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Reviews

Protein kinase biochemistry and drug discovery

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ABSTRACT

Protein kinases are fascinating biological catalysts with a rapidly expanding knowledge base, a growing appreciation in cell regulatory control, and an ascendant role in successful therapeutic intervention. To better understand protein kinases, the molecular underpinnings of phosphoryl group transfer, protein phosphorylation, and inhibitor interactions are examined. This analysis begins with a survey of phosphate group and phosphoprotein properties which provide context to the evolutionary selection of phosphorylation as a central mechanism for biological regulation of most cellular processes. Next, the kinetic and catalytic mechanisms of protein kinases are examined with respect to model aqueous systems to define the elements of catalysis. A brief structural biology overview further delves into the molecular basis of catalysis and regulation of catalytic activity. Concomitant with a prominent role in normal physiology, protein kinases have important roles in the disease state. To facilitate effective kinase drug discovery, classic and emerging approaches for characterizing kinase inhibitors are evaluated including biochemical assay design, inhibitor mechanism of action analysis, and proper kinetic treatment of irreversible inhibitors. As the resulting protein kinase inhibitors can modulate intended and unintended targets, profiling methods are discussed which can illuminate a more complete range of an inhibitor's biological activities to enable more meaningful cellular studies and more effective clinical studies. Taken as a whole, a wealth of protein kinase biochemistry knowledge is available, yet it is clear that a substantial extent of our understanding in this field remains to be discovered which should yield many new opportunities for therapeutic intervention.

Contents

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1. Protein phosphorylation

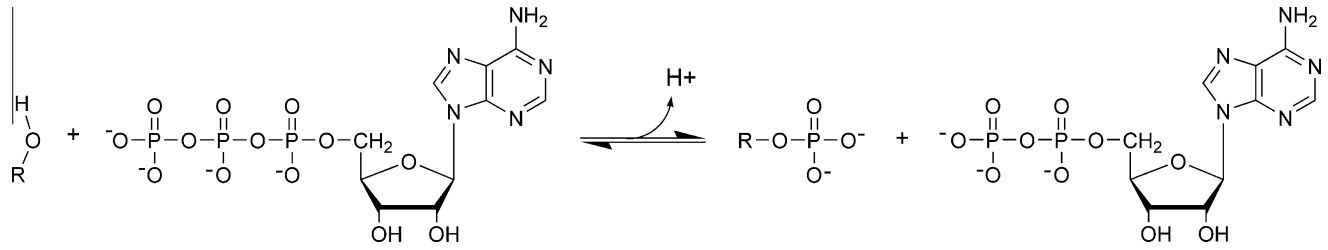
The study of protein phosphorylation encompasses over 100 years of research and yet our knowledge continues to expand rapidly with new challenges continually emerging. Protein phosphorylation traces its origin back to the discovery of phosphorylated proteins by Levene and Alsberg in 1906 [1] with subsequent mapping to serine residues [2,3]. The repertoire of phosphorylated eukaryotic residues expanded to tyrosine by Hunter and coworkers in 1979 [4]. Since the initial discoveries, phosphorylation has been found to be a very common post-translational modification with 500,000 potential phosphorylation sites in the human proteome and 25,000 phosphorylation events described for 7000 human proteins [5]. These phosphorylated residues are asymmetrically distributed: 85% serine, 11.8% threonine, and 1.8% tyrosine residues [6]. Protein phosphorylation specificity is dependent on many factors: the residue itself (serine, threonine, tyrosine), the surrounding primary sequence, and the conformation of the phosphorylated motif [7–10]. Recent evolutionary genomic studies have illuminated other levels of complexity. Protein phosphorylation occurs in both ordered and disordered regions. Disordered regions typically mediate protein–protein interactions (e.g. protein complex formation), contain many more phosphorylation sites than ordered regions [11,12], and are enriched in serine/threonine residues. Ordered regions typically code for catalytic, structural, or conformational interactions and are enriched in tyrosine residues [13]. These genomic findings indicate that there are systemic differences in the functional roles for Ser/Thr/Tyr phosphorylation events [12,14–16]. Further analysis reveals that the exact positions of serine and threonine residues in proteins are not evolutionarily conserved, illuminating a plasticity of the coded protein–protein interactions [16]. Another level of complexity is that the types of phosphorylated residues are treated differently through the course of evolution. The number of threonine residues is inversely correlated with an organism's complexity while the number of serine residues is not. The number of tyrosine residues and tyrosine kinases is also inversely correlated with the complexity of the organism [13]. Thus the acquisition of additional tyrosine kinases through evolution required a system-wide response to eliminate deleterious tyrosine residues. Signaling specificity of tyrosine and threonine (but not serine) residues may be conserved to protect against aberrant signaling. Taken in the whole, phosphorylation of serine, threonine, and tyrosine residues are common post-translational modifications with critical and distinct biological roles.

2. Why phosphorylation?

The phosphate group has special properties that are utilized to regulate critical biological functions. Phosphates are the salts of phosphoric acid with three discrete ionization states (pK_a 2.15, 7.20, 12.33). At physiological pH, phosphate groups are predominantly dianionic which is a property not found in any naturally occurring amino acid. The protein phosphorylation post-translational modification is chemically stable under physiological conditions which allows for reliable control of a biological function [17]. Using a model system, uncatalyzed alkyl phosphate monoester hydrolysis is very slow ($k = 2 \times 10^{-20}\ s^{-1}$ at 25 °C for methylphosphate) [18]. Electrostatic arguments have been made to explain the chemical stability of the phosphate monoester bonds to proteins as the ionized phosphate group is proposed to repel anionic nucleophiles [17]. In contrast, enzymatic dephosphorylation of phosphoproteins can be quite facile with 17 orders of magnitude rate acceleration affected through enzymatic catalysis [18]. As such, the phosphorylation post-translational modification can be precisely regulated by enzymatic reactions. Another factor which makes the phosphorylation reaction suitable is that ATP is ubiquitous and present at high cellular concentrations: typically reported to be between 1 and 4 mM but can vary widely and be as high as 12 mM [19–22]. Energetically, transfer of the phosphate group from ATP to the hydroxyl group of a phosphoacceptor is favorable ($ATP + H_2O \rightarrow ADP + Pi$; $\Delta G = -7.3$ kcal/mol) (Scheme 1, R = H). Thus the source of the phosphate group is readily available and the transfer reaction is thermodynamically favorable. Once incorporated into a protein, a single phosphate group is capable of making many strong hydrogen bonds that can affect intramolecular and intermolecular interactions [23]. The phosphate group is particularly suited for interacting with the guanidino group of arginine residues, which has a rigid, planar structure that can make multiple directed hydrogen bonds to the dianionic phosphate group at physiological pH [23,24]. Thus the simple phosphate group is an ideal molecular "switch" to affect extremely complex signaling networks through flexible, rapid, transient phosphorylation events which direct the formation and reorganization of dynamic protein interaction networks.

3. Protein kinase overview

Protein kinases catalyze a simple reaction: the transfer of the terminal (gamma) phosphate of ATP to the hydroxyl group of an amino acid residue (Scheme 1, R = Ser/Thr/Tyr). The origin of



Scheme 1. γ-Phosphoryl transfer reaction from ATP.

protein kinase research traces back to the discovery of ATP-dependent, divalent metal ion-dependent enzymatic activity in 1955 by Fischer and Krebs [25], which ultimately led to the discovery of the serine/threonine kinase phosphorylase b kinase [26,27]. Tyrosine kinases were added to this enzyme class with the discovery of the viral protein vSrc (see Table 1 for kinase abbreviations) [28] which was correctly characterized as a tyrosine kinase in 1979 [4,29]. The vertebrate homolog of vSrc (cSrc) was subsequently characterized as the first proto-oncogene [30]. Since the discovery of protein kinases, the field of research has rapidly expanded. In 1988, there were approximately 100 protein kinases known [31] and the number increased to 205 by 1995 [7]. The landmark study of kinases in the human genome revealed 518 protein kinases (the kinome) of which 478 had catalytic domains with related primary sequences [32]. Thirty-two atypical kinases (e.g. PI3K, ATM, RIOK) with divergent primary sequences have been identified [32]. Subsequent studies have added only two other kinases to the kinome [33]. A recent re-evaluation of the human kinome has disqualified 3 of the original 518 kinases and added 23 new kinases for a total of 538 human protein kinases (Gerard Manning, personal communication). As such, protein kinases make up almost 2% of the human genome and control most important biological processes. Although there are 90 tyrosine kinases (58 are receptor tyrosine kinases) in the human kinome, the majority of protein kinases phosphorylate serine or threonine residues. In addition, there are a small number of kinases that can act as both tyrosine and serine/threonine kinases which are termed dual specificity kinases (e.g. MEKs, CLKs, Mps1, DSTYK, TESKs, DYRKs) [34]. The dual specificity of protein kinases may be more of a general phenomenon than originally thought as a well-known tyrosine kinase, Syk, has been recently shown to also have a serine/threonine kinase activity too [35]. From these studies, the broad landscape of O-phosphorylation has been defined.

N-Phosphorylation of residues with amine-containing side chains (His, Lys, Arg) is emerging as a frontier in protein phosphorylation research. Histidine protein kinases have a well-defined role in prokaryotic and simple eukaryotic species utilized in simple two-component and multi-component phospho-relaying signaling systems which link extra-cellular inputs to gene regulation [36,37]. Roles for N-phosphorylation in humans are poorly understood presumably due to technical issues stemming from the inherent chemical instability of the phosphoramidate bond. Nonetheless, a more transient phosphorylation event, N-phosphorylation, may have utility in more complex signaling networks found in more evolved eukaryotic organisms. There have been reports of phosphorylated histidine residues on human proteins. Nucleoside diphosphate kinase phosphorylation of a potassium channel histidine residue [38] is the best studied case but there are other examples (e.g. P-selectin [39] and other proteins [40]). New methods for detecting these types of phosphorylation events are emerging which should facilitate growth in the understanding of the role of N-phosphorylation in human biology [41]. As the bulk of protein kinase research has focused on hydroxyl-containing amino acid residues, the review will concentrate on O-phosphorylation biology and biochemistry.

Despite the genomic classification of the human kinome, only a small fraction has been studied in detail and an even smaller fraction yielding drugs in the clinic. Fifty protein kinases (<10% of the kinome) have been the subject of three quarters of all of the kinase citations through 2009 [42]. Over 100 kinases have unknown function and 50% of all kinases are largely uncharacterized [43]. This is not to say that research has been focused on the critical kinases. RNAi knockdown kinome screening studies reveal cellular functional effects which are not biased towards kinases previously studied [44,45]. These findings indicate that a significant fraction of kinase biology and the attendant drugs remain outstanding. Nonetheless, protein kinases are essential biological catalysts known to affect signaling by a myriad of mechanisms: enzyme activation, enzyme inactivation, protein localization, protein stabilization, protein degradation, and protein–protein interactions. They impact this diverse array of biology, in part, by being efficient catalysts. Recent studies show that kinases can affect a 10^{12}–10^{14} fold rate enhancement over non-enzymatic rates [46]. Since the maximal rate enhancement achievable through substrate co-localization and proper alignment is eight orders of magnitude (10^8) [47], protein kinases affect catalysis through additional contributions [46]. In addition, phosphorylation of kinases themselves is known to affect their catalytic properties [25]. Autophosphorylation effects can be profound as observed for Tie2 which has a 460-fold rate enhancement upon autophosphorylation and is transformed into a more "perfect" catalyst (chemical step less rate-limiting) [48]. As will be seen in this review, catalysis of this "simple" chemical reaction by protein kinases is actually quite intricate.

4. Structural overview of the catalytic domain

As a consequence of being part of a large gene family, specificity and regulation of protein kinase catalytic activity is essential for proper function. Structural studies have contributed to the current understanding of regulatory mechanisms. The first protein kinase catalytic domain crystal structure solved was PKA in 1991 [49]. Since then ~1500 structures of ~200 unique kinases have been deposited into the Protein Database (PDB) [50]. Many comprehensive structural reviews are available [24,51–56] as well as descriptions of the molecular recognition elements that assist inhibitor design [57,58]. A rudimentary overview is included in this review as a framework for biochemical and biophysical analysis. The catalytic domain ("kinase domain") has two major subdomains: a smaller N-terminal "lobe" comprised of five β-strands with one critical α-helix (αC-helix) and a larger C-terminal "lobe" that is primarily α-helical. The two subdomains are joined by a peptide strand (the hinge) with the cleft formed between the subdomains constituting the active site (Fig. 1). This cleft has a front pocket containing the residues directly involved in either catalysis or ATP binding while the hydrophobic pocket (back pocket) supports regulatory functions [59,60].

In the front pocket, the adenine ring of ATP hydrogen binds to the amide backbone of the hinge region (Fig. 1). A flexible N-terminal loop (G-loop, P-loop), which contains three conserved glycine residues (G–X–G–X–X–G), binds to the nontransferable α- and β-phosphate groups of ATP through ionic interactions. The conformation of the G-loop is dynamic and depends on the activation state of the catalytic domain and the presence of ligands. Enzymatic activation is regulated, in part, through the orientation of the αC-helix (inward towards the active site in the active state) and frequently interacts with the C-terminal activation segment to alter the alignment of catalytic residues. The activation segment has multiple elements (DFG, activation loop, P + 1 loop) with multiple functions. The activation loop contains a conserved DFG sequence (Mg^{2+} orienting loop) critical for catalysis, in part, because the aspartate residue binds directly to the magnesium ion cofactor orienting the γ-phosphate of ATP for transfer. The activation loop typically contains phosphorylation sites critical to the regulation of catalytic activity. Incorporation of a phosphate group on an activation loop residue creates a network of strong hydrogen bonds which facilitates the organization of catalytic active site residues and the phosphoacceptor binding site. An alternative allosteric activation mechanism is invoked by a subset of kinases (e.g. EGFR, cyclin-dependent

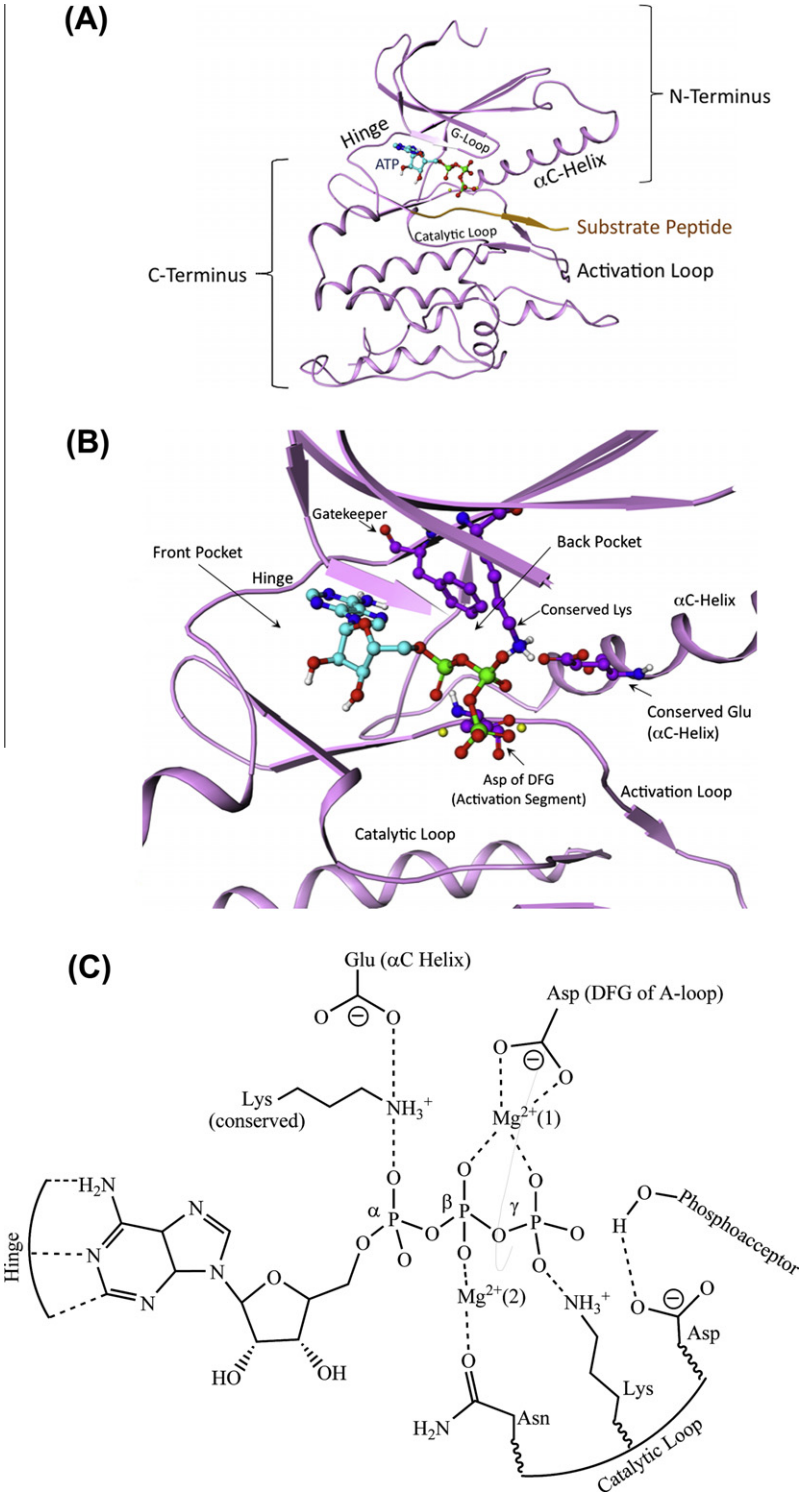


Fig. 1. Protein kinase structure and molecular interactions with substrates (Protein Data Bank ID code 2PHK). (A) Ribbon representation of phosphorylase kinase (magenta), bound with an ATP analog (AMP–PNP), two Mn^{2+} ions (yellow) and peptide substrate (orange) [235]. Structural features are annotated: N-terminus, C-terminus, C helix, hinge, A-loop. (B) Phosphorylase kinase catalytic region bound with ATP analog and Mn^{2+} ions (yellow). Key residues and binding pockets are highlighted. (C) Simplified illustration of the molecular contacts between the substrates and conserved active site residues and cofactors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

kinases) that involves intermolecular interactions affecting conformational dynamics [61–63]. The conserved lysine residue (K72 in PKA) that helps orient ATP for catalysis is not in a flexible loop as are many of the other essential catalytic residues. In inactive conformations, the αC helix is rotated away from the ATP binding site, thus preventing a necessary ionic interaction between a conserved glutamate residue in the helix (E91 in PKA) with the conserved lysine residue. This inter-residue binding positions the lysine residue to align the non-transferable phosphates of ATP in the active conformation. The C-terminal subdomain contains several other residues critical for catalysis which are contained in the catalytic loop (Fig. 1C).

The understanding of the other active site region (back pocket) has recently expanded from a site utilized in drug design to one that contains kinase regulatory elements. Access to the hydrophobic pocket is controlled by two residues: the conserved lysine residue, described above, and a "gatekeeper" residue. The gatekeeper residue can have either a large amino acid side chain (e.g. Phe in Cdk2) which blocks ligand (inhibitor) access to the back pocket or a small side chain (e.g. Thr in EGFR) which allows access. Recently, a regulatory mechanism facilitating inter-subdomain interactions has emerged: hydrophobic spines. Hydrophobic spines are an array of internal (not solvent-exposed) hydrophobic residues that transverse the two subdomains providing a flexible connection for inter-subdomain communication [60]. Hydrophobic spines, which contain portions of the activation segment, the αC-helix, and the gatekeeper residue, are assembled during activation of the catalytic domain [60,64]. As such, hydrophobic spines may control conformational dynamics of the catalytic domain. As illustrated in this brief overview, the catalytic domain is a dynamic structure that has evolved sophisticated mechanisms for regulation of catalytic activity. Structural studies of individual conformations of isolated kinase catalytic domains are quite informative yet capture a subset of protein kinase biochemical richness.

5. Kinetic mechanism

An initial step in understanding an enzymatic mechanism is the characterization of the kinetic mechanism which is defined by (1) the relative rates of chemical and physical steps and (2) the obligate order, if any, of substrate addition (A and B) and product release (P and Q) (Fig. 2). Sequential mechanisms, that is, reactions in which all reactants must be bound to an enzyme before catalysis occurs, fall under a continuum of specific cases dependant on the location of the slow step(s). In one mechanistic extreme, the rapid equilibrium case denotes a reaction in which an equilibrium between the enzyme–reactant complex and free reactant is established in a reaction cycle; in other words, the off-rate constants for A and B from the EA and EB binary complexes are both much greater than k_{cat}. The steady-state mechanism occurs between the extremes of the rapid equilibrium case and the case in which chemical conversion of the central complex (E·A·B ↔ E·P·Q) and release of the first product is so fast that there is essentially no build up of the central complex (Theorell–Chance mechanism). Overwhelming kinetic evidence indicates that protein kinases react via sequential mechanisms, where interconversion of the central ternary complex (E·ATP·S) occurs via direct phosphotransfer from the donor to acceptor in a single-displacement reaction (Fig. 2). Characterization of the kinetic mechanism generally utilizes steady-state kinetic approaches in the presence or absence of added inhibitors [65]. These kinetic studies use a range of phosphoacceptors including: physiological target proteins, short peptides comprised of a kinase-specific recognition sequence, and random copolymers. Although structurally similar, the protein kinase enzyme class exhibits a range of kinetic mechanisms whose rates are dominated by a variety of steps. This diversity was previously reviewed [8] and a decade of subsequent research confirms the varied kinetic nature of these enzymes.

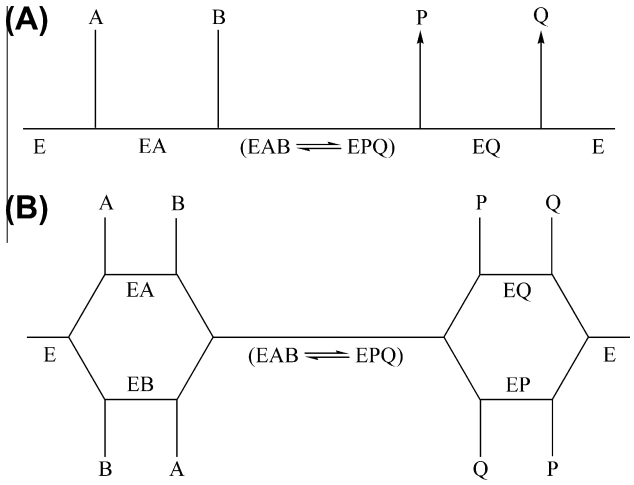


Fig. 2. Cleland diagrams for sequential mechanisms of phosphoryl group transfer reactions in protein kinase catalysis. (A) Ordered sequential mechanism. For kinases exhibiting this mechanism, A is commonly but not necessarily ATP. (B) Random sequential mechanism. In this mechanism both substrates can form a productive binary complex with enzyme.

The substrate addition order is also important in the understanding of the kinetic mechanism. There is a continuum of mechanisms between a random one with independent substrate binding and a highly synergistic one where the binding appears ordered. Most ordered kinetic mechanisms are limiting cases of random mechanisms where there is high synergism between the binding of the substrates. The synergism makes the more tightly bound one appear to bind first. Changing the substrates can alter the degree of synergism and thus the apparent kinetic mechanism. The order of substrate addition varies among protein kinases; however, random binding is the most commonly reported observation (Fig. 2B). For example, both in JNK2α2 catalysis employing the activating transcription factor 2 (ATF2) protein substrate and WNK1-mediated peptide phosphorylation random binding is observed [66,67]. Frequently, these random mechanisms operate under rapid equilibrium conditions (e.g., Aurora B and Cdk5/p25) [68,69]. Nevertheless, observations of ordered substrate addition have been reported (e.g. VEGFR2, PKCθ, AKT and S6K1) (Fig. 2A) [70–73]. In these examples, ATP is the first substrate to bind; a common characteristic for protein kinases exhibiting ordered mechanisms. As such, a diversity of substrate addition orders are possible with a random addition most frequently observed.

The steps governing catalytic rate are similarly varied, but the kinase reactions studied to date are generally limited by the chemical step, ADP product release, or internal conformational motions. Frequently, catalysis is only partially limited by a single step. Use of slow reacting ATP analogs, pre-steady-state kinetic observations, catalytic trapping experiments (quench-flow methods), and viscosity effects resulting from slowed product release are helpful in assessing factors contributing to catalytic rate [8]. For example, phosphorylation of the random copolymer poly(Glu_4Tyr) by Csk is limited by the chemical step, whereas the reactions of Tie2, FAK1 and PKCθ are partially limited by chemistry and an internal conformational change or ADP product release [48,71,74,75]. Pre-steady-state analysis of PDK1 indicates that the rate of this reaction is dominated by internal conformational changes, while similar observations combined with catalytic trapping experiments demonstrate that S6K1 catalysis is limited by ADP product release [73,76]. Turnover by the epidermal growth factor receptor HER2 is partially limited by both ADP product release and a conformational change [77]. Thus kinase catalytic efficiencies are governed by many factors in a kinase-specific manner.

The interpretation of a kinetic mechanism can be influenced by the choice of phosphoacceptor. Out of convenience or necessity, small peptide substrates devoid of distal recognition elements are routinely used in kinetic studies. In addition to altering the phosphoacceptor's affinity for enzyme, use of a larger substrate might impact catalysis by blocking association of ATP or by promoting conformational changes through interactions at locations remote to the active site. Substitution of peptide with full length native protein substrate can result in an altered kinetic profile for *in vitro* kinase reactions. A number of cases demonstrate that in protein kinase catalysis, the kinetic behavior is affected by the

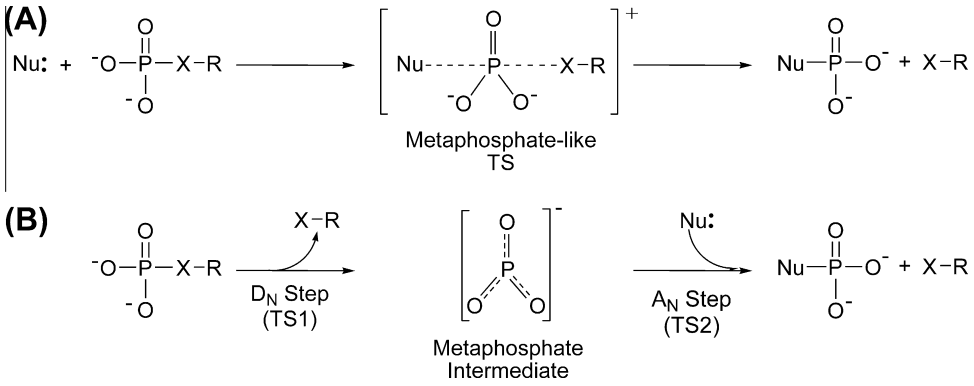


Fig. 3. (A) Concerted and (B) stepwise phosphotransfer mechanisms.

phosphoacceptor used. For example, PDK1-catalyzed phosphorylation of an extended peptide substrate containing a single distal recognition element reacts through a rapid equilibrium random mechanism [76]. When the phosphoacceptor is replaced with a native downstream target of PDK1, the S6K1 protein kinase, a switch to steady-state ordered mechanism is observed, where binding of S6K1 precludes association of ATP [78]. For the mitogen-activated protein kinase p38α, peptide reacts through an ordered mechanism with ATP binding first [79]. However, when catalyzing phosphotransfer to the protein ATF2, the reaction is shown to be ordered with protein binding first [80]. It should be noted that subsequent work on the latter reaction using an improved enzyme preparation suggests a random addition of substrates is more likely [81]. These studies reinforce the importance of distal recognition sites for a subset of protein kinases. Changing the peptide substrate is also sufficient to affect the kinetic profile of the reaction, even in the absence of distal recognition elements. *In vitro* EGFR catalytic domain reactions utilizing poly(Glu_6Ala_3Tyr) copolymer or [Val^5]-angiotension II peptide exhibit kinetic behavior consistent with random sequential mechanisms, but the same reaction using an unrelated 12 residue peptide is ordered with peptide binding first [82,83]. Whether the use of artificial substrates accurately reflects *in vivo* behavior is unresolved.

An added complication to kinetic analysis can be substrate inhibition by the phosphoacceptor. As the peptide becomes saturating, a kinase may form a non-productive ternary substrate complex with ATP. For PKA, a slow conformational change converts the nonproductive ensemble into a productive central complex [84]. For PKCθ, addition of NaCl ablates the observed substrate inhibition, without affecting ATP affinity [71]. The biochemical nature of these non-productive complexes between enzyme, ATP, and peptide are not fully understood and the biological relevance of substrate inhibition *in vivo* has yet to be determined. The importance of distal recognition elements, however, is known to be biologically important. Interactions at conserved docking regions in mitogen-activated protein kinases are required for the efficient enzymatic reactions of their physiological processes [85]. Not surprisingly, in a reaction catalyzed by the Tec family kinase ITK, addition of a single distal recognition element to the peptide decreases the K_m by fifteen-fold and increases k_{cat}/K_m fivefold [86]. As observed from a diversity of protein kinase kinetic studies, the choice of phosphoacceptor can be critical when determining the kinetic mechanism.

6. Chemical mechanism of phosphoryl group transfer

6.1. Mechanistic nomenclature

The mechanistic terms "associative" and "dissociative" have been applied to both the stepwise and concerted nucleophilic substitution reactions of phosphoryl group transfer (Fig. 3). Stepwise reactions are frequently referred to as either associative or dissociative mechanisms (Fig. 4). In stepwise phosphotransfer reactions, an associative mechanism proceeds via addition and elimination events, passing through a discrete adduct (e.g., pentavalent phosphorane intermediate) and is symbolized by $A_N + D_N$[1] [87,88]. A dissociative stepwise mechanism proceeds through an intermediate not covalently bonded to either the nucleophile or the leaving group (e.g., monomeric metaphosphate intermediate) and is symbolized as $D_N * A_N$. Concerted bimolecular reactions (A_ND_N) can also be described as associative (tight) or dissociative (loose), but in these cases the descriptor refers to the position of a single transition state (Fig. 4). In concerted phosphoryl transfer reactions, an associative transition state is one that is phosphorane-like (sum of bond orders to the nucleophile or leaving group >1) and a dissociative transition state is one that is metaphosphate-like (sum of bond orders to the nucleophile or leaving group <1).

6.2. Aqueous model chemistry

The chemistry of aqueous phosphotransfer reactions provides the foundation for the study of protein kinase catalysis. While still debated, there is a general consensus on the elements of solution-phase mechanisms that serve as a framework for characterizing the corresponding enzyme-catalyzed processes [89]. A brief synopsis of solution phosphoryl group transfer chemistry is provided to facilitate an examination of the molecular underpinnings of enzymatic catalysis [90–92].

The study of protein kinase chemical mechanism, in the simplest sense, is the study of the energetic, yet kinetically stable, phosphoanhydride bond and the reactivities of the singly substituted phosphoryl groups (e.g., alkyl and aryl phosphates) with phosphate monoesters most relevant. The rate of phosphate monoester hydrolysis greatly depends on both the pK_a of the leaving group and the ionization state of the phosphate group. Phosphate monoesters exist either as the dianionic **1**, monoanionic **2**, or

[1] The IUPAC nomenclature for reaction mechanisms will be used in place of ambiguous descriptors or the Ingold system (S_N1, S_N2, etc.) [87,88]. A reaction mechanism is symbolized by dividing the reaction into elementary steps. The symbol A_N is used to represent nucleophile addition while the symbol D_N is used to represent nucleofuge dissociation. A nucleofuge is defined as a leaving group that retains the bonding electron pair. When D_N and A_N occur in separate steps and a discrete intermediate is formed (stepwise mechanism), the terms are separated. In solution chemistry, the terms are separated by a plus sign ($D_N + A_N$) if the intermediate is long-lived enough to diffusionally separate from the leaving group. If the intermediate is high-energy and too short-lived to diffusionally separate (instead reacting within its solvation sphere) the terms are separated by an asterisk ($D_N * A_N$). In enzymatic reactions involving stepwise mechanisms it is generally accepted that intermediates do not diffuse from the reaction center and an asterisk (*) separates the terms for elementary steps. In a bimolecular reaction, when A_N and D_N occur in the same step (concerted mechanism), the terms are not separated (A_ND_N).

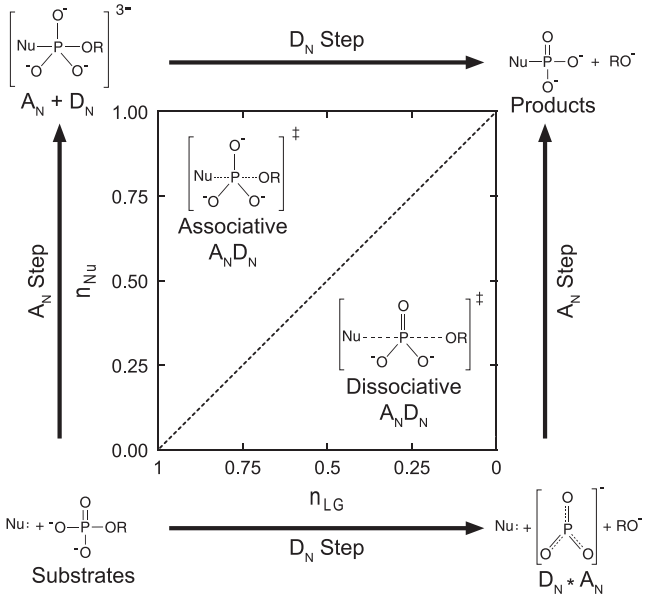
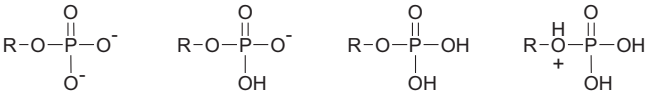


Fig. 4. Moore O'Farrell Jencks plot showing possible transition states and intermediates for phosphoryl group transfer reactions. Stepwise mechanisms proceed along the perimeters as they move across the reaction coordinate (passing through an intermediate at the corners) and concerted mechanisms proceed through the interior of the plot, with synchronous processes occurring on the diagonal line between substrates and products (- - - -). For concerted processes, as the transition state becomes either more associative or more dissociative, it falls closer to corners containing the corresponding intermediate. The diagonal line orthogonal to the dotted line (- - - -) would be all possible symmetrical transition states.

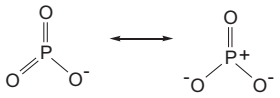
neutral, **3** species with a cationic species **4** occurring at very low pH. The monoanionic and dianionic species both occur in the physiological pH range, with hydrolysis proceeding through cleavage of the bridging P–O bond. For leaving groups with higher pK_a values, such as methylphosphate (p$K_a \sim 15.5$), the monoanionic species reacts much faster than the dianionic species: for hydrolysis $k_{monoanion}/k_{dianion} > 10^4$. However, as the leaving group becomes more activated, the difference in rate constants between the two reactivities decreases, with the dianionic species reacting faster for leaving groups whose pK_a is less than 5.5 [93]. These model systems are explored in detail as a framework for the subsequent analysis of protein kinase catalysis.



(**1**) (**2**) (**3**) (**4**)

6.3. Phosphate monoester dianions

Central to the understanding of phosphoryl group transfer is an understanding of the highly electrophilic metaphosphate intermediate species **5** [94]. This metaphosphate intermediate is extremely reactive, does not diffuse freely, and reacts within its solvation sphere [91]. The preponderance of evidence suggests that monoester dianions react by highly dissociative A_ND_N (concerted) mechanisms through metaphosphate-like transition states, switching to $D_N * A_N$ or $D_N + A_N$ (stepwise) mechanisms under special conditions (e.g., exceptionally good leaving group or solvolysis with a poor, non-solvating nucleophile) (Figs. 3 and 4).



(**5**)

A classic approach to understanding the transition state structure is measurement of Brønsted coefficients (β values) for the nucleophile (β_{nuc}) and leaving group (β_{lg}). The β value describes the net effective charge developing at the (rate-limiting) transition state on either the entering (nucleophile) or departing (leaving group) atoms by measuring linear free energy relationships between reaction rate and basicity of a set of substrates [95,96]. This allows one to infer the extent of bond-making and bond-breaking at the transition state. The reactions of the monoester dianions exhibit a small rate dependence on the basicity of the nucleophile (low β_{nuc}) and a large dependence on the basicity of the leaving group ($\beta_{lg} = -1.2$ for hydroxylic nucleophiles), indicating little bond making and significant bond breaking at the transition state [93,97,98]. As such, these phosphotransfer reactions appear to react through highly dissociative transition states (Fig. 3A) with little participation from the nucleophile and near complete departure of the leaving group. Additional evidence for these dissociative A_ND_N mechanisms are observations of near zero entropies of activation [93,97], large normal primary ^{18}O kinetic isotope effects (KIEs) for the bridging oxygen ($^{18}k = 1.0189$), and slightly (but significantly) inverse ^{18}O KIEs ($^{18}k = 0.9994$) for the non-bridging oxygen atoms [99]. Phosphotransfer reactions involving chiral phosphorus (using ^{16}O, ^{17}O, and ^{18}O labels) proceed with inversion of configuration, which is consistent with an A_ND_N mechanism (see below) [100]. While these observations alone are not sufficient to distinguish between highly dissociative A_ND_N and $D_N * A_N$ mechanisms, the existence of a discrete metaphosphate intermediate is unlikely (Fig. 3) [101].

Special cases exist in which the typical dissociative concerted reaction becomes a true stepwise process (Fig. 3). Reactions of arylphosphates using non-solvating, weak nucleophiles are reported to be stepwise. For solvolysis reactions in *t*-butanol, nucleophilic substitutions at chiral phosphorus proceed with complete racemization [100,102,103] and in one case with a significantly more positive entropy of activation [104]. This demonstrates that these reactions proceed through stepwise $D_N + A_N$ mechanisms, forming a freely diffusible metaphosphate intermediate in the absence of a nucleophile within its solvation sphere. In reactions of μ-monothiopyrophosphate trianion $(HO_3P–S–PO_3)^{3-}$ the weak P–S bond cleaves fast enough to form the metaphosphate intermediate, with attack of a solvating nucleophile occurring in a separate step from $HO_3P–S^-$ departure (Fig. 3B) [105,106]. Other more relevant special cases exist such as phosphate monoester monoanions.

6.4. Phosphate monoester monoanions

The presence of a proton on the non-bridging oxygen (phosphate monoanion **2**), under certain conditions, provides an improved kinetic route to phosphoryl group transfer. The non-bridging oxygen can transfer its proton to the leaving group, likely through the intermediacy of a water molecule (Fig. 5) [93,94]. When the leaving group is sufficiently basic, the proton can transfer in a step preceding nucleophilic attack (establishing a pre-equilibrium) (Fig. 5A). While highly unfavorable because of the weak basicity of the bridging oxygen, the resultant zwitterionic species is an excellent leaving group. With superior reactivity and a lower barrier to nucleofuge (footnote 1) dissociation,

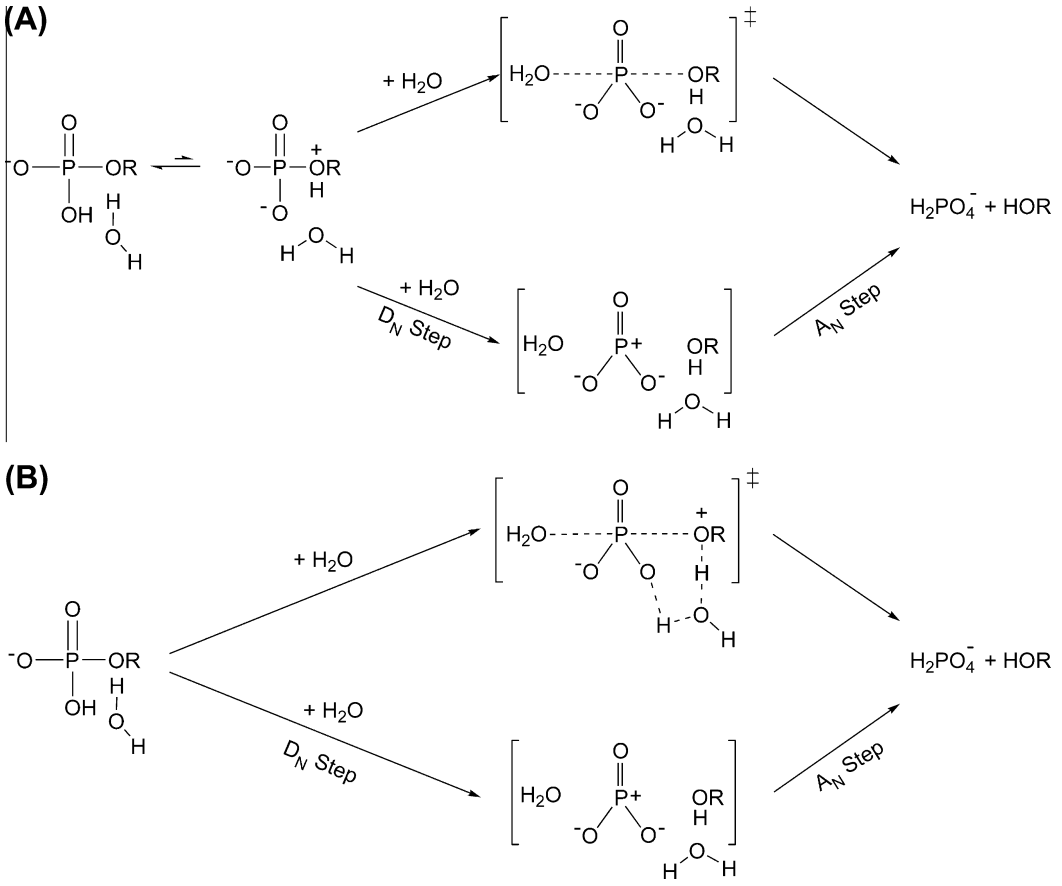


Fig. 5. Possible chemical mechanisms of aqueous phosphoryl group transfer for phosphate monoester monoanions Ref. [92]. (A) Mechanisms for leaving groups with high pK_a. (B) Mechanisms for leaving groups with low pK_a.

the reactions of the monoanion are much faster than those of the dianion when the pK_a of the leaving group is high. The rate of hydrolysis for the monoanions shows only a small dependence on the pK_a of the leaving group ($\beta_{lg} = -0.27$), suggesting that the leaving group is protonated at the transition state [93]. The weak inductive effect is due to the opposing contributions of increasing leaving group effectiveness (lowering its pK_a) and decreasing the basicity of the bridging oxygen (diminishing its ability to abstract the proton on the non-bridging oxygen). Furthermore, when the pK_a of the leaving group is decreased below a certain threshold, proton transfer becomes partially rate-limiting and occurs in concert with leaving group departure (Fig. 5B) [93,107]. Solvent isotope effects (^{D}k) for model reactions support this hypothesis. For the hydrolysis of methyl phosphate monoanion at 100 °C an inverse ^{D}k of 0.87 was observed, suggesting that protonation occurs in a pre-equilibrium step and not at the rate-limiting transition state (Fig. 5A) [108]. For the hydrolysis of 2,4-dinitrophenyl phosphate at 39 °C, a large normal ^{D}k of 1.45 was observed, consistent with proton transfer occurring concurrent with leaving group departure (Fig. 5B) [93]. Further evidence supporting the concerted proton transfer of leaving groups with lower pK_a's is found in the heavy atom KIEs for the hydrolysis of *p*-nitrophenyl phosphate monoanion [99]. A lower normal ^{18}O KIE for the bridging oxygen ($^{18}k = 1.0087$) indicates that proton transfer is advanced in the transition state and bond order to the phenolic oxygen is only slightly decreased. A large normal ^{18}O KIE for the non-bridging oxygen atoms ($^{18}k = 1.0184$) suggest it is no longer a secondary isotope effect, but rather a primary one resulting from proton transfer to the leaving group at the transition state.

A discrete metaphosphate intermediate in the reactions of the phosphate monoesters (Fig. 5), i.e., whether the reaction is a dissociative A_ND_N or a true $D_N * A_N$ mechanism, is still debated. Evidence indicates that these reactions are unimolecular with a rate dependence on the concentration of the phosphoester monoanion [93,94]. The stereochemistry of chirally labeled phosphorus in the solvolysis of monoanions of phenyl phosphate and phosphocreatine proceeds with inversion of configuration [100]. However, this may not be the result of a bimolecular mechanism but instead may indicate the high energy nature of the metaphosphate intermediate and reactivity by way of a "pre-association" mechanism. In this stepwise mechanism, the nucleophile is in an encounter complex prior to generation of the metaphosphate intermediate with product formation occurring more rapidly than any rotation leading to racemization. It has been suggested that hydrolysis of the monoanion of 2,4-dinitrophenyl phosphate is concerted [109], but for esters having more basic leaving groups (and therefore full protonation of the leaving group in a pre-equilibrium step) the reaction is stepwise [91,92].

6.5. Metaphosphate as a driving force for reactivity

An essential feature of phosphotransfer reactions is that they have a dissociative, metaphosphate-like transition state when reactants can form the dianionic phosphoryl species. When a dianionic state is unavailable, a different mechanistic solution is necessary (e.g., phosphomonoester monoanions). This effect is further exemplified in the reactions of higher substituted phosphate esters. Hydrolytic reactions involving phosphate diester monoanions **6** and phosphate triesters **7** are much slower than phosphate

monoester dianions containing the same leaving group (10^4 times slower in the case of the diester) [90]. Because forming metaphosphate or a metaphosphate-like transition state is not possible, phosphodiesters and phosphotriesters instead react through more associative A_ND_N mechanisms (Fig. 1 region 2). This is evident from large β_{nuc} values (>0.3, frequently much greater), indicating substantially more bond making to the nucleophile [92,110]. Due to the increased participation of the nucleophile in the transition state, more reactive nucleophiles can significantly increase the rate of these reactions (not the case for the highly dissociative A_ND_N mechanisms of the monoester dianions) [90]. As such, specific properties of the phosphoryl group can affect the phosphotransfer mechanism.

(**6**) (**7**)

6.6. γ-Phosphoryl group transfer for ATP

Like the phosphate monoesters, the terminal phosphate (γ-phosphoryl group) of ATP possesses a single substituent. However, the substitution is through a phosphoanhydride bond lending the molecule unique properties. In protein kinase-mediated processes, reactions of ATP proceed with transfer of the γ-phosphoryl group to a "phosphoacceptor" nucleophile (Scheme 1). While thermodynamically favorable, at 25 °C and near-neutral pH values, non-enzymatic γ-phosphoryl transfer from ATP is slow. In 2 M methanol, methanolysis proceeds with second order rate constants ranging from $3.9 \times 10^{-9}\ M^{-1}\ s^{-1}$ ($ATP^{4-}{\cdot}Mg^{2+}$) to $3.4 \times 10^{-10}\ M^{-1}\ s^{-1}$ (ATP^{4-}) [46]. A wealth of information indicates that, as phosphate monoester dianions, reactions of phosphoanhydrides occur by dissociative A_ND_N mechanisms. Reactivity is also highly dependent on the dianionic driving force necessary to form the metaphosphate-like transition state [111,112]. The β values for reactions of the phosphoanhydrides are similar to those of phosphate monoester dianions, consistent with a similar reaction mechanism. ATP^{4-} solvolysis by water and a series of simples alcohols yields a β_{nuc} of 0.07, indicating very little nucleophilic participation at the transition state [113]. Furthermore, a β_{lg} of −1.1 was observed for a series of phosphoanhydrides, establishing substantial bond breaking at the transition state. Thus for the uncatalyzed hydrolysis and phosphorylation reactions of biological alcohols (e.g., Ser/Thr/Tyr residues or sugars), the chemical mechanism is largely independent of the phosphoacceptor.

Model chemistry provides insight into how protein kinases may utilize divalent metal ions to promote efficient catalysis. Under a variety of solvolytic conditions, complexation to different divalent metal ions including Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Mn^{2+} show varied rate enhancements with respect to the uncatalyzed process [46,113,114]. Whether divalent metal ions enhance the rate by altering the reaction mechanism or by simply stabilizing the transition state has been investigated. A β_{nuc} of 0.06 for the solvolysis of ATP^{4-} in complex with Mg^{2+}, similar to the process without a divalent metal ion, indicates that there is minimal perturbation of the transition state structure along the reaction coordinate [113]. This result is in agreement with the lack of perturbation by Mg^{2+} and Ca^{2+} coordination to the transition state in nucleophilic substitution reactions of p-nitrophenyl phosphate [115]. In solution chemistry, divalent metal ions participate in the reaction as electrophilic catalysts through charge neutralization.

7. Chemical mechanism of protein kinases

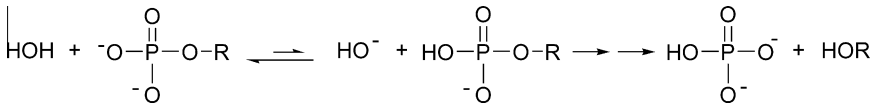
Enzyme-catalyzed γ-phosphoryl transfer reactions of ATP have been calculated to effect 10^{12}–10^{14} fold rate enhancements [46]. For protein kinases, a mechanistic explanation for this rate enhancement has been the source of considerable debate. Central to the debate is whether kinases facilitate catalysis by lowering the activation energy required for reaction or by fundamentally changing the chemical mechanism and altering the nature of the transition state. If the former is true, then enzyme-catalyzed γ-phosphoryl transfer should exhibit similar physical characteristics to the non-enzymatic reactions of phosphate monoesters and phosphoanhydrides. Because of the kinase domain structural similarity and use conserved catalytic residues, it has been suggested that all protein kinases share a common chemical mechanism differing only in their substrate and distal recognition elements [8]. Tyrosine kinase reactions have been used to study the chemical reactivity of this enzymes class because the electronic structure of its phosphoacceptor can be systematically perturbed.

7.1. Kinases and the dissociative transition state

As used for mechanistic analysis of aqueous phosphoryl group chemistry, pH-rate and Brønsted analysis have been essential in the characterization of the protein kinase mechanism. Systematic fluorine atom substitution on the phenol moiety of tyrosine kinase substrates for Csk and IRK was used to probe both the chemical mechanism and ionization state of reactants. The pH-rate profile analysis of IRK and Csk catalysis reveals a downward break on the basic limb of the curve for fluorinated substrates corresponding to the pK_a of the phenolic phosphoacceptor on the fluorotyrosyl peptides, indicating a preference for the neutral form [116,117]. In addition, the phenoxide ion form of the substrate is a weak competitive inhibitor of IRK. Elevated pK_a values for the phenol hydroxyl in fluorotyrosyl peptides bound to IRK and a failure of peptides with a negative charge *ortho* to the hydroxyl to be catalyzed by Csk demonstrates an electrostatic environment in the active site that prefers neutral charge on the nucleophilic tyrosine [117,118]. Neutral charge on the nucleophile is expected for reactions only weakly dependent on nucleophile reactivity, as significant electrostatic charge repulsion with the anionic phosphate would dominate reactivity.

Structure–function analysis was performed on Csk and IRK using tyrosine and fluorotyrosine containing peptides [116,117,119]. The β_{nuc} values of 0.08 (k_{cat}/K_m) and 0.07 (k_{cat}) in the reaction catalyzed by Csk and ≈0.1 (k_{cat}/K_m) in the reaction catalyzed by IRK are strikingly similar to those found for the dissociative A_ND_N mechanism governing ATP solvolysis. In addition, a linear free energy relationship was measured for the reverse reaction (ATP synthetic direction; Scheme 1) using phosphorylated tyrosine and fluorotyrosine peptides with Csk. A plot of log (k_{cat}/K_m) against pK_a of the leaving group exhibits a β_{lg} of −0.3, similar to the value for the non-enzymatic hydrolysis of phosphate monoester monoanions, indicating that the leaving group (the nucleophile in the physiological direction) is at least partially protonated at the transition state. Considering the preference for neutral charge on the nucleophilic oxygen, these results are consistent with a loose transition state exhibiting significant metaphosphate-like character and little bonding of phosphorus to either the nucleophile or the leaving group (dissociative A_ND_N). In terms of chemical mechanism, the findings for the enzymatic reactions are similar to those of related non-enzymatic processes.

An alternative model has been proposed to explain the low β_{nuc} values (and near unity γ non-bridging oxygen secondary KIE) observed with enzymatic and aqueous phosphoryl group transfer



Scheme 2. Substrate-assisted mechanism for phosphomonoester dianion hydrolysis.

reactions of kinases: substrate-assisted general base catalysis [120]. In this model, a non-bridging oxygen on the dianionic substrate phosphate acts as a catalytic base by first abstracting a proton from the nucleophile in a tautomeric equilibrium (Scheme 2). The deprotonated nucleophile then proceeds with nucleophilic displacement of the leaving group through an associative transition state analogous to the non-enzymatic phosphate diester reactions. The low β_{nuc} value thus results from the reduced capacity to abstract a proton from substrates with increasing pK_a values, offsetting the expected reaction rate increase for more reactive nucleophiles. Quantum mechanical calculations support this hypothesis as a possible alternative to the dissociative A_ND_N mechanism. Nonetheless, substrate-assisted general base catalysis remains controversial and was shown to be inconsistent with the reactivity or KIEs of model compounds in solution [99,107,109]. In addition, a mutant of Csk in which a catalytic aspartate residue in hydrogen bonding distance to the nucleophilic hydroxyl (see below) had been mutated to an asparagine residue shows a preference for the negatively charged phenolate ion nucleophile (albeit at a reduced rate compared to wild type) [121]. The linear free energy relationship for the fluorotyrosyl peptides in the anionic form, which are incapable of transferring a proton to the γ-phosphoryl group of ATP, still exhibit a small β_{nuc} value suggesting the tyrosine kinase reacts through a dissociative transition state. Taken as a whole, this alternative model does not seem to be a likely mechanism for phosphoryl group transfer reactions.

7.2. Structural insights into protein kinase mechanism

Crystallographic studies have identified key components in protein kinase active sites, provided insight into their mechanistic roles, and support a highly dissociative A_ND_N process [49,122]. When considering structural interpretation of transition states, it is imperative to understand the bond distances along the reaction coordinate. Contrary to the implication from the nomenclature, a highly dissociative A_ND_N mechanism for phosphoryl group transfer does not occur with a greatly expanded reaction coordinate; in solution, the reactions proceed with a very small volume of activation [123]. When the reactants start at van der Waals contact, the nucleophile and leaving group oxygen atoms do not significantly move during the reaction [89,124]. The non-bridging oxygen atoms on the transferred phosphate move only slightly toward the nucleophile, with the largest movement occurring for the phosphorus atom. In other words, during nucleophilic substitution the phosphorus moves from the leaving group to the nucleophile with little other motion. In the non-enzymatic model system, a symmetric transition state results in bond orders of only 0.05 to the attacking and departing oxygen atoms, with bond distances of 2.5 Å[2] [125]. For protein kinases, the reaction likely occurs with slight compression of the reaction coordinate and bond distances of 2.4 Å or 2.3 Å at the transition state, resulting in high dissociative character and bond orders of 0.08–0.11, respectively (see below). At bond orders greater than 0.15, the non-bridging γ-phosphoryl oxygen atoms do not move along the axis parallel to the reaction coordinate as phosphorus is transferred from leaving group to nucleophile. By convention, associative transition states begin with distances of $\leqslant$1.9 Å to the nucleophile and leaving group when the bond orders are more than 0.5.

Structural studies of the PKA catalytic domain have provided insight into the likely phosphotransfer transition state. Co-crystal structures representing the substrate complex were solved in the presence of ATP/ATP analogs and an inhibitory peptide containing alanine in place of the nucleophilic serine (substrate analog) [49,126,127]. Another critical co-crystal structure representing the product complex with a phosphopeptide was solved and used in the superpositioning of reactant-like and product-like co-crystal structures to calculate an approximate distance traversed by the phosphorus atom during the reaction (1.5 Å) [122]. Assuming a symmetrical transition state and in-line transfer, the bond orders are estimated at 0.084 to the nucleophilic and leaving group oxygen atoms (4.8 Å along the reaction coordinate) [124]. This distance is within error of a previously determined value derived from an NMR study using Co(III) and Cr(III) complexes of substrate peptide and an ATP analog [128]. In addition, recent co-crystal structures of PKA crystallized with ADP, a serine peptide substrate, and AlF_3 (a trigonal planer molecule that serves as an analog of metaphosphate) show serine aligned for an in-line transfer of the phosphate moiety and Al–O distances of 2.3 Å for the nucleophilic and leaving group oxygen atoms; this allows a bond order of 0.11 to be estimated at the transition state [129]. Crystallographic studies of protein kinase catalytic domains are consistent with a highly dissociative transition state.

7.3. Catalyzing the dissociative enzymatic mechanism

Knowledge of the protein kinase transition state provides insight into mechanistic strategies that facilitate enzymatic catalysis. Detailed analyses of the molecular underpinnings for dissociative A_ND_N reaction mechanisms are reviewed elsewhere and briefly summarized here [90,113,130,131]. A common strategy to affect enzymatic catalysis is to stabilize developing charge in the transition state through electrostatic interactions with active site residues and cofactors. With a variety of basic and acidic residues and two metal ion cofactors in the active site of protein kinases, there is ample opportunity to apply this strategy. How these factors participate in the mechanism has been the subject of focused research. In the dissociative transition states of phosphotransfer reactions involving ATP, the nucleophilic alcohol is largely unchanged from the ground state and therefore electrostatic interactions applied for the purpose of stabilizing charge on these atoms are ineffective [113]. The non-bridging γ-phosphoryl oxygen atoms experience a slight decrease in charge compared to the ground state, and potential interactions with positive charge at the transition state would be anti-catalytic. The β–γ bridging oxygen experiences significant development of negative charge, as bond breaking is nearly complete in the transition state; application of a positive charge at this position would provide a significant stabilizing force. Likewise, coordination of a positive charge by the non-bridging β-phosphoryl oxygen atoms would provide a stabilizing force to the leaving group by reducing charge buildup on the bridging oxygen.

[2] Bond order to the nucleophile and leaving group at the transition state is described as $D(n) = D(1) - 0.60 \log (n)$ where $D(n)$ is the bond distance to the fractional bond with bond order (n) and $D(1)$ is the single bond distance, which is 1.73 Å for a P–O bond.

Other mechanistic strategies are invoked by kinases to affect catalysis. In a discussion of chemical mechanism, the key factor is orientation. Juxtaposition of substrates by aligning them in the active site in a configuration appropriate for catalysis can have profound influence on reactivity [47,132]. Attacking nucleophiles do not want to line up anti to the leaving group, but rather prefer to hydrogen bond to the non-bridge oxygens. This gives a geometry not conducive to reaction. Thus an enzymatic function in catalysis is forcing the correct geometry to occur. This is what also drives ATPases and ATP synthase enzyme catalysis. Force the correct geometry and the non-bridge oxygens and the attacking and leaving groups do not have to move, but only the phosphorus jumps back and forth. Another strategy enzymes can use is the desolvation of substrates, as hydrogen bonding in aqueous solution can decrease reactivity for electronic and steric reasons [131]. Removal of these solvent interactions concomitant with replacement by specific electrostatic interactions in the active site can be a significant driving force in many enzymes. Key interactions are examined in detail in the following section.

7.4. Role of conserved active site aspartate residue

Protein kinases contain a highly conserved aspartate residue (D166 in PKA) in the catalytic loop that is within hydrogen bonding distance of the hydroxylic nucleophile [7,31,122,126] (Fig. 1). Mutation of this residue significantly reduces the reaction rate; however, it does not completely abolish activity. Replacement of the active site aspartate with alanine in PKA and phosphorylase kinase reduces k_{cat} by 2–3 orders of magnitude [133,134]. In Csk, mutation of aspartate to glutamate reduces k_{cat} by almost 10^4 [135]. In all cases, little effect is observed on substrate K_m values, suggesting that aspartate enhances the catalytic rate but is not essential for substrate binding. The role of the active site aspartate has been the focus of significant debate. While it has been proposed to act as a general base [122], facilitating catalysis through deprotonation of the nucleophilic alcohol, a number of arguments have shown this is unlikely [90,113,116,117,119,121,130,136,137]. For instance, pH-rate analyses of protein kinases frequently show a break on the acidic limb with pK_a values between 6 and 7, which has been used to support the hypothesis that the aspartate residue behaves as a general base [68,116,138]. However, other studies show that the ionizable group is not directly involved in the chemical step and more likely serves a role in substrate binding [137]. Pre-steady state measurements of the phosphoryl transfer step in PKA show that the break on the acidic limb of the pH rate profile is eliminated [136]. In Csk, the rate dependence on an ionizable group with this pK_a is substrate dependant; it is not observable when ionizable groups on the phosphoacceptor are removed [116]. If the protein kinase catalytic mechanism involves a general base, its pK_a must be outside the usual pH range employed in these studies (~5–10).

Conclusions drawn from analogous aqueous model reactions, as well as the establishment of their similarity to enzymatic processes, provide a rational basis to doubt the role of the conserved aspartate residue as a general base. Formal deprotonation of the hydroxyl group prior to nucleophilic attack seems unlikely based on linear free energy relationships measured for Csk. The β_{lg} value for the reverse reaction (Scheme 1) suggests that in the forward direction the nucleophile is protonated as it begins to attack ATP [116]. Furthermore, phosphoryl group transfer in protein kinases exhibits a low rate-dependence on substrate nucleophilicity. Based on the low β_{nuc} value, the slightly elevated rate garnered from increased substrate reactivity after deprotonation of the nucleophilic alcohol (around 50-fold from nucleophilicity alone) would not be enough to overcome the rate reduction resulting from electrostatic repulsion between the negatively charged oxyanion and phosphate moieties; the resultant overall effect should be anti-catalytic [96,116]. Early deprotonation by the aspartate residue during displacement is also questionable. Over the course of the reaction there is a large decrease in pK_a as the nucleophilic hydroxyl substrate is converted to the phosphate monoester product, but in a highly dissociative transition state the pK_a of the nucleophilic hydroxyl is largely unchanged from the ground state. While potentially achievable with a tyrosine residue (pK_a 10), it is unlikely that the active site aspartate residue is basic enough to abstract a proton from a serine or threonine hydroxyl (pK_a 13.6–15) [139]. It seems more likely that proton abstraction from the nucleophile happens much later along the reaction coordinate (after the transition state), when the pK_a of the reacting oxygen nucleophile is much lower and equal to that of the aspartate residue.

Although the catalytic aspartate residue may not serve the role of a general base (proton transfer at the transition state), it may be important in positioning the substrate hydroxyl for in-line nucleophilic attack [136]. An important rotamer for attack on the γ-phosphoryl group of ATP may be isolated on the nucleophilic alcohol by hydrogen bonding to the carboxyl group of aspartate. Co-localizing substrates in the active site of an enzyme and positioning them in correct alignment for catalysis may provide upwards of 10^8 in rate enhancement, well above the expected effect from this residue [47]. Alternatively, the catalytic aspartate residue may assist in substrate dissociation through repulsion of the negatively charged phosphate ester product [122] (see Table 1).

Computational analyses of the PKA reaction have been employed to shed light on the roles of the catalytic residues. Initial semi-empirical and DFT (density functional theory) methods suggest that the reaction proceeds by way of a substrate assisted mechanism (Scheme 2), with the catalytic loop aspartate residue potentially mediating proton transfer to the γ-phosphate [140–142]. These conclusions, however, are inconsistent with experimental results as well as more sophisticated computational analysis. For the phosphotransfer reactions catalyzed by protein kinases, semi-empirical methods may not be adequate and calculation of reaction barriers by DFT methods are highly dependent on the model size and crystal structure used in the analysis [143,144]. More sophisticated DFT calculations suggest a role for the aspartate residue as late proton acceptor, with the reaction proceeding through a dissociative transition state containing a largely intact O-H bond in the serine nucleophile [145,146]. The hydroxylic proton on the serine residue transfers to the aspartate residue late in the nucleophilic attack, after the transition state is reached. These results were consistent with a combined DFT QM/MM (quantum mechanics/molecular mechanics) study of the entire catalytic domain of PKA, which find that the serine retains its hydroxyl proton at the transition state, and the aspartate residue functions as a base late along the reaction coordinate [147]. Interestingly, changing the aspartate residue to alanine *in silico* does not significantly affect the configuration of serine leading the authors to believe that the residue does not act to orient the nucleophile. The conclusions drawn from the more recent DFT calculations are consistent with experimental results. Taken in the whole, the catalytic aspartate residue may be critical to catalysis but its participation as a general base acting at or before the transition state for phosphotransfer is unlikely. The main role of the catalytic aspartate residue would be as a proton acceptor after the phosphotransfer transition state which would reduce the rate of the back reaction.

7.5. Leaving group stabilization

The presence of positively charged residues and Mg^{2+} cofactors within hydrogen bonding distance of the ADP leaving group may

Table 1
List of kinase acronyms cited in text.

Kinase Acronym	Name	Common alternate name
Akt	AK-transforming	PKB
ALK	Anaplastic lymphoma kinase	
ATM	Ataxia telangiectasia mutated	
AurB	Aurora kinase B	Aur1
BCR-Abl	Break point cluster-Abelson tyrosine kinase	
BLK	B-cell lymphocyte kinase	
BMX	Bone marrow kinase X-linked	
B-Raf	Rapidly accelerated fibrosarcoma kinase B	
BTK	Bruton's tyrosine kinase	
Cdk2,	Cyclin-dependent kinase	
CLK	CDC-like kinase	
cMet	Mesenchymal–epithelial transition proto-oncogene	HGFR
Csk	C-terminal Src kinase	
DSTYK	Dual serine/threonine and tyrosine protein kinase	RIPK5
DYRK	Dual specificity tyrosine phosphorylation-regulated kinase	
EGFR	Epithelial growth factor receptor	ErbB1
EML4-ALK	Echinoderm microtubule-associated protein-like 4 ALK	
ErbB2	v-erb-a erythroblastic leukemia viral oncogene homolog 2	HER2
ErbB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	HER4
ERK	Extracellular-signal-regulated kinases	MAPK
FAK	Focal adhesion kinase	
FGFR	Fibroblast growth factor receptor	
HER2	Human epidermal growth factor receptor 2	Neu, ErbB-2
IRK	Insulin receptor kinase	IR
ITK	IL2-inducible T-cell kinase	
JAK	Janus kinase	
JNK	c-Jun N-terminal kinase	
Lck	Lymphocyte kinase	
MEK	MAPK/ERK kinase	MKK, MAP2 K
MKK7	Mitogen-activated protein kinase kinase 7	JNKK2, MAP2K7
Mps1	Monopolar spindle 1	TTK
PAK	p21-activated kinase	
PDK	3-Phosphoinositide-dependant protein kinase	
PI3K	Phosphoinositide 3-kinase	
PKA	Protein kinase A	cAMP-dependent kinase
PKC	Protein kinase C	
RIOK	Right open reading frame (RIO) kinase	
S6K1	70-kDa 40S ribosomal protein S6 kinase-1	p70 S6 kinase 1
Src	Sarcoma kinase	
Syk	Spleen tyrosine kinase	
TEC	Tyrosine kinase expressed in hepatocellular carcinoma	
TESK	Testis-specific protein kinase	
Tie2	Tyrosine kinase with Ig and EGF homology domain-2	TEK
TXK	T-cell-restricted protein tyrosine kinase	Rlk
VEGFR2	Vascular endothelial growth factor receptor	KDR
Wnk	With no lysine kinase	

provide a means of facilitating nucleophilic substitution at the γ-phosphate of ATP. In the dissociative transition state, considerable negative charge develops on the β–γ bridging oxygen, as well as a significant increase in negative charge on both β-nonbridging oxygen atoms [113]. General acid and electrophilic catalysis is expected to promote bond cleavage in these reactions [130].

Overall factors contributing to stabilization of the ADP leaving group have been difficult to assess, however interactions with the β-non-bridging oxygen atoms have been proposed. Protein kinases contain two Mg^{2+} ions in contact with non-bridging oxygen atoms in the active site (Fig. 1). The essential Mg^{2+} (which is present in crystals formed at low Mg^{2+} concentrations) is in complex with a non-bridging oxygen atom on the β- and γ-phosphates and is required for catalytic activity. For PKA, this is termed the activating Mg^{2+}, as the second one (which complexes the α- and γ-phosphates and is present in crystals formed at high Mg^{2+} concentrations) is inhibitory [148]. In other protein kinases, such as IRK, the Mg^{2+} present at high concentrations may only complex the β-phosphate and is not inhibitory [149]. The effect of the essential Mg^{2+} has been estimated through mutation of the DFG catalytic loop aspartate residue (Asp-184 in PKA), a residue responsible for positioning the divalent metal ion. Metal ion catalysis in PKA is estimated to contribute at least a factor of $10^{2.5}$ to the rate enhancement based on the alanine mutant, with a factor of $\geqslant 10^{4.5}$ more likely [124]. Additional roles for the essential Mg^{2+} have been proposed but not defined experimentally: positioning the γ-phosphate for nucleophilic attack and masking charge in order to limit charge repulsion with the incoming nucleophile [8]. The second Mg^{2+} ion's role in catalysis is poorly understood as it has a variety of effects for different protein kinases. Csk and Src show an increase in k_{cat} with no effect to the K_m of ATP upon binding of the second Mg^{2+} [150]. In IRK and v-Fps, this event results in a decrease to the K_m of ATP but no effect on k_{cat} [151,152]. While with FGFR and Cdk5, both an increase to k_{cat} and decrease to the K_m of ATP is observed [68,150].

The strictly conserved lysine residue complexes with the non-bridging oxygen atom(s): Lys-72 in PKA binds the α- and β-phosphates [31] while Lys-1030 of IRK binds only the α-phosphate (Fig. 1) [149]. For a number of protein kinases, this lysine residue is characterized as essential to catalysis but not ATP binding. This lysine residue may be important for transition state stabilization, orienting ATP for catalysis, or both. In PKA, replacement with alanine results in an 800-fold decrease in k_{cat} but only a 6-fold increase in the K_m of ATP [133]. Substitution with arginine in the

non-receptor tyrosine kinase Lck results in no detectable turnover or effect on ATP binding as judged by its affinity for the ATP analog 8-azido-ATP [153]. Replacement by either arginine or alanine in the ERK2 yields mutant enzymes exhibiting a two-order of magnitude reduction in k_{cat} but no more than a twofold increase in the K_m of ATP for a variety of phosphoacceptors [154].

The transition state for phosphotransfer reactions in protein kinases is likely stabilized by a number of different interactions working in concert to provide the large observed catalytic rate enhancement. In a DFT QM/MM computational study of the entire PKA catalytic subunit, contributions to catalysis from the essential Mg^{2+} and lysine residues are elucidated from the calculated transition state model [147]. These factors are found to contribute significantly in reducing the transition state barrier by applying strong electrostatic interactions to the ADP leaving group, with lysine and the essential Mg^{2+} reducing the barrier by approximately 18 and 32 kcal/mol, respectively. Furthermore, the combined effect from nucleotide complexation by conserved glycine-rich loop residues provide an additional ~8 kcal/mol stabilization to the transition state barrier. Differential transition state stabilization methodology (MP2 calculations) applied to this transition state model for the PKA reaction are consistent with the original *ab initio* calculations, indicating that of the conserved lysine residue and the essential Mg^{2+} are responsible for stabilizing the transition state by 22.7 and 32.4 kcal/mol, respectively [155].

8. Kinases as drug targets

Commensurate with their dominant role in the regulation of a wide array of cellular functions, protein kinases have emerged as an important enzyme class for the discovery of therapeutic agents that significantly impact human disease. As of early 2011, there are 14 regulatory approved kinase inhibitor drugs with additional approvals likely (Table 2). The expected global market for kinase directed therapies is estimated to be $35 billion in 2013 with further growth expected. Despite success on a small subset of kinases, the scope of this research remains limited. One source cites that of the 149 kinase inhibitors that have entered clinical trials target only 42 are protein kinases [43]. The narrow research focus is compounded by the fact that about 50% of these inhibitors target kinases which already have approved drugs [43]. Basic kinase research is focused on only a small fraction of the kinome, which likely contributes to the lack of novel kinase drugs currently in clinical studies. As such, there is significant promise for breakthrough therapies directed towards previously untargeted kinases. From this analysis, kinase drug discovery is still ascendant.

Early in the field of kinase drug discovery research, high cellar ATP concentration (1–4 mM) [19–22] and relatively low protein kinase $K_{m,ATP}$ values (typically 5–200 μM) [156] caused many to question the utility of kinases as drug targets. These early skeptics reasoned that ATP-saturated active sites would not available for drug binding (poor cellular potency). In addition, there were concerns that ATP-competitive inhibitors would lack sufficient kinase selectivity because all kinases have evolved to bind ATP. Two underlying assumptions for these concerns were that: (a) small molecule inhibitors and ATP bind equivalently (same molecular contacts and and/or binding properties), (b) the ATP Michaelis constant ($K_{m,ATP}$) is equivalent to the dissociation constant (K_d). We now know that these assumptions have limitations and that protein kinase architecture and dynamics are more complex than initially thought. As was illustrated in the structural description of the catalytic domain, a subset of the active site is evolved to facilitate phosphate transfer when the protein is in an active conformation. However, regions in the active site not directly involved in catalysis are structurally diverse presumably because there is no evolutionary pressure to maintain uniform geometric arrangement of amino acid residues. In addition, inactive conformations of the catalytic domain may be structurally diverse for similar reasons. As such, small molecule, ATP-competitive inhibitors with starkly different kinetic properties from ATP are possible and can achieve vastly different interactions with protein kinases: potent binding (e.g. axitinib $K_i = 28$ pM [157]), low half-lives of the protein–inhibitor complex (axitinib $t_{1/2} > 2$ h [157]), and kinase selectivity (PF-04217903 > 1000-fold selective for cMet relative to 208 kinases [158]). Long inhibitor residence times [159] are derived from slow off-rates which may contribute to successful competition with ATP. As for the $K_{m,ATP}$, it will be equivalent to a dissociation constant (K_d) when the enzyme operates under rapid equilibrium, conditions. For Bi Bi sequential mechanisms of protein kinases, there are multiple kinetic mechanisms possible. Interestingly, the arguments against kinase as drug targets are being raised again to explain the mechanism of acquired resistance of protein kinases (e.g. EGFR, EML4-ALK) towards successful ATP-competitive drugs (e.g. erlotinib) [160].

Table 2
Approved protein kinase inhibitor drugs.

Generic name	Brand name	Number	Year approved	Known targets	Disease
Fasudil	–	HA-1077	1999	ROCK	Cerebral vasospasm
Sirolimus	Rapamune	Rapamycin	2000	mTOR	Organ rejection (kidney) prevention
Imatinib	Gleevec	STI571	2001	Abl, Arg, PDGFR, cKit	CML, GIST, ALL, other (MDS/MPD, ASM, HES, CEL)
Nilotinib	Tasigna	AMN107	2007	Abl, Arg, cKit, PDGFR, others	CML with resistance to imatinib or intolerance
Dasatinib	Sprycel	BMS-354825	2007	Abl, Arg, cKit, PDGFR, Src and others	CML
Gefitinib	Iressa	ZD1839	2004	EGFR	NSCLC
Erlotinib	Tarceva	OSI-774	2004	EGFR	NSCLC, PC with gemcitabine
Lapatinib	Tykerb	GW572016	2007	EGFR (ErbB-1, ErbB-2)	Breast cancer (Her2-positive)
Sorafenib	Nexavar	BAY 43-9006	2006	B-Raf, VEGFR, PDGFR, FLT3, cKit	RCC
Sunitinib	Sutent	SU11248	2006	VEGFR, PDGFR, FLT3, cKit	RCC, GIST, pancreatic NET
Temsirolimus	Torisel	CCI-779	2007	mTOR	RCC
Everolimus	Afinitor	RAD001	2009	mTOR	Advanced RCC, pancreatic NET, SEGA, Organ rejection prophylaxis,
Pazopanib	Votrient	GW786034	2009	VEGFR, PDGFR, cKit	RCC
Vandetanib	Zactima	ZD6474	2011	Ret, VEGFR, EGFR	Thyroid cancer
Crizotinib	–	PF-2341066	–	ALK, cMet, Ros	NSCLC (ALK positive)

Abbreviations: CML, chronic myeloid leukemia; RCC, renal cell carcinoma; GIST, gastrointestinal stromal tumors; NSCLC, non-small-cell lung cancers; ALL, acute lymphoblastic leukemia; NET, neuroendocrine tumors ; MDS/MPD, myelodysplastic/myeloproliferative diseases; ASM, ; HES, hypereosinophilic syndrome; CEL, chronic eosinophilic leukemia; SEGA, subependymal giant cell astrocytoma; PC, pancreatic carcinomas.

Proper biochemical assessment of the mechanism of action (MOA) of kinase inhibitors requires careful analysis using appropriate experimental conditions. To achieve this end, many fundamental questions need to be addressed. (a) Is the protein appropriate for meaningful studies? (b) What is the biologically-relevant biochemical potency? (c) What is the inhibitor mechanism of action? (d) Is the inhibitor effective towards clinically relevant mutations? A detailed treatment is beyond the scope of this review but is briefly discussed in order to illustrate the need for high-quality biochemical analysis of kinase inhibition. The protein (construct and quality) is the foundation for meaningful biochemical analysis with a diversity of approaches reported in the literature: full-length protein, isolated catalytic domain, affinity tagged protein, phosphorylated protein, unphosphorylated protein, properly folded protein (100% of active sites available). Kinetic findings for kinases can be dependent on the protein selected for the assay. For instance, studies of a GST fusion with VEGFR2 cytoplasmic domain report a k_{cat} value of 2.7 s^{-1} [161] while a k_{cat} value of 27.5 s^{-1} has been reported for the isolated, untagged catalytic domain with over 90% of the active sites intact [157]. The k_{cat} value for the hexahistidine-tagged cytoplasmic domain of cMet was reported as 0.0095 s^{-1}, [162] quite different from the untagged catalytic domain: 1 s^{-1} (unphosphorylated cMet) and 31 s^{-1} (autophosphorylated cMet) [158]. The difference in k_{cat} values (a specific activity surrogate) could be due to many factors including uncleaved affinity tag, the phosphoacceptor used, the enzyme purity, or the number of competent active sites (conformational purity). Low specific activity protein decreases confidence in the relevance of kinetic findings. High activity protein has the added benefit of a lower enzyme level in biochemical assays which extends the useful range in inhibitor assessments typically to K_i value determinations in the high pM to low nM range. In addition, the context of the kinase catalytic domain is commonly overlooked. Many biochemical studies of protein kinases treat catalytic domains as modular units that are independent of the rest of the protein. This simplification has utility but it also has limitations. For VEGFR2, addition of a 19 residue juxtamembrane domain to the catalytic domain has a profound effect on the measured potency of a highly optimized drug (K_i = 28 vs 1100 pM) [157]. Although differing protein constructs exist, all effective biochemical assays predict kinase behavior in more complex systems (e.g. cellular or *in vivo*). Choosing the protein construct that predicts cellular activity is a minimum requirement for effective assay design.

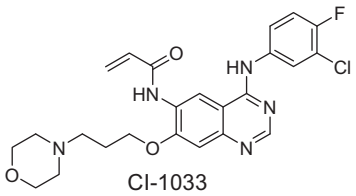
Another significant contribution to meaningful biochemical analysis is the type of enzyme assay employed. There are a myriad of kinase activity assays available [48,163–167]. Some are more relevant for high throughput screening of large numbers of compounds (potency estimation) while others are better for detailed enzymatic analysis and biochemical inhibitor characterization. Real-time, continuous assays offer advantages over discontinuous assays including that enzyme reaction velocity is defined through multiple experimental observations, not just a single value. Irregularities in enzymatic activity (e.g. non-linear product formation timecourse) and inhibition (potency time-dependence) are readily detected. Fitting of kinetic data can also affect the determined inhibitor potency. Ideally, inhibitor potency should be calculated by fitting the kinetic data to the equation that describes the mechanism of inhibition (simple competitive inhibition, tight-binding competitive inhibition) [168,169]. A K_i potency value should provide an assay-independent parameter that directly correlates with binding energetics (ΔG). Reporting IC_{50} values masks useful information (e.g. tight-binding). For the same reason, simple Cheng–Prusoff conversions of IC_{50} values to K_i values [170] can also be misleading. Another limitation in measuring IC_{50} values is that the useful range is truncated because analysis does not account for enzyme depletion. IC_{50} values hit a potency plateau when potent inhibitors fully titrate the enzyme in the assay. These limitations of IC_{50} determination can be overcome in a number of ways. K_i values are a true measure of affinity and as such assay conditions can be manipulated [157,169]. For example, the concentration of the competitive substrate (e.g. ATP) can be increased to decrease the apparent inhibitor potency and thus extend the assay's useful range [169]. Inhibitors bind to enzyme free of the competitive substrate, not total enzyme, so increasing the substrate concentration will decrease the amount of free enzyme and increase the lower limit of the assay's useful range. Another strategy is to use an equation that accounts for enzyme depletion (tight binding phenomenon): the quadratic equation (Morrison equation) [157,168,169]. Another element in assay design is the phosphoacceptor used. Typically, there are only modest phosphoacceptor specific effects on ATP-competitive inhibitor potency. The potency (K_i) of the VEGFR2 inhibitor axitinib using a common receptor tyrosine kinase substrate, poly(Glu_4,Tyr) random copolymer, is 1.1 nM while it is 0.9 nM for a VEGFR2 activation loop peptide [157]. Although not typical, the phosphoacceptor can affect observed biochemical potency. A VEGFR2 peptide substrate (minigastrin) was shown to increase an ATP-competitive inhibitor's potency (axitinib) by over an order of magnitude (Brion, Murray, unpublished observation). In addition, peptide substrate has been reported to alter the inhibitor potency of a PAK inhibitor by 5-fold [171]. As seen in this brief overview, there are many factors that can affect the measured enzyme activity and inhibitor potency.

Exploring the inhibitor mechanism of action can further refine the level of biochemical understanding, facilitate meaningful structure–activity assessments (e.g. similar substrate competitiveness), and properly defined potency (e.g. time-dependence) to enable assessments in more complex systems (cellular potency, pharmacodynamic/pharmacokinetic *in vivo* studies, etc.). The majority of kinase inhibitors reported to date are reversible inhibitors but irreversible inhibition as a drug modality is also an effective strategy (see irreversible inhibition section). The first step in MOA studies is confirmation of biochemical potency in an orthogonal assay. A second biochemical assay relying on a different detection modality using a different phosphoacceptor can facilitate the confirmation of potency. Alternatively, direct binding methods (surface plasmon resonance, isothermal calorimetry) are powerful tools because they are simple systems that minimize assay-specific artifacts. Ideally, K_i values determined through kinetic methods should correlate with K_d values derived from direct binding methods [157]. When potency differences occur, detailed analysis is prudent [171]. The next step in inhibitor characterization is determination of the time-dependency for the observed inhibition in order to insure that the measured potency is invariant. Simple inhibitor pre-incubation studies can illuminate time-dependent behavior while lower throughput inhibitor washout experiments (dilution, filtration) can confirm the findings. If no time-dependence is observed, then an inhibition can be characterized as a function of both substrate and inhibitor concentrations (double-reciprocal analysis [171]). This allows one to determine against which substrate an inhibitor is competitive. Double reciprocal analysis also illuminates how an inhibitor interacts with the enzyme (e.g. linear, hyperbolic) and thus gives an additional degree of confidence on the mechanistic assignment. A more modern approach involves global fitting of the progress curves measured under varying substrate and inhibitor concentrations with different kinetic models readily available in kinetic analysis programs (e.g. KinTeck Explorer, Dynafit). Although most kinase inhibitors are ATP-competitive, there are multiple sub-classes. Type I binds to the active form of the catalytic domain (DFG-in) (erlotinib, gefitinib, dasatinib, lapatinib, and sunitinib) [58]. Type II binds to an inactive, DFG-out conformation (imatinib, nilotinib, sorfenib and axitinib)

[58]. Unfortunately, this active/inactive conformation nomenclature is insufficient as the Type I inhibitor sunitintib has been shown to bind to a DFG-out cKit conformation [172]. An alternate conformation currently being targeted (DFG-in + αC-helix out) which is in between Type I and Type II is called Type $I_{1/2}$ [58]. New classes of ATP-competitive inhibitors are emerging (potent inhibition through an inhibitor halogen atom with the hinge region [173,174]) which have not yet been mechanistically assigned. The multiple conformations targeted by ATP-competitive inhibitors underscore the richness of the catalytic domain dynamics [175]. Detailed inhibitor characterization should encompass potency assessments with regard to conformation (e.g. active, inactive) which sometimes can be assessed by measuring the potency toward different phosphorylation states of the kinase [157]. Biochemical methods have been developed to distinguish between different types of ATP-competitive inhibitors by assessing activation state specific inhibitor potency either through binding [176] or kinetic assays [157]. In addition, there is another class of kinase inhibitor that binds to an allosteric site (non-ATP competitive) responsible for regulating kinase activity. The classic example for this mode of inhibition is the MEK inhibitor PD098059 [177] but the strategy continues to be utilized for other kinases such as Akt, KDR, Tie2, and Raf [178]. As observed from the study of known kinase inhibitors, there is a diversity of inhibitor interactions which continues to expand.

9. Irreversible Inhibition

Irreversible inhibition of protein kinases is an emerging field in kinase drug design. Inhibition is achieved through covalent modification of a kinase residue which blocks catalytic activity. These inhibitors have been primarily confined to ATP-competitive, EGFR family inhibitors with crotonamide Michael-acceptor electrophiles (e.g. CI-1033) strategically oriented to react with the hinge region cysteine residue nucleophiles (Fig. 1) [179].



CI-1033

To date, clinical trials of irreversible kinase inhibitors have had mixed success. EGFR drug discovery efforts began in the 1990s with CI-1033 as the first irreversible to enter clinical trials. Although CI-1033 was discontinued after Phase II studies [180], four EGFR irreversible inhibitor drugs reside in late-stage clinical trials: PF-299804 [181], Neratinib (HKI-272) [182], Pelitinib (EKB-569) [183], and Afatinib (BIBW-2992) [184]. The outlook for these second-generation drugs is promising. Originally, irreversible inhibition was thought to be a niche approach as only eleven of the 538 protein kinases have a nucleophilic cysteine residue in the hinge region: TEC family (BTK, BMX, ITK, TEC, RLK), EGFR family (EGFR, ErbB2, ErbB4), and other kinases (BLK, JAK3, MKK7). More recently, 193 kinases were reported to have active site cysteine residues accessible in at least one catalytic domain conformation [185].

Irreversible inhibition provides an excellent opportunity for biochemical knowledge to guide drug discovery endeavors. Cell-based assays generally lack the temporal resolution necessary to independently characterize inhibitor reactivity and binding (Fig. 6). Thus reactivity and binding are combined into a single potency value masking the individual contributions of each step to the overall potency. This limitation also occurs in biochemical assessments that occur at a single time point (e.g. IC_{50} values). A re-evaluation of EGFR inhibitors previously reported in the literature is instructive. In simple biochemical studies, wild-type EGFR potency of CI-1033 (IC_{50} = 1.8 nM) is greater than the second-generation inhibitor PF-299804 (IC_{50} = 6.0 nM) [186]. The reported cellular activities of CI-1033 and PF-299804 show that CI-1033 more potently inhibits EGFR autophosphorylation (IC_{50} = 1.6 nM) compared to PF-299804 (IC_{50} = 5.8 nM) [186]. These data would indicate that CI-1033 has superior potency yet the simple, biochemical and cellular analysis of potency may be misleading. Preliminary findings on the detailed kinetic analysis of these molecules are discussed to illustrate the potential benefit of proper kinetic analysis (Brion, Murray, unpublished results). Kinetic values describing EGFR inactivation are derived by monitoring product formation as a function of inhibitor concentration and globally fitting the resulting progress curves to rate equations describing the contributing processes (e.g. product formation, inhibitor binding, covalent bond formation) through the use of a numerical integration method (e.g. Dynafit, KinTek Explorer). The first-order rate constant measuring the contribution of reactivity is k_{inact}, an analogous parameter to k_{cat} in simple single substrate Michaelis–Menton kinetics, describes the maximum rate of inactivation at an infinite concentration of inhibitor. Differences in k_{inact} values can be due to inherent reactivity or proper alignment for the reaction (steering effects). The K_i value is the dissociation constant for inhibitor binding while the K_I is analogous to the Michaelis constant (K_m). The ratio of k_{inact}/K_I is analogous to k_{cat}/K_m because both values describe overall reaction efficiency. Both CI-1033 and PF-299804 are very effective wild-type EGFR inhibitors (k_{inact}/K_i 2.9×10^7 vs 2.5×10^7 $M^{-1}s^{-1}$). Characterization of the individual contributions to overall potency reveals important distinctions between the two molecules. Although CI-1033 and PF-299804 have high affinity for wild-type EGFR, PF-299804 exhibits superior binding affinity (K_i = 0.5 and 0.1 nM respectively). However, the reactivity of CI-1033 is greater than PF-299804 (k_{inact} = 0.015 s^{-1} vs 0.003 s^{-1} respectively). Thus potency is derived through differential contributions; PF-299804 has more binding affinity and less reactivity to achieve equivalent overall effectiveness. In addition, these kinetic values can be used to estimate the amount of reactivity necessary for effective inhibition. A dynamic linkage exists between binding affinity and effective inhibitor reactivity. The amount of time a molecule resides in the binding site (residence time) is derived from the inverse of off-rates ($1/k_{off}$) [159]. The minimum time a molecule takes to achieve covalency (capture time) is derived from the inverse of reactivity ($1/k_{inact}$). A molecule with a long residence time will require less reactivity. Conceptually, the residence time should be longer than the capture period.

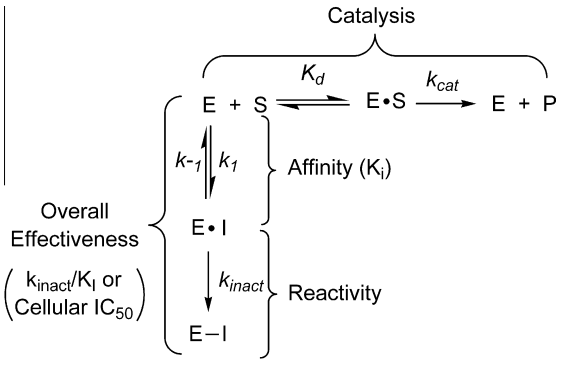


Fig. 6. Inactivation reactions that affect product formation in the inactivation of kinase enzymatic activity.

Reactivity above this threshold should not result in additional overall potency and may bring about unwanted off-target reactivity. The off-rate constant (k_{off}) can be estimated by measuring the off-rate of a non-reactive analog by surface plasmon resonance (SPR) or by measuring the off-rate of the drug to a non-reactive kinase (Cys/Ser mutation). From EGFR, it is apparent that proper biochemical analysis is uniquely qualified to impact irreversible drug design.

9.1. Breadth of inhibitor biological impact

Understanding the true scope of biochemical interactions an inhibitor is capable of making is essential for meaningful cellular analysis and effective drug discovery. This goal is complex because there are 538 human protein kinases and over 20,000 other proteins in humans. The complexity is magnified if there are mutations in the intended kinase target. Off-target activities can cause unwanted safety liabilities [156] or beneficial effects. Many successful small molecule kinase drugs treat patients utilizing unintended kinase inhibitor activities. The first FDA approved ATP-competitive kinase inhibitor (imatinib, Gleevec) was designed against BCR-Abl kinase for chronic myeloid leukemia but was also FDA-approved for use in cKit kinase driven diseases such as gastrointestinal stromal tumors [187,188]. Sorafenib was initially reported to be a selective Raf kinase inhibitor [189] but was later reclassified as a multi-kinase inhibitor for use in advanced renal cancer [190]. Off-target activities of kinase inhibitors should be expected and relying on serendipity should be avoided. Additional activities can be quite beneficial if they are proactively known. Crizotinib (PF-2341066) was designed as a cMet kinase inhibitor but broad biochemical kinase profiling revealed a potent anaplastic lymphoma kinase (ALK) inhibitory activity [191,192]. This attribute was exploited when the EML4-ALK translocation mutations were subsequently identified in lung cancer [193] and allowed a rapid progression of crizotinib through clinical trials [194]. Inhibitor on-target potency does not necessarily predict kinase selectivity. This point can be illustrated with two highly optimized cMet drugs. These inhibitors have equivalent biochemical and cellular potencies yet very different kinase and mutant selectivity profiles [158]. Identifying relevant inhibitor activities requires broad profiling with an array of approaches in a multi-tiered process. The first step is to utilize readily available, broad biochemical kinase inhibition screening services. As kinase inhibitors can bind to other proteins, inhibitor characterization should include biochemical binding studies using a panel of non-kinase proteins: broad ligand profiling. For reasons delineated in earlier sections, biochemical screening data should serve as a starting place for understanding inhibitor specificity (hypothesis generating). Not all biochemical potencies observed in screening assays will translate to more complex systems. For example, PF-3758309 inhibited Src family kinases and PKC kinases in isolated kinase assays [171]. Global cellular high content evaluation of PF-3758309 compared to *bona fide* Src family and PKC inhibitors revealed distinct global cellular signatures indicating divergent biological profiles [171]. Follow-up studies to biochemical screening should utilize full length kinase proteins to characterize inhibitor binding (e.g. ActivX, Ambit). Ambit technology uses phage-displayed recombinant kinases in the evaluation of an inhibitor's capacity to competitively displace a kinase from an immobilized ligand [195]. ActivX technology evaluates an inhibitor's capacity to protect endogenous (native) kinases from irreversible modification by an ATP-competitive affinity probe with detection through mass spectrometric quantitation [196]. The source of kinases can be from cell line or tissue samples. Assays can provide relative potency because the affinity probe is applied to cell or tissue lysate containing a large subset of the kinome (100's of kinases). Ideally, the candidate off-target biochemical activities should be confirmed in validated, kinase-driven cell-based assays. The next level of understanding is gained through target-agnostic approaches which are capable of measuring a wide breadth of biological activities (proteomics). Currently, one of the most advanced approaches is an antibody-based proteomic method (e.g. reverse phase protein array) which allow quantitation of levels and activation states for a wide array of proteins (phosphoproteins) [197,198]. This approach measures direct and indirect inhibitor effects on individual proteins from *in vitro* or *in vivo* drug treatment. Mass spectrometry-based phosphoproteomics is a maturing technology capable of measuring effects on a large range of kinase-driven events [199,200]. These proteomic approaches are currently limited as interconnectivity of cellular events is not fully understood. This makes precise interpretation of observed changes in protein abundance patterns challenging. Also, proteomic analysis is specific to the context (cell type or tissue) and time-point studied. Another interesting target-agnostic approach is high content global cellular testing which measure effects at the individual protein level [171,201] and cellular functional outputs [202–204] in the context of living cells. High content cellular testing was used to determine a previously unknown mechanism of action for a clinical agent: DNA damage induced PAK4 activation and subsequent p53 activation [171]. Another unbiased approach to characterize inhibitor interactions is chemogenomic analysis. Many years ago, the protein kinase p38 was discovered through affinity purification using an immobilized small molecule inhibitor known to inhibit production of interleukin-1 and tumor necrosis factor from stimulated human monocytes [205]. This chemogenomic approach could be used to identify proteins that bind a given inhibitor. Understanding the full range of kinase inhibitor effects is a complex endeavor but an essential one for modern drug discovery.

10. Mechanisms of acquired drug resistance

Clinical success for kinase inhibitor drugs has a limited duration due to the development of resistance to therapy. It is understandable that resistance arises as kinase inhibitor drugs create strong selection pressures for mutations or alternate pathway signaling that can bypass the drug's effects. This presents new challenges in kinase drug discovery: understand acquired resistance to kinase inhibitor drugs and develop strategies to overcome it. Acquired resistance has been observed for drugs directed towards the first targeted kinases: BCR-Abl and EGFR. For BCR-Abl inhibitors (e.g. imatinib), the initial clinical benefit is impressive: 80% of chronic myelogenous leukemia (CML) patients respond to imatinib with under 3% progressing to advanced disease within 5 years [206]. In 2001, the first imatinib resistance mutant was reported as a T315I BCR-Abl gatekeeper mutation (Fig. 1) [207]. Subsequent analysis revealed that reoccurrence arises with over 50 documented point mutations occurring throughout the catalytic and regulatory domains, with a large percentage located in the G-loop and the gatekeeper position. The mechanistic understanding of gatekeeper mutation-mediated resistance has evolved. Originally, resistance was thought to arise from a steric clash between imatinib and the more bulky I315 residue and the loss of a critical hydrogen bond between the drug and the T315 residue found in the wild-type enzyme [208,209]. Subsequent reports downplay the importance of losing this hydrogen bond and alternatively suggest that changes to the conformational dynamics of the mutant protein play a considerably more important role [210,211]. More recently, the conformational effects of the BCR-Abl gatekeeper residue have been attributed to its role in a "hydrophobic spine" that links N- and C-lobes of the kinase domain [64]. Presently, it is commonly believed that imatinib resistance to the BCR-Abl T315I mu-

tant arises from both disruption of specific drug interactions in the active site (steric interactions and to a lesser extent loss of hydrogen bonding) and changes in the conformational dynamics of the protein [206].

The mechanism of acquired resistance to EGFR inhibitors is less well understood and may be different than for BCR-Abl. To start the evaluation of acquired resistance, it is useful to begin with the mechanism of activation mutations. EGFR is mutated to achieve oncogenic activity while Abl is activated through a BCR fusion event. Activating mutations of EGFR are not evenly distributed on the gene but confined to "hotspots". In the catalytic domain, two main mutation sites are clinically observed. The N-terminal region preceding the αC-helix (exon 19) is targeted by a series of in-frame deletion mutations (e.g. Del746-750) comprising 45% of the activated EGFR mutation population (Fig. 1). The C-terminal activation loop (exon 21) is targeted by 40–45% of the clinical activation mutations through L858R mutation. The remainder (10%) occur in exon 18 (G-loop) and exon 20 [212]. The in-frame deletion mutations are thought to activate EGFR by moving the αC-helix towards a more active-like state [212,213]. Leu858 is a hydrophobic residue on the activation loop that helps stabilize the inactive conformation [206] but it may have other functions. Mutation of this residue to arginine is hypothesized to destabilize the inactive conformation leading to constitutive activity. The L858R mutation may also affect the coordinated assembly of the hydrophobic spine [213]. Interestingly, although most believe that the two main activating mutations are functionally equivalent, patients fare differently. Patients with tumors harboring the exon 19 in-frame deletion survive longer than patients with the exon 21 L858R point mutation [214,215]. Acquired resistance to EGFR inhibitors typically occurs within one year in non-small cell lung cancer patients [216]. Resistance largely arises through a single gatekeeper mutation (T790M) with approximately 50% of responsive patients acquiring this second mutation. Compensatory cMet signaling is another major mechanism of resistance [216]. For this review, we will only address the molecular mechanism of target-based acquired resistance. Emerging data suggests that EGFR drug treatment serves to select this second mutation as over 40% of treatment-naïve patients had low levels of T790M mutant EGFR [217]. Many proposed mechanisms have been invoked to explain target-driven acquired resistance: alteration of the ATP affinity [206,216,218,219], steric hindrance to inhibitor binding [220–222], altered active site topography [216], disruption of favorable inhibitor–EGFR interactions [223], altered protein dynamics [213], and increased oncogenicity [216]. The T790M mutation by itself has been reported to increase the oncogenic activity of EGFR [224]. Although there are findings that support a role for steric interactions in acquired resistance [220–222,225], recent molecular dynamics studies of EGFR provide evidence against the steric clash model for T790M resistance because the methionine has the flexibility to adopt a different conformation [223]. Many of the EGFR inhibitors attain high potency through utilization of binding energy from association with the deep pocket (Fig. 1) [206]. Alterations in the kinase can attenuate the binding of the drug. Recent studies on the mechanism of the EGFR gatekeeper mutation report that resistance is not due to steric interactions and/or loss of a hydrogen bond but rather a lower $K_{m,ATP}$ (higher occupancy of ATP in the active site) [160,219]. In support of this view are studies that report that the activating mutations (L858R, exon 19 del) have an increased $K_{m,ATP}$ and therefore are easier to inhibit due to less competition from ATP [226,227]. Although the authors found similar wild-type $K_{m,ATP}$ values (5.0–5.3 μM), reported values for the L858R mutant vary substantially (10.9–148 μM). It must be noted that binding interactions may not be simple because different EGFR inhibitors are shown to have varied off-rates which do not correlate with potency measurements (K_i): lapatinib ($t_{1/2}$ = 300 min and K_i = 3 nM) compared to gefitinib and erlotinib ($t_{1/2}$ < 10 min and K_i 0.4–0.7 nM) [228]. In addition, only a small part of the EGFR active site has optimized constituents and geometry to bind ATP; the rest of the active site residues can mutate without disabling catalysis. As such, ATP competitive inhibitors may bind very differently than ATP and achieve high active site occupancy even at high cellular ATP concentrations. A recent hypothesis based on EGFR modeling studies attributes acquired resistance to altered conformational dynamics [213]. The authors evaluated the conformational landscape during the activation process to show differences in wild-type and mutant forms of EGFR. The authors hypothesize that L858R facilitates the activation process through coordinated assembly of the hydrophobic spine and movements of the αC-helix toward an active-like position. Furthermore, the hydrophobic spine is established in the transition state conformation with T790M affecting this process [213]. The EGFR T790M mutation has been reported to affect acquired resistance by many different mechanisms without a general consensus. As observed for EGFR and BCR-Abl, the molecular mechanism(s) of acquired resistance to kinase inhibitors is a challenging and exciting scientific frontier for mechanistic biochemistry and drug discovery.

11. Additional frontiers in kinase research

The existence of alternative splicing in kinases likely implies that the kinome is more complex than genomic analysis predicts. For example, there are 10 splice variants of three distinct JNK genes [229]. More recently, functional differences for alternate splice variants have emerged. Alternative B-Raf splice variants are necessary for learning and memory but dispensable for development [230]. An alternate splicing of JAK2 has been implicated in myeloproliferative neoplastic disease [231]. DYRK4 sub-cellular localization and catalytic activity are dependent on splice variation [232]. It should be expected that kinase splice variation will play an increasing role in our understanding of kinase-driven biology.

Due to the importance of the enzyme family, protein kinases are regulated at many levels: phosphorylation state, sub-cellular localization, protein complex context, proteolysis, and recycling. Kinases exist in complex systems (cell, animal) that are in rapid flux. A new type of regulation is emerging. There is a class of proteins related to protein kinases, pseudokinases, which possess the structural features of a catalytic domain but do not perform catalysis. Recent findings indicate that these proteins may act as regulators of catalytically active kinases [233]. The existence of pseudokinases underscores the fact that even true protein kinases can have both catalytic and non-catalytic functions. Another emerging field of kinase research explores the relationship between catalysis and systems control. Differential kinase activity can affect bistable signaling networks that are central to complex signal transduction and cell function [234]. These signaling networks may be tuned to the system through alteration of catalytic activities as was observed for cMet kinase [158]. The importance of kinase activity regulation cannot be over emphasized and will continue to be the subject of essential biological research.

As outlined in this review, there is a wealth of knowledge pertaining to protein kinase biochemistry and drug discovery. More importantly, it is apparent that the much of the rich complexity and fine details of protein kinase knowledge remains undiscovered. Many protein kinases are uncharacterized or poorly characterized. The regulation of catalytic activity continues to yield new discoveries. We know that intervention of protein kinase function can yield effective therapies, yet most of the human kinome remains unexplored.

The breadth of our protein kinase knowledge has expanded extensively, but substantial intellectual development is still required.

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