis and peptide binding. An important structural element of the large lobe is the catalytic loop connecting the β strands 6 and 7. The loop is preceded by the conserved Arg165, the side chain of which forms a tight electrostatic contact with phosphorylated Thr197. This
Figure 2. Ribbon diagram of PKA catalytic subunit cocrystallized with MgATP and peptide inhibitor PKI[5-24].
interaction is essential for maintaining the active conformation of the kinase. The neighbouring amino acid, the conserved Asp166, is proposed to act as catalytic base in the reaction of phosphoryl transfer [95].

β-strand 8 is followed by a conserved Asp184-Phe185-Gly186 (DFG) motif or the Mg\(^{2+}\) binding loop. The Asp184, correctly positioned by the anchoring hydrophobic interactions of the neighbouring phenylalanine, is one of the ligands of the magnesium ion coordination sphere, which in turn coordinates the β- and γ-phosphates of ATP. The DFG motif is followed by β-strand 9 and the activation loop. The activation loop contains the previously mentioned Thr197, which phosphorylation by autophosphorylation or the PDK1 kinase [13] is essential for the activity of PKA. Most protein kinases possess threonine or tyrosine residues in the activation loop that must be phosphorylated, either autocatalytically or by an activating kinase in order to be active (reviewed in [66]). The three additional residues in the large lobe of the kinase core, which are conserved throughout the superfamily act as general stabilizers of the structure. The Asp220 forms hydrogen bonds to the backbone nitrogens of Arg165 and Tyr164, and thereby stabilizes the catalytic loop, while Glu208 and Arg280 form a buried ion pair [77].

The large lobe also contains the binding region of the peptide and protein substrates, whose structural details determine the peptide specificity of protein kinases and thus are particularly interesting in relation to the practical aims of the current thesis. The specific binding of peptide pseudosubstrate inhibitor TTYADFIASGRTRRRNA\(^*\)IHD, or PKI(5-24), is facilitated by several specific contacts between consensus motif amino acid side chains, highlighted in bold in the preceding sequence, and the amino acids in the binding site at the catalytic subunit. In the current thesis we have adopted a nomenclature that designates the phosphorylation site as P-site. In the case of PKI the P-site is occupied by a non-phosphoacceptor amino acid alanine (*). The amino acids carboxy-terminal to this site are denoted as plus and the amino terminal residues as minus. The residues that are important for recognition of the main basic specificity determinants in PKI and peptide substrates are Glu170 and Glu230 for P-2 arginine, Asp328 and Glu127 for P-3 arginine, Glu203 and Tyr204 for P-6 arginine. The hydrophobic pocket accommodating the side chain of P+1 Ile is formed by Leu198, Pro202 and Leu205 of the P+1 loop, which follows the activation loop. The phenylalanine of P-11 located in the N-terminal amphipathic helix, a critical residue responsible for high affinity binding of PKI, lies in the hydrophobic pocket that consists of Tyr235, Pro236 and Phe239.

The kinetic mechanism of PKA-catalysed phosphorylation of peptide substrates is formally random sequential, but under standard assay conditions and presumably also in physiological conditions, the mechanism is predominantly an ordered sequential steady-state, with ATP binding first [1]. In the same study it was found that \(k_{on}\) was very sensitive to viscosity, indicating that ADP release and not the chemical step is rate limiting.
3. Regulation of protein kinase activities

There are several control mechanisms for protein kinase catalytic activity. The first and the most essential is the control by additional subunits or domains that may function in response to second messengers. PKA is a specific example of regulation of a protein kinase by regulatory subunits. The regulatory subunit contains a pseudosubstrate type sequence with the substrate consensus sequence RRGAI, which at low levels of cAMP occupies the substrate binding site and thereby hinders the binding of the substrate. So far, both the catalytic subunit and the regulatory subunit of PKA have been crystallized but the absence of a crystal structure for the regulatory and catalytic subunit complex hampers the precise determination of the structural basis of the regulatory mechanism. Downregulation by the pseudosubstrate regions is also the case for the regulation by regulatory domains, with the simple difference that the pseudosubstrate motif in these cases is found in the same polypeptide chain as the catalytic domain. The two characteristic examples of this type of regulation, are the protein kinase C (PKC) and calcium dependent protein kinase (CDPK) from plants.

Another type of regulation is the control mechanism by additional subunits whose level of expression varies depending on the functional state of the cell. Examples of such regulation are the cyclin-dependent kinases, activated through binding of different cyclins and thereby controlling the timing of the cell cycle progression. The crystal structures of inactive cyclin-dependent protein kinase 2 (cdk2) [20] and the same enzyme activated by cyclin A have been solved explaining the control mechanism is sufficient detail [64].

The third most important type of regulation is the regulation by phosphorylation of the activation loop. In PKA and also several other studied kinases, a significant component of activation comes from the interaction of basic residues of catalytic loop with a phosphate covalently attached to the activation loop. In case of PKA this absolutely essential phosphorylation occurs at the Thr 197 residue. In other kinases several individual varieties of this regulatory mechanisms have been discovered (reviewed in [66]).

4. Protein kinase C

Protein kinase C (PKC) was initially discovered as a histone kinase which could be activated by limited proteolysis by a Ca\(^{2+}\)-activated protease [61]. The protease cleaves off the regulatory domain of the inactive enzyme releasing the active catalytic kinase core. Later it was found that the discovered kinase could also be activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca\(^{2+}\)-dependent manner and additionally by tumor promoting phorbol esters such as PMA (reviewed in [111]). Until now, a number of PKC isoforms have been discovered, defining a PKC subfamily, consisting of at least twelve distinct genes [100]. There are three main groups in this subfamily, the conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs.
The most studied and understood of them are the conventional PKCs, which comprise of the α, βI, βII and γ isoforms and are activated by Ca\(^{2+}/PS\) and also by binding of DAG. The PKC\(β\) gene is alternatively spliced yielding the two isoforms, which differ only in their C-terminal ends. The nPKCs, consist of the ε, δ, η and θ isoforms and are Ca\(^{2+}\) insensitive but activated by DAG or phorbol esters in the presence of PS [118]. The aPKC (isoforms 1 and ζ) are both insensitive to Ca\(^{2+}\) and PMA/DAG [119].

The schematic view of the functional domain building blocks for cPKC group is given in Fig. 3. For cPKCs, the N-terminal regulatory domain starts with the pseudosubstrate sequence followed by a cystein-rich C1 domain, which contains two repeated zink-finger motifs and has been shown to accommodate the binding of phorbol ester [117] and DAG [137]. The C1 domain is present also in nPKCs, but the aPKCs contain only one zink-finger motif. The C2 domain that follows the C1 domain confers Ca\(^{2+}/PS\) binding to the cPKCs. Initially it was thought that there was no C2 domain in nPKC and aPKC, but later analysis showed that the N-terminal regions preceding the C1 domain, or the single zink-finger motif in the case of aPKC, reveal homology with the C2 domains, but lack certain conserved aspartate residues, which explains why these PKCs are incapable to bind Ca\(^{2+}\) but are still stimulated by PS [100].

PKCs have a multitude of substrates in cells and the general physiological pathway for cPKCs and nPKCs starts with the stimulation of phospholipase C activity by particular cell surface receptors, which is followed by the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns4,5P\(_2\)) yielding DAG and as a consequence activated PKC. Upon activation, DAG binds to the PKC/Ca\(^{2+}\)/phospholipid unproductive complex, formed by association with the cell membrane (or vesicle), forming the tight active ternary complex, where the pseudosubstrate region is removed from the active site groove [122].

The kinase core of mammalian PKCs shares identity in the functionally important conserved residues listed for PKA above, except for several differences in amino acids located in the substrate peptide binding pockets, these being the structural
origin of the differential substrate specificity of these two kinase groups. Within the PKC family, the substrate specificity requirements are broadly overlapping due to high homology of the kinase core [47], and therefore the complete understanding of the specificity factors governing the isoenzyme specific signalling is not yet completely attained.

Figure 4. Schematic view of the CDPK structure. The calmodulin-like domain contains four calcium-binding EF-hand motifs.

5. Calcium-dependent protein kinase from plants

Calcium-dependent protein kinases (CDPKs) form a family of protein kinases in plants [42, 130]. The molecule of CDPK consists of a single polypeptide with a typical protein kinase catalytic core, a linker region containing the pseudosubstrate autoinhibitory sequence and an adjoining calmodulin-like domain with four calcium-binding EF-hand motifs (Fig. 4). CDPK activity is regulated by cytosolic Ca\(^{2+}\), which act, similarly to mammalian cells, as second messengers in plants.

The CDPKs belong to the CaMK group of the eukaryotic protein kinase superfamily, where they form a large family together with Ca\(^{2+}\)/calmodulin-activated protein kinases including CaMKII, CaMKIV, PhK-γ, SmMLCK and SkMLCK [38]. The physiological roles and protein targets for CDPKs have yet been poorly documented, however a few reports prove their involvement in such central mechanisms as stress signal transduction, regulation of cell growth, metabolism and gene expression [22, 57, 139]. As one of the putative physiological roles for CDPK, also studied by our group, is the regulatory phosphorylation of one of the key enzymes of sucrose metabolism in maize, the sucrose synthase 2, which is phosphorylated \emph{in vivo} on its Ser15 by a CDPK from maize seedlings [58, 91].

A number of recent discoveries have revealed a complex isoenzyme pattern of CDPKs expressed in plant cells and many of these closely related kinase forms have been isolated or cloned from a number of plant sources [7, 23, 25, 62]. The high degree
of the isoform versatility indicates to a sophisticated network where one of the key factors maintaining the exactness of signal transduction must be the substrate specificity of particular isoenzymes.

An interesting aspect making the CDPKs attractive for research is its functional similarity to mammalian PKCs. There are no structural PKC analogues found in genomic data on plant model organisms and therefore it has been proposed that CDPKs may be the functional analogues of PKCs in plants. CDPKs, similarly to PKCs, are regulated by intracellular Ca$^{2+}$ signals. But strikingly, there is also experimental evidence that some of the CDPK forms are additionally activated by phospholipids, making them candidate mediators of the inositol phospholipid-based signalling in plants [149].

6. Studies on the substrate specificity of protein kinases

Substrate specificity of protein kinases is controlled on three main structural levels. Firstly, most of the protein kinases exhibit a certain degree of site-specificity, which means that they are able to recognize primary structure motifs in the vicinity of the phosphoacceptor amino acid. Secondly, the recognition of a substrate protein may be mediated by a high-affinity interaction between a site of the kinase other than the P-site accommodating region, and a short sequence of the substrate protein called “docking site”. Thirdly, the targeting of a kinase to its substrate may be achieved by specific anchoring protein(s) containing “anchoring motif”, which binds the kinase, and the “targeting domain”, which directs the subcellular localisation of the kinase to the vicinity of its substrate. A protein kinase may use all of these three levels of recognition for different phosphorylation events, but a certain degree of phosphorylation site specificity is the minimum necessary factor needed for correct docking and positioning of the phosphoacceptor residue into the active site. In many cases the other two levels are not absolutely required to be present, but their presence may decrease the importance for the substrate to possess the full P-site recognition consensus motif. In the present thesis, emphasis is put on the study of phosphorylation site primary structure specificity factors of different model protein kinases.

6.1. Phosphorylation site specificity of protein kinases

Study of the phosphorylation site specificity of protein kinases started about 40 years ago with phosphorylase kinase, which was shown to phosphorylate both phosphorylase [28] at Ser14 and the chymotryptic peptide containing the same site [112]. Due to growing evidence that, in a number of cases, synthetic peptide substrates based on the phosphorylation site sequence in proteins, showed $K_m$ values in the same range or even lower than their protein progenitors, a series of studies was conducted in order to
characterise the primary structure preferences for different kinases (reviewed in [72, 124, 183]). The overview of the specificity studies given below is mainly focused on PKA and PKC, since these two kinases are the model systems used in the current thesis work. The current knowledge on the phosphorylation site-specificity consensus sequences of other protein kinases are taken together in Table 1.

6.1.1. Structurally varied peptides for study of substrate specificity of protein kinases

The first systematic studies using structurally varied synthetic peptide substrates, based on the sequence of identified phosphorylation sites in protein substrates were performed for PKA [27, 70, 98, 181, 182]. It was found that the short peptide substrates based on the phosphorylation site of L-type pyruvate kinase were just as good substrates or even better than the pyruvate kinase itself and it was found that the minimal short sequence RRASV was still efficiently phosphorylated by PKA with \( K_m \) in the low micromolar range [182]. Further shortening of this structure led to dramatic decreases in reactivity [182]. Thus the structure RRASV, was denoted “minimal substrate” for PKA, i.e. the shortest substrate structure containing the whole set of important specificity determinants for efficient phosphorylation by the protein kinase. The two arginines were found to be exclusive substrate specificity determinants for recognition by PKA since they could not be replaced by even lysine without serious detriment to substrate efficiency [70]. These studies resulted in the definition of a minimal consensus sequence RRXS/TB, for PKA, where X denoted any amino acid and B is a large hydrophobic amino acid [74]. More recently, the determination of three-dimensional structures of PKA-pseudosubstrate inhibitor peptide complexes [152, 161] have shed light on the peptide recognition mechanism of PKA in atomic detail and the system has become a model of substrate recognition for the whole protein kinase superfamily.

Extensive studies using peptide substrates have also been performed on PKC (reviewed in [47]), however, the site-specificity of this kinase was not as defined as for PKA. The studies resulted in definition of several possible consensus sequences (Table 1), the most frequent of which was RXXS/TXR. In addition, a favourable role of hydrophobic residue in position +1 was found [176], indicating the similarity to PKA, but later systematic studies showed that also basic amino acids are specificity determinants in the position P+1 [68].

However, despite of these extensive efforts, no systematic positional analysis of the structural impact of each possible amino acid on protein kinase substrate reactivity was done until the early nineties. The attempt to rationalise and systematize the collected peptide data was performed for PKA by Järv and Ragnarsson [67]. In this study the amino acid positions from –3 to +2 around the serine in synthetic peptide analogues were analysed by linear free energy relationships. Different structural parameters for each amino acid side chain were correlated with logarithmic values of kinetic constants obtained for the positionally varied peptide analogues and the
structural specificity of each position was characterized by the intensity factors obtained from these correlations. A similar approach was used, and developed further in two studies where the structural specificity of PKC and PKA was studied using the peptide analogues derived from a phosphorylation site of myelin basic protein, varied in position P+1 [68, 92]. It was found that the specificity requirements of these kinases in position P+1 could be quantitatively described by the obtained correlations. Hydrophobicity, bulkiness and charge were all important factors for recognition by PKC while only the hydrophobicity and bulkiness were important for PKA. From these studies it was understood that it is not possible to express the primary structure site specificity of protein kinases by a defined and rigid consensus sequence, since it may have a far more complex character, involving different structural factors, which may have positive or negative influences on substrate reactivity.

6.1.2. Use of peptide libraries for study of substrate specificity of protein kinases

Despite the extensive knowledge obtained from the studies described above, the approach using the separately synthesized peptide substrate models for determination of specificity requirements for protein kinases is time consuming and costly. As an alternative, in the last decade, different combinatorial peptide libraries techniques were applied for determination of the consensus sequences and optimal substrates for several protein kinases [11, 12, 59, 82, 84, 85, 94, 109, 110, 114, 116, 141-145, 154, 156, 177, 178, 180]. These innovations were introduced to the protein kinase studies following the development of automated combinatorial peptide synthesis methods [32] and successful applications of peptide libraries for development of new bioactive peptides [51, 52]. The pioneering attempt to apply these techniques for study of kinase recognition motifs was done by Songyang and co-workers, where the new strategy was used to determine optimal sequences of phosphopeptides recognised by SH2 domains [146]. One of the first studies on the protein kinase substrate specificity was done by the same group for PKA, cyclin B-Cdc2, cyclin A CDK2 and SLK1 kinases, using a random peptide library with sequence M AXXXXSXXXXAKKK, where X indicates all amino acids except tryptophan, cystein, tyrosine, serine and threonine [143]. The methodology developed in this work has become a standard protocol for several later kinase specificity studies [109, 110, 114, 116, 141, 142, 144, 145]. According to this protocol, after partial phosphorylation of the library, the phosphopeptides are isolated using Fe-chelating chromatography and submitted to peptide sequencing. The specificity profiles are constructed using the relative abundances of each amino acid in each subsequent positions. The optimal peptide consensus motifs obtained in these studies were generally in agreement with the known phosphorylation sites in proteins, showing that such library methods can be used for qualitative substrate specificity studies.

In one of these studies the substrate specificity profiles for PKC isoenzymes were investigated [110] using the positionally oriented (fixed phosphorylation site) library with general structure MAXXXXRXXSXXXXAKKK. The arginine was
“locked in” since previous studies had shown the importance of Arg at P-3. This detailed study allowed determination of the optimal substrate sequences for nine PKC isoenzymes. It was also possible to design optimal peptide substrates, which showed a degree of selectivity between the isoenzymes, rebutting the standard opinion that the isoenzymes of PKC are too homologous to allow discrimination between them using peptide substrates. However, certain disadvantages of these random libraries are evident when the specificity diagrams are examined. The specificity profiles in several positions do not show strict structural specificity, i.e. in several positions the amino acids of different chemical nature show only minor differences in relative abundance, likely indicating that the specificity is not strictly determined by defined binding pockets on the enzyme molecule. The reason for this may be that the assay of such a large library was not made under the initial velocity conditions, leading to library “overphosphorylation”. Application of the initial velocity conditions is impossible due to low sensitivity and separation efficiency of the detection methods used. It could be proposed that the correct sensitivity of the positional structure-activity relationships can be achieved by using the step-by-step library design where every generation of libraries has only a subset of amino acid positions varied. This can be combined with the rational design of libraries, where the structural variations are diminished to a minimum, but still cover a sufficiently wide range of structural details.

Another drawback, affecting the random library study in the opposite way, was pointed out by Pinna [124]. It was shown that the results from the library study for PKA [143] revealed 13 fold preference for arginine over alanine in position P–1, while the same substitution performed on individual peptide analogues showed almost equal reactivity for the two peptides. This was explained by the situation where the arginine in P–1 position, in course of library phosphorylation starts to compensate the absence of arginines in positions P-2 and P-3 and therefore becomes more abundant than the P-1 noncharged alanine in the collected phosphopeptide pool.

As an alternative to the soluble peptide library strategies, peptide libraries attached to solid matrix have been developed. In the first attempt at this approach, random synthetic combinatorial peptide libraries were generated by the split peptide synthesis on polystyrene beads of 90 μm, where each bead expressed only one peptide entry [177]. This "one bead one peptide" approach, also called the Selectide Process, had been previously described in [83] as a general method for peptide ligand search. Using this method, several optimal substrates for PKA were “fished out” from the large pool of 500000 beads with random hexapeptide sequences. These substrates contained the PKA consensus sequence RRXS. However, the shortcoming of this approach is that only few optimal sequences are selected and no real knowledge about the detailed positional structure-activity relationships and of the specificity profiles can be obtained.

Another powerful method was developed, where peptide libraries were simultaneously and separately synthesised on cellulose membranes as SPOTs™ peptides [29]. The successful utilisation of the SPOTs peptides for protein kinase assay was demonstrated in our laboratory [157] and it was shown that this method was efficient and less time consuming for selection of optimal peptide substrate for PKC from a number of candidate sequences based on the substrate sites of proteins [158].
more systematic approach for determination of kinase specificity profiles using the SPOTs peptide arrays, was developed by Tegge et al. [154], synthesizing several generations of libraries. The first generation had the structure AcXXX12XXX, where X represents mixtures of all twenty natural amino acids and the numbers 1 and 2 represent individual amino acids, each fixed on a separate spot. After phosphorylation by [γ-32P]ATP the sublibraries AcXXXRRXX and AcXXXRKXXX were identified as the best substrates for PKA and PKG. In the next library generations, these two positions were fixed and the rest of the positions of the octapeptides were analysed in a similar way resulting in the optimal peptide structures KRAERKASIY and TQKARKKSNA for PKA and PKG, respectively, with K_m values in low micromolar range when measured in solution. However, this method suffers from the relatively high background binding of [γ-32P] ATP (unpublished results from our laboratory) and also due to the possibility of formation of inhibitor structures causing the affinity docking of the enzyme to the spot area. Furthermore, the kinetic meaning of the radioactive spot intensities remains unclear, as the values did not correspond to the K_m values estimated for the peptides in solution [157].

In general, the following different aims in the peptide library studies on protein kinase specificity can be outlined:

- First, selection of some of the most optimal substrate structures from the large pool of possible substrate sequences.
- Secondly, determination of the substrate specificity profiles, where structure-activity relationships for each critical position around the phosphorylatable amino acid are obtained. This approach provides detailed knowledge about the specificity requirements of closely related kinase forms and thus can be used for design of selective substrates discriminating between different kinase forms.
- Thirdly, the obtained consensus motifs may be valuable in predicting the potential phosphorylation sites using protein sequence databases [69, 114, 142, 143] However, this perspective must be analysed separately, because of the known flexibility of the consensus motifs [81] and also in respect to the question how the sites predicted and tested in vitro, reflect the real physiological sites in vivo.

6.2. Overview of the substrate specificity of serine/threonine protein kinases

With few exceptions, most of the studied serine/threonine kinases are markedly “sequence specific”. The serine/threonine kinases can be categorized into three main groups with respect to their specificity determinants [124],

- basophilic kinases, using basic and often also hydrophobic residues as specificity determinants
- proline-directed kinases, some of which also require basic residues besides proline and
Table 1.
Phosphorylation specificity consensus motifs of serine/threonine protein kinases. The table is based on reviews [123, 124] and peptide library studies [114, 145]. B denotes the hydrophobic amino acid, Z any amino acid except arginine and X any possible amino acid. The phosphoacceptor residues are underlined.

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basophilic protein kinases</strong></td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td>R-(R/K)-X-S/T-B</td>
</tr>
<tr>
<td>PKG</td>
<td>R-(R/K)-X-S/T-B</td>
</tr>
<tr>
<td></td>
<td>(R/K)-(R/K)-X-S/T-B-(R/K)-(R/K)-(X)<em>{2,0}-S/T-(X)</em>{2,0}-(R/K)_{1,3}</td>
</tr>
<tr>
<td>AKT/PKB kinase</td>
<td>R-X-R-X-S/T</td>
</tr>
<tr>
<td>CaM kinase I</td>
<td>B-X-R-X-X-S/T-X-X-S/T-B</td>
</tr>
<tr>
<td>CaM kinase II</td>
<td>B-X-(R/K)-X-X-S/T-B</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>K/R-X-X-S-B</td>
</tr>
<tr>
<td>CDPK maize</td>
<td>L/A-X-R-X-X-S/F-X-X-X-R-Z-R</td>
</tr>
<tr>
<td>sm MLCK</td>
<td>K-K-R-X-X-S-X-B</td>
</tr>
<tr>
<td>HSV protein kinase</td>
<td>R-R-R-R-X-S/T-X</td>
</tr>
<tr>
<td>P70^{6k}</td>
<td>(K/R)-X-R-X-X-S/T-B</td>
</tr>
<tr>
<td>MAPKAP kinase 2</td>
<td>X-X-B-X-R-X-X-S-X-X-S-X</td>
</tr>
<tr>
<td>NIMA</td>
<td>F-R-X-S-R/B</td>
</tr>
<tr>
<td>HRI (Haem-regulated eIF-2α) kinase</td>
<td>(E)-X-S-R-X-X-R</td>
</tr>
<tr>
<td><strong>Proline-directed protein kinases</strong></td>
<td></td>
</tr>
<tr>
<td>Cdc2</td>
<td>X-S/T-P-X-K/R</td>
</tr>
<tr>
<td>CDK2</td>
<td>X-S/T-P-X-K/R</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>P-X_{1,2}S_{1,2}-T-P-P</td>
</tr>
<tr>
<td><strong>Acidophilic protein kinases</strong></td>
<td></td>
</tr>
<tr>
<td>CK1</td>
<td>(Sp/Tp)-X-X-(X)-S/T-B</td>
</tr>
<tr>
<td></td>
<td>(D/E)_{2,0}-X-X-S/T-B</td>
</tr>
<tr>
<td></td>
<td>X is preferably acidic</td>
</tr>
<tr>
<td>GSK3</td>
<td>S/T-X-X-X-Sp</td>
</tr>
<tr>
<td>βARK</td>
<td>(D/E)_{n}S/T-X</td>
</tr>
</tbody>
</table>
• acidophilic/phosphate directed kinases, which require carboxylic and phosphorylated residues.

A short overview of the consensus sequences of these three specificity groups is given below, and more precisely taken together in Table 1.

6.2.1. Basophilic protein kinases

This group is comprised mainly of members of ACG and CaMK groups of protein kinases and also of several kinases not falling into major groups according to the Hanks and Hunter nomenclature [38]. All three model enzymes used in the current work: PKA, PKC, and plant CDPK, are representatives of this specificity group. By examination of the determined consensus sequences collected in Table 1, it can be concluded that the most conserved basophilic determinant is the requirement of a basic amino acid, and mostly arginine, in position –3. Secondly, hydrophobic residues are outlined as important specificity determinants for basophilic kinases, and most importantly requirement for the large hydrophobic residue in position +1. Alternatively, the hydrophobic residues can be determinants, which provide selectivity between closely related basophilic kinase forms, as has been shown in the case of PKA and PKG [16]. In this study it was found that the phenylalanine in position P+4 was a detrimental factor for PKG, but not for PKA, being thus an illuminating example of the promising and still hidden opportunities for selective substrate design for closely related kinases.

As a negative determinant for the basophilic group of kinases, the proline in position P+1 should be mentioned. This crucially required determinant for the second kinase specificity group overviewed below, is shown to be universally detrimental for basophilic kinases [124].

6.2.2. Proline-directed protein kinases

The two main proline-directed kinase groups are the cyclin-dependent protein kinases and the MAP kinases, both belonging to the CMGC group of protein kinase superfamily. As the general substrate motif for both subgroups is Ser/Thr-Pro, the CDK kinases additionally require the basic residues around the phosphorylation site. For MAP kinases a second proline is often present in position P-2 [35] or even the third at position P+2 as shown for Erk1 [145]. The peptide substrates derived from the phosphorylation site containing the motif PXT/SP, were poor substrates for MAP kinase p38, compared to its corresponding protein substrate [43]. This finding was expected since docking site interactions between the MAP kinases and their substrates has been shown to play an important role in substrate phosphorylation [138]. Similarly, the substrate recognition of cyclin-dependent protein kinases is in large part determined by the nature of cyclin associated with the kinase subunit [36].
6.2.3. Acidophilic and phosphate directed protein kinases

The acidophilic kinases are a considerably smaller group compared to the basophilic kinases. The CK2 kinase (formerly casein kinase II) and GSK3 belong the CMGC group, while two other examples, the ß-adrenergic receptor kinase and the rhodopsin kinase belong to ACG group. The casein kinase 1 (CK1) is a family of enzymes not falling into any one of the major groups according to the Hanks and Hunter classification. An interesting example of substrate specificity of an acidophilic kinase is the glycogen synthase kinase GSK3, whose crystal structure in complex with pseudosubstrate region was solved by two separate groups recently [18, 155]. GSK3 requires the prephosphorylation of the substrate at position P+4 in a consensus motif S/TXXXXSpX by CK2. Prior to the phosphorylation by GSK3, the glycogen synthase is phosphorylated at Ser 656 by CK2. After this event the GSK3 will sequentially phosphorylate Ser652, Ser648, Ser644 and Ser640, each of which locates four positions upstream from the previous one. Both solved crystal structures provide the structural explanation for this peculiar priming mechanism, showing that the phosphate added by CK2 kinase localises at the same site as the covalently attached phosphate in the activation loop of many other protein kinases. This is possible because the GSK3 does not require phosphorylation of any residues in the activation loop for its activity, and as for alternative, displays the described substrate-coupled activation mechanism. The phosphate directed phosphorylation is also observed in case of CK1, but only in rare cases for CK2, which generally prefers carboxylic acids in recognition of its substrates [124].

6.3. Docking sites and anchoring proteins as secondary specificity determining factors of protein kinases

The specific recognition of substrates by a protein kinase may be partly achieved by high affinity docking sites located away from the phosphorylation site. Such a mechanism is used by the MAP kinases [49, 138]. These docking motifs are rich in basic amino acids and often contain a characteristic motif LXL. Depending on substrate, these motifs may be located 50-150 amino acids upstream or downstream from the phosphoacceptor motifs. An additional docking motif FXF is also present in many MAP-kinase substrates. The phosphoacceptor motifs are usually sandwiched between a docking domain and FXF domain, forming the MAP-kinase recognition module. However many substrates contain only either the docking domain or FXF motif, and there are substrates which do not contain these domains.

The “targeting hypothesis” proposes that the specificity of phosphorylation events is controlled in part by the specific localisation of the kinases and phosphatases in the cell [55]. This is determined by association of these enzymes with “targeting subunits” or “anchoring proteins” [103]. Until now many of such anchoring proteins have been isolated. For PKA they are called AKAPs (A kinase anchoring proteins) [26].
AKAPs contain two conserved structural modules: (i) a targeting domain that serves as scaffold and membrane anchor, and (ii) PKA specific domain interacting with the regulatory subunits. There are several variants of AKAPs, with different targeting specificity and it has been shown that they may also bind other molecules, mainly phosphatases, forming multiprotein complexes called “transduceosomes”, capable of assembling and integrating signals from multiple pathways [26]. Similarly, in the case of PKC, the anchoring proteins help to translocate the isoenzymes to specific subcellular sites after stimulation with hormones or phorbol esters. Several proteins that bind PKC have been cloned including several annexins, cytoskeletal proteins and nuclear proteins [103]. A group of 30-36 kDa proteins collectively termed as RACKs (receptos for activated C-kinase) bind PKC through sites in the region of the C2 and V5 domains [104, 148]. Differential localisation of PKC isoenzymes is determined by isoenzyme specific RACKs [75, 131]. Several PKC binding proteins are also substrates like the myristoylated alanine rich C-kinase substrate (MARCKS) [60].

7. Inhibitors of protein kinases

In recent years much of attention has been paid to the design of the selective and potent inhibitors of protein kinases. The rise in interest has been caused mainly by the discoveries revealing the involvement of protein kinases in several diseases.

There are five main classes of reversible protein kinase inhibitors:

a) ATP-site inhibitors are compounds competing with ATP for its binding pocket in the enzyme active site cleft
b) Peptide-site inhibitors are compounds competing with the peptide or protein substrate for their binding site on the enzyme molecule
c) Bi-substrate inhibitors are inhibitors directed simultaneously into the ATP and peptide binding sites
d) Inhibitors targeting regulatory domains by acting on the sites of allosteric effectors
e) Inhibitors blocking the docking sites for anchoring proteins, inhibiting the specific localisation of a kinase (localisation inhibitors).

7.1. Protein and peptide site directed inhibitors

This class of inhibitors competes with the peptide or protein substrates for their binding sites on the protein kinase molecule and is mostly comprised of peptides containing the elements of substrate binding motifs. The peptide inhibitors containing the protein kinase P-site consensus motif are generally denoted as pseudosubstrate inhibitors. According to the definition, any peptide chain that folds back into the active site to turn
a protein kinase off using substrate like recognition groups can be termed as pseudosubstrate [71].

The first discovered and the most thoroughly studied members of the pseudosubstrate class of inhibitors are the heat stable protein kinase inhibitors (PKIs) of PKA [165]. The PKIs contain the substrate-like sequence RRNAl, where the phosphoacceptor serine is replaced by alanine, and they possess nanomolar $K_i$ values [165, 171]. Low nanomolar affinity is retained in a shorter synthetic variant PKI(5-24), but a variant containing only the short substrate consensus motif, the PKI(16-24), has several hundred fold higher $K_i$ than the PKI protein [33]. According to the crystal structure of the PKA-ATP-PKI(5-24) complex [79] and studies on structurally altered variants of PKI, it was found that arginine in position P–6 also plays important role in tight binding of the inhibitor [136]. The same studies prove the importance of phenylalanine in position P–11, which is the determinant not generally present in known peptide substrates, and therefore, proposed to determine the tight binding of the inhibitor [34].

As described for the PKA, PKC and CDPK, in many protein kinases there are autoinhibitory regions outside the catalytic core, which act by mimicking the features of the protein substrate phosphorylation site. These regions have been identified by searching for the consensus motifs in the sequences of regulatory domains, by truncation mutagenesis or point mutations and proteolysis. Several synthetic peptides corresponding to these sequences have been found to act as potent competitive inhibitors with $K_i$ values in the nanomolar or low micromolar range, including inhibitors for PKC [53], CaM-II [17] and soybean CDPK [41] and smooth muscle MLCK [78].

However, these inhibitors do not work in all cases. For example, the peptides from the regulatory regions of PKA and PKG show very weak inhibitory effects with $K_i$ values in the millimolar range [73]. The mechanism of a kinase autoinhibition can be illustrated with the crystal structure of twichin kinase, a member of the MLCK subfamily [54]. On the carboxy-terminal tail of the catalytic core of twichin kinase there is a 60-residue autoinhibitory sequence which extends over the surface of the cleft between the two lobes of the catalytic core. There are three different functions in this inhibitory interaction: (i) substrate-like binding contacts, (ii) steric superpositioning with the ATP and (iii) contacts with residues crucial for catalysis. This shows that downregulation by a pseudosubstrate peptide chain is more complex than a simple binding via a few interactions of the pseudosubstrate consensus motif, but involves functional interactions all over the active site. Such a multi-contact binding mode may explain why in some cases shorter pseudosubstrate peptide fragments are not high affinity inhibitors.

Sequences of the autoinhibitory regions have been used as templates for combinatorial libraries, designed to obtain selective and potent inhibitors for MLCK [93]. Another recent library study describes the design of highly specific peptide inhibitors for the cGMP-dependent protein kinase (PKG) by a rational stepwise approach using arrays of octameric peptide libraries on cellulose paper, screened for the binding of $^{32}$P-autophosphorylated PKG [21]. The obtained peptide FLLKKKKKKHHK, did not contain the defined pseudosubstrate motif, but did contain
the basic specificity determinants and showed selectivity relative to PKA. This result suggests that the active site of protein kinases hides many possibilities for design of highly efficient ligands/inhibitors, and that the pseudosubstrate consensus motif is not an absolutely necessary element in these structures.

Due to their peptidic nature, the pseudosubstrate peptide inhibitors of protein kinases are not classified as drug-like molecules and the main efforts in the field of protein kinase inhibitor design, largely promoted by pharmaceutical companies, have been applied to development of small-molecule ATP site directed inhibitors. However, quite a breakthrough for the practical application of peptide based inhibitors is the above quoted recent work by Dostmann et al. [21]. The fusion peptides designed by combining the peptide inhibitors for PKG and membrane translocation signals from HIV-1 tat protein and *Drosophila* antennapedia homeo-domain, proved to be efficiently delivered through the cell membrane. As an *in vivo* effect of the inhibitors, it was shown that the NO-induced dilation was substantially decreased in pressurized cerebral arteries. The described library-based inhibitor design combined with novel methods for intracellular delivery is a promising way for modulation of protein kinase activities in *in vivo* systems.

In several cases, the peptide substrates designed on the basis of the pseudosubstrate sequences have been shown to be very potent substrates. The PKI (14-22)S21 analogue is the best known peptide substrate for PKA with $K_m$ of 0.11μM [102]. On the other hand, the alanine substituted peptide based on the optimal peptide substrate Kemptide, LRRASLG, with $K_m$ value of 16μM [70], has very low inhibitory effect, with $K_i$ of 320μM [101, 170]. This was not surprising since the Kemptide itself had relatively low affinity to the PKA catalytic subunit ($K_d = 210\mu M$) [170], which is an order of magnitude lower than would have been expected from the $K_m$ value. The discrepancy between the $K_m$ and $K_d$ was found to be at least in part due to the fast chemical step of the phosphoryl transfer reaction [1]. The finding may seem discouraging with respect to the use of substrate specificity data for inhibitor design. However, this $K_m$ vs $K_d$ difference is not general and was shown only for a very specific example, the minimal short substrate Kemptide, not containing the specific arginine in P–6. On the other hand, Walsh and co-workers [33, 102] have shown that the $K_i$ for PKI(14-22)amide, a peptide containing the P-6 arginine, is 4-fold lower than the $K_m$ for the corresponding serine containing substrate. In order to reduce the possibility of being misled in the substrate specificity based inhibitor design, the use of aromatic and secondary alcohols as phosphate acceptor groups in substrates was proposed [169]. These substrates were less efficiently phosphorylated but displayed $K_m$ values comparable to their dissociation constants since the altered phosphoacceptor site caused the phosphoryl transfer to be the rate limiting step.

Proceeding from the optimal structures for the peptide-competitors, several attempts to obtain irreversible (covalent) inhibitors have been reported. The irreversible inactivation of PKC via S-thiolation of an active site cysteine in peptide RKRCLRRL was shown [166]. The bound peptide hindered sterically catalysis of peptide and protein substrates and even the PKC catalysed ATP hydrolysis [167]. A selective affinity label that inactivates PKG but not PKA was constructed using D-alanine instead of the
phosphoacceptor-site serine in a pseudosubstrate peptide with an aminoterminal cystein[3-nitro-2-pyridinesulfonyl-(Npys)]-amide [179].

For protein kinases from the MAP kinase cascades, the peptide inhibitors based on the docking domain motifs and FxF motifs in substrates have been designed [50, 63, 138]. Finally, nonpeptidic peptide/protein site directed inhibitors have been described, comprising mainly of different polyanionic or polycationic compounds, inhibiting basophilic or acidophilic protein kinases, respectively [4, 45, 120, 164]. An interesting example is the inhibition of CK2 kinase by heparin. The kinetic mechanism of this inhibition system has been previously studied by our group [113]. Despite the negative charges in heparin, which most likely mimic the P-site substrate recognition determinants for CK2, it was found that the inhibitor binding region does not overlap with the binding site for the peptide substrate. On the other hand, it competitively hindered the binding of the protein substrate casein. This example shows that in the vicinity of the minimal peptide substrate binding site of a protein kinase, there may be other possible sites, which may be considered to be the docking sites of inhibitors.

7.2. ATP site directed inhibitors

ATP binding site in protein kinases is in the cleft between the two lobes of the enzyme catalytic domain. Due to the high conservation of the ATP binding pocket region, the initial assumption was that it is not possible to design ATP-site directed inhibitors that differentiate between different protein kinases. However, when several protein kinase crystal structures became available, this opinion changed. Using crystallographic studies coupled to computer modelling, regions inside the nucleotide binding pocket were identified, which allowed the design of more highly selective compounds [31]. Until now, both computer modelling and pharmacophore-based approach have helped to design several classes of inhibitors which have an already noteworthy degree of selectivity between closely related protein kinases [24, 30, 105, 127, 134, 160].

The first discovered potent ATP-site directed inhibitor was staurosporine, a microbial alkaloid, isolated from *Streptomyces staurosporeus* [150]. This inhibitor has $K_i$ values in the low nanomolar range for several protein kinases including PKA and PKC [99], and it has remained in wide use in research as a potent universal inhibitor of protein kinase activity. Various related compounds, developed on the basis of staurosporine have relatively good selectivity for several kinases. These include bisindolyl maleimides, indolcarbazoles, and bisindolylmaleimide macrocycles [65, 96, 159, 172]. Another historically important class of protein kinase inhibitors is the H-series inhibitors, include different derivatives of isoquinolinesulphonamide, first reported by Hidaka et al. [46]. Today, the crystal structures of the kinase-inhibitor complexes are available for the staurosporine in complex with PKA [126] and Cdk2 [88] and three variants of complexes of the H-series of inhibitors with PKA [24]. These crystallographic studies together with several other structures confirm the existence of three specificity regions in the adenosine binding pocket: the “hydrophobic pocket”, the
“ribose pocket”, and the “linker region”. The existence of the hydrophobic pocket is confirmed by crystal structures of the p38 and FGFR protein kinases in complex with pyridinylimidasole and PD173074 inhibitors [105, 174]. The “ribose pocket” is used by the isoquinoline and dianalinophthalamide inhibitors [24]. The diversity of the structural environment of the “linker region” is utilised by the purine based class of olomoucine inhibitors [134]. In general, there are two key components of the protein kinase structure that contribute to the specificity of ATP-site directed inhibitors. (1) amino acid sequence diversity within the ATP binding cleft, and (2) conformational diversity, which allows inhibitors to “adopt” and “lock” the protein kinase into a specific conformation.

7.3. Bi-substrate inhibitors

Combination of the structural elements of the peptide-site inhibitors and ATP-site inhibitors into one molecule by connecting them via a linker is the basic principle of the bi-substrate inhibitor design. The general principle of the bi-substrate inhibitor approach for enzymes catalysing bimolecular reactions has been described in [8, 37]. A classical successful example of a bisubstrate inhibitor is the P\textsuperscript{1}, P\textsuperscript{5}-Di(adenosine-5')pentaphosphate, a molecule combining two adenosines connected via a pentaphosphate linker, which inhibits the adenylate kinase with K\textsubscript{i} values in low nanomolar range [90]. The first attempt to apply the bi-substrate approach for design of inhibitors for serine/threonine protein kinases was performed by Ricouart et al. [128] by combining one of the derivatives of a H-series inhibitor to a tetra- and hexaarginine peptide anchors via different linkers. The best inhibitor for both PKA and PKC, with IC50 values of 3 and 300 nM, respectively, was found to be a isoquinoline-5-sulfonamide coupled to a Ser-Arg\textsubscript{6} peptidic moiety via a –NH(CH\textsubscript{2})\textsubscript{2}NH(CH\textsubscript{2})\textsubscript{2}CO-linker. Further, an attempt was made to design transition-state analogue inhibitors, resembling the bi-substrate reaction complex of PKA, by coupling the serine residue of the specific PKA substrate Kemptide to adenosine nucleotides via phosphate linkers of different length [97]. The most potent of these compounds had an inhibitory effect in micromolar range. Similarly, a moderately successful attempt, with inhibitory effects in the low micromolar range, was performed to generate selective inhibitors for cdc2 kinase using the bi-substrate approach by coupling potent ATP-site inhibitor 3,4-bis(indol-3-yl)maleimide analogues to a pseudosubstrate peptide Ac-C(S)PKK-NH\textsubscript{Me} via ethyloxy linkers of different length [133]. Thus, the exact structural requirements for successful bi-substrate inhibitor design are still undefined and according to the knowledge available, the most crucial variable seems to be the length and chemical nature of the linker region. A recent crystallographic study has shed some light on several of the unclear aspects of the bi-substrate approach [121]. In this study a bi-substrate inhibitor for the insulin receptor tyrosine kinase was synthesised by linking ATP\gammaS to a peptide substrate analogue via a two-carbon spacer. The design of this molecule was inspired by the knowledge about the dissociative transition state of the
protein kinase catalysed phosphoryl transfer. The crystal structure confirmed the predicted bi-substrate mode of binding and showed also that the linker takes part in the octahedral coordination of an active site Mg$^{2+}$.

Knowledge obtained from the studies quoted above, reveals that the idea to connect two highly specific structural elements into one molecule via a linker region is a powerful method for potentiation of inhibitory effects. While the studies carried out so far have used the ATP-site binding blocks originated from inhibitor structures of medium potency and the peptide parts with lower affinities compared to the most optimal pseudosubstrate structures, it is most probable that when combining the recently designed ultrapotent ATP-site inhibitors via a suitable linker to the best inhibitor peptides at an optimal connection point, the unprecedented inhibitory affinities could be achieved.

8. Affinity isolation of protein kinases using specific ligands targeted to substrate sites

Specific ligands that bind to the substrate sites in the protein kinase active site groove have been used for affinity purification of protein kinases. These attempts have shown some success, but there has been no general breakthrough. A method using peptide substrates for isolation of glycogen synthase kinase and protein kinase CK2 has been reported [175], but the low affinity of the kinases to the column did not allow stringent washing conditions. Peptide inhibitor PKI has been used with relative success for purification of catalytic subunit of PKA [115]. This method, required a long incubation time for protein kinase binding and gave low yields of purification. The ATP binding pocket directed affinity adsorbent γ-ATP-Sepharose has been tested for isolation of several kinases [19, 44]. This method, however, cannot be strictly specific for protein kinases, due to the high number of different enzymes utilizing adenosine nucleotides as substrates and cofactors.
Present investigation

Aims of the thesis
This thesis focuses on study of the specificity of different model protein kinases at three different levels using
a) novel bi-substrate analogue inhibitors
b) synthetic peptide substrates
c) mutated protein substrates.

The specific aims for the projects were:
- to investigate the possibilities to obtain high affinity inhibitors and affinity ligands for protein kinases by combination of structural elements of both substrates into one bi-substrate analogue inhibitor molecule.
- to isolate the 59 kDa form of CDPK from maize seedlings and determine the substrate specificity consensus motif for this kinase
- to develop the positionally oriented peptide library methodology for quantitative substrate specificity study of protein kinases using ESI-MS for detection of phosphopeptides. To investigate the substrate specificity of PKCβ using the new method
- to study the phosphorylation site specificity determinants for PKA using the site-directed mutagenesis into the consensus motif positions of the L-type pyruvate kinase (L-PK) phosphorylation site and compare the kinetic data obtained with the data on similar alterations performed on peptide substrates in previous studies.

Paper I

Adenosine 5'-carboxylic acid peptidyl derivatives as inhibitors of protein kinases
In collaboration with a research group in Tartu University, we have designed and developed a novel class of inhibitors for protein kinases. These compounds contain the structural elements of both substrates of protein kinases: the peptide part and the adenosine part. These two parts are coupled to each other via a linker. A set of eleven inhibitors was synthesised at the organic synthesis facility of Tartu University, by Dr. Asko Uri. The novel compounds were potent inhibitors of PKA and PKC with IC50 values in nanomolar range. It was also shown that the two main building blocks of these compounds, the adenosine and hexaarginine peptide had inhibitory effects in micromolar range indicating that the coupling of these two structural elements into one bi-substrate analogue has a positive synergistic effect on the inhibitory efficiency. The selective effect was also gained as these compound were relatively poor inhibitors for protein kinases with acidic substrate specificity determinants CK1 and CK2 (IC50>30μM). A third basophilic protein kinase, CDPK from maize, was also inhibited by the new compounds but the inhibitory effects were in micromolar range. It was
found that the hexaarginine peptide moiety was necessary for the high affinity binding
of the inhibitors, since the compounds with four and two arginines showed ten and one
hundred-fold weaker inhibitory effects, respectively. The main focus of this study was
to optimise the requirement of the linker region of the novel bi-substrate inhibitors of
general structure AdoC-Linker-Arg₆. The linkers of different charge and length were
tested. For introducing negative charges, the aspartic acids were used, and for positive
charges the amino acids containing secondary amines were included. Thirdly, the
aliphatic chains of different length were tested as linkers. It was found that the optimal
linker structures for PKA and PKC were the aliphatic chains comprised of six and eight
carbons, respectively. Interestingly, in spite of much weaker inhibitory effects for
CDPK compared to PKA and PKC, the structural preferences for the linker region were
closely similar for all three enzymes, pointing to the common structural features of the
region locating the linker chain of the bound inhibitor. Since CDPK is quite a distant
kinase relatively to PKA and PKC, belonging to the CaMKII group of the protein kinase
superfamily, the results obtained for linker optimisation may apply universally to the
kinase superfamily. In this case, the AdoC-linker part could stay unchanged in the
design process and the peptide anchor could be varied according to the peptide substrate
specificity of the kinase of interest resulting in selective inhibitors. For such variations
the peptide substrate libraries studies are a potential source of valuable information.

Paper II

Bi-substrate analogue ligands for affinity chromatography of protein kinases

Novel purification methods are needed to characterize the growing number of
discovered protein kinases. Relatively slow progress in this area can partly be related to
complex purification protocols, which use conventional chromatographic steps for
protein isolation (ion-exchange chromatography, hydrophobic chromatography, size
exclusion chromatography, etc.). Attempts to use affinity chromatography for this
purpose have still not lead to a general breakthrough. In this paper we proposed a novel
affinity medium for purification of PKA. This medium was based on the application of
a new type of protein kinase bi-substrate inhibitor, described in paper I of the current
thesis. Two ligands, AdoC-Aoc-Arg₄-Lys (ligand A), with Kᵢ value of 330 nM for PKA
and AdoC-Aoc-Arg₄-NH(CH₂)₆NH₂ (ligand B), with Kᵢ value of 83 nM, were designed
for purification of PKA. The ligands A and B were coupled to the epoxy-activated
Sepharose and NHS-activated Sepharose, respectively. The efficiency of the media was
tested for purification of recombinant catalytic subunit of PKA from bacterial extract.
Both MgATP and L-arginine were successfully used as eluting agents yielding
homogeneous PKA preparation. The binding of PKA was not distorted by 1 M NaCl
confirming the specific mode of binding. PKA was also isolated from pig heart using
the column with ligand B. Since PKA is a very sparse enzyme in tissues, a preliminary
step of DE-52 was included for elimination of a part of the proteins present in the
supernatant of the heart homogenate and thereby the PKA was enriched. After the
subsequent affinity chromatography step a preparation of PKA was obtained with no essential contaminants as visualised in SDS-page. Additionally it was found that the affinity column did not bind the purified protein kinase CK2, which belongs to protein kinase class with acidic specificity determinants. No binding of proteins possessing nucleotide binding site (the L-type pyruvate kinase), or sites for wide variety of different ligands (the bovine serum albumin) was observed. These findings point to high selectivity of the bi-functional binding mode of the affinity ligand.

Paper III

Peptide phosphorylation by Ca\(^{2+}\)-dependent protein kinase (CDPK-1) from maize seedlings
A 59 kDa form of Ca\(^{2+}\)-dependent protein kinase (CDPK-1) was partially purified from maize seedlings and the substrate specificity of this enzyme was studied using a set of synthetic peptides, derived from the phosphorylatable sequence RVLSRLHS(15)VRER of maize sucrose synthase 2. The isolated enzyme was activated by Ca\(^{2+}\)-ions at low micromolar range, but its activity was not stimulated by the phospholipid-diacylglycerol mixture. The preparation showed both a single band of autophosphorylation and a single immunoreactive band with the monoclonal antibody rised against the calmodulin-like domain of the soybean CDPK, confirming that the CDPK preparation contained only one protein kinase. The molecular size estimated from both autophosphorylation and blotting experiments was close to the predicted molecular mass value of 59.4 kDa for a cloned CDPK from maize seedlings [132]. The predicted molecular mass of 60 kDa has been reported also for a cloned maize pollen-specific CDPK [25]. The purified CDPK was different from the 54 kDa CDPK form stimulated by phospholipids [149]. Besides the molecular size and the activation conditions, the characteristic difference between these two major forms of CDPK in maize seedlings was the different ionic strength required for elutions of the two enzyme forms from the DEAE ion exchanger.

For study of the substrate specificity of the newly isolated kinase we applied two sequential approaches. First, a series of synthetic peptides with different lengths, shortened by one amino acid at a time, was used for determination of a minimal peptide substrate which would be efficiently phosphorylatable by the kinase. In this way, the deca-peptide LARLHSVVRER, was identified as a minimal unit containing the critical determinants for recognition by kinase and showing \(V_{\text{max}}/K_m\) value still in the same order of magnitude as the parent peptide. The same set of peptides was tested as substrates for mammalian PKCβ, and it was found that the phosphorylation motif from sucrose synthase 2 also contains a specific substrate motif for this kinase. Furthermore, PKC had similar optimal peptide length requirement to CDPK-1. Conversely, these peptides revealed low reactivity in the case of PKA.

In the second step of the study a systematic analysis of the positional specificity of CDPK-1 within the minimal substrate structure LARLHSVVRER was performed using positionally varied substrate sets synthesised as spots on cellulose membranes
(SPOTs™ membranes). This method has been developed and used previously by Frank and co-workers [29] and also partly in our laboratory by Toomik et al. [157]. The systematic variation of the hydrophobic (Ala, Leu) and ionic (Arg, Glu) amino acids subsequently in each position of the minimal peptide lead to the definition of the consensus sequence motif A/LXRXSXRXZR, for CDPK-1, where X denotes a position with no strict amino acid requirements and Z a position strictly not tolerating arginine compared to the other three varied amino acids. The consensus motif for maize CDPK-1 was in agreement with the results of specificity studies made with CDPK forms from wheat germ [125], soybean [89] and from a lower plant Mougeotia [129], where the location of basic amino acids in position –3 from the substrate phosphorylation site seemed to be critical for substrate recognition. The leucine in position –5 has been shown to be an important substrate recognition element for two forms of CDPK from spinach leaf [2]. However, the CDPK forms from these plants did not exhibit any strict requirements for basic or acidic amino acids at the C-terminal side of the serine. Thus the fragment RZR, in positions from +2 to +4 seems to be specifically characteristic for maize CDPK-1. The recognition of this motif was probably based on electrostatic interactions, as the enzyme did not tolerate amino acids of opposite charge in this region. However, this fragment alone was not sufficient for efficient phosphorylation of peptides. For example, the peptides that lack the Leu in position –5 but possess the fragment RER, were poor substrates for CDPK-1. From these observations it can be concluded that CDPK-1 has complex and rather original substrate specificity. Interestingly, the obtained consensus motif was close to the analogous sequence K/RXXS/TXK/R proposed for mammalian PKC, but different from the consensus motif RXXS/TX for PKA.

Paper IV

Systematic analysis of substrate specificity profile of protein kinase C using a positionally oriented peptide library approach coupled with electrospray mass-spectrometry

In this study a positionally oriented peptide library approach based on ESI-MS detection of phosphopeptides in initial velocity conditions was designed for quantitative kinetic characterization of protein kinase specificity profiles. The leading structure of the peptide library, LLRLHSLRER, is a slightly modified sequence of a phosphorylation site of maize sucrose synthase. Peptides derived from this sequence were previously shown to be potent substrates for maize calcium-dependent protein kinase (CDPK) and also for mammalian PKC.

Each subsequent position in the peptide library was successively substituted with a mixture of amino acids resulting in nine different libraries. PKC was used as a model enzyme for optimization of the method. For quantitative detection of the phosphopeptides in true initial velocity conditions, the ESI-MS was used. It was shown that five amino acid positions upstream and four downstream from the serine had
defined and sensitive structural specificity for PKC, and that the positively charged arginines showed strong preference over negatively charged glutamic acid in all varied positions. On the basis of the data obtained, an optimal peptide substrate for PKC, FRRRRSFRRR, was designed, showing the $K_m$ value in nanomolar range.

Additionally, the possibilities to use the obtained quantitative specificity profile for prediction of phosphorylation sites for PKC by screening the protein sequence databases were analysed. It was found that the optimal substrate structures for PKC are very rare in known protein substrates and the majority of phosphorylation site sequences in proteins contain only a minimal number of substrate specificity determinants.

Paper V

**Phosphorylation of peptide and protein substrates by protein kinase A**

The results from papers III and IV showed that the substrate specificity profiles of protein kinases obtained from the studies with synthetic peptide substrates corresponding to the sequences of phosphorylation sites in protein substrates may reveal a sensitive profile of structural requirements for amino acid positions surrounding the phosphorylatable residue. However, the discussion in the paper IV revealed that these specificity profiles are not strictly present in many phosphorylation sites in proteins. Therefore, for validation of the possibilities for prediction of phosphorylation sites in proteins it is important to estimate the impact of both the primary structure site specificity and that of secondary determinants, such as protein-protein surface contacts outside of the phosphorylation site. In the paper V, we addressed this question by studying a kinase P-site specificity using site-directed mutagenesis in a phosphorylation site of a physiological substrate of PKA, L-type pyruvate kinase (L-PK). Amino acids with basic, acidic, small and large hydrophobic side chains, and also with hydrophilic side chains were systematically introduced in the positions P-3, P-2 and P+1 of phosphorylation motif RRASV of L-PK and the purified mutated proteins were tested as substrates for PKA. The obtained kinetic data confirmed the basic substrate specificity requirements for PKA, i.e. the preference of arginine in P-2 and P-3 and hydrophobic residue in P+1 position. Comparison of the relative logarithmic values of the obtained kinetic data with the available data on similar structural alterations in separately tested peptide substrates collected from literature revealed that amino acid alterations in peptides cause stronger effects on the phosphorylation rate than the corresponding alterations in protein substrate L-PK.

This finding indicates that the importance of the primary structure specificity profile around the phosphorylation site in protein substrates may be overestimated and similarly to the conclusion from paper IV, other structural factors play an important role in the efficiency of protein phosphorylation reactions. These factors may be the docking sites or additional docking proteins for protein kinases. But, importantly, in the case of L-PK, these factors are most plausibly the nonspecific interactions between the surfaces of two encountering proteins, since there are no specific docking sites known,
which would mediate the recognition of L-PK and PKA. Since the phosphorylation rates of the wild type L-PK and wild type peptide RRASVA did not differ very much, it may conceived that these additional interactions between the surfaces of the two encountering proteins compensate the negative effect of unfavorable residues in consensus motif positions, which otherwise when introduced in peptide substrates would be detrimental for the reactivity. In this context, the number of potential physiological phosphorylation sites should be very high, possibly making the kinases too promiscuous mediators for signaling systems requiring exactness. However, this drawback is overcome and the specificity of kinase action is shown to be determined by compartmentation and specific subcellular targeting of kinase, as shown for PKA system exemplified by the A-kinase anchoring proteins (AKAPs).

Future perspectives

Further development of the bi-substrate analogue inhibitors by varying the structure of the peptide anchor may lead to design of selective inhibitors for protein kinases. The structure of the peptide anchor could be optimised according to the results from the ESI-MS coupled peptide library studies for different kinases. By this way even minor substrate specificity differences can be used for modulation of selectivity of the bi-substrate molecules. Such substrate specificity based inhibitor design would combine the two main themes of the current thesis. The obtained inhibitors may be used for further optimisation of the group specific affinity isolation methods for protein kinases.

On the other hand, the highly detailed specificity data possible to obtain from the peptide library methodology, designed for kinetic measurements in initial velocity conditions, opens new perspectives for isoenzyme specific substrate design. The designed substrates can be used for high-throughput protein kinase activity assays for drug screening or for validation of protein kinase activity profiles as potential biomarker panels for different diseases.
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