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Review

Coupling phosphoryl transfer and substrate interactions in protein kinases

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Abstract

Protein kinases control cell signaling events through the ATP-dependent phosphorylation of serine, threonine and tyrosine residues in protein targets. The recognition of these protein substrates by the kinases relies on two principal factors: proper subcellular co-localization and molecular interactions between the kinase and substrate. In this review, we will focus on the kinetic role of the latter in conveying favorable substrate recognition. Using rapid mixing technologies, we demonstrate that the intrinsic thermodynamic affinities of two protein substrates for their respective kinases (Csk with Src and Sky1p with Npl3) are weak compared to their apparent affinities measured in traditional steady-state kinetic assays (i.e.—$K_m < K_d$). The source of the high apparent affinities rests in a very fast and highly favorable phosphoryl transfer step that serves as a clamp for substrate recognition. In this mechanism, both Csk and Sky1p utilize this step to draw the substrate toward product, thereby, converting a high K_d into a low K_m. We propose that this one form of substrate recognition employed by protein kinases is advantageous since it simultaneously facilitates high apparent substrate affinity and fast protein turnover.

Keywords: Clamping; Kinase; Kinetics; Phosphorylation; Substrate; Recognition

1. Introduction

Since their initial discovery several decades ago, many biochemical studies have been performed to characterize the substrate specificities of protein kinases. Early kinetic experiments showed that protein kinases will phosphorylate short peptide sequences based on known phosphorylation sites in physiological substrates. This approach led to the consensus sequence model where the kinase recognizes residues directly flanking the site of phosphorylation [1,2]. Some protein kinases prefer positively or negatively charged residues flanking the phosphorylation site while others prefer hydrophobic side chains at discrete positions. Traditionally, the affinity of the substrate is measured in a steady-state kinetic assay where the initial velocity of the reaction is followed as a function of total substrate concentration under conditions of limiting substrate consumption. The concentration at which 50% of the maximal velocity is achieved is defined as the substrate K_m. While there are some exceptions, it is generally found that peptides derived from these consensus sequences have higher K_m values (i.e.—weaker apparent substrate affinities) for the kinase compared to the full-length protein substrates. Differences of about two orders of magnitude between the K_m's for full-length proteins and the derivative peptide substrates are not uncommon (Fig. 1B).

To explain the large discrepancies between the apparent affinities of physiological protein substrates and their peptide counterparts, residues outside the limited consensus sequence must participate in substrate recognition (Fig. 1A). Prior studies showed that several transcription factors are not effectively phosphorylated by their kinases unless specific residues 50–100 residues away from the consensus sequence are present [3–5]. While a three-dimensional structure of a full-length protein substrate bound to a protein kinase has

Abbreviations: Cbp, Csk binding protein; Cbp peptide, phosphotyrosine peptide derived from Cbp with sequence ISAMpYSSVMK; Csk, COOH terminal Src kinase; Kemptide, peptide substrate with sequence LRRASLG; Npl3, nuclear protein localization 3; PKA, cAMP-dependent protein kinase; Sky1p, SR protein kinase in yeast; SH2, Src homology 2 domain; SH3, Src homology 3 domain; Src, SFK from Rous Sarcoma virus

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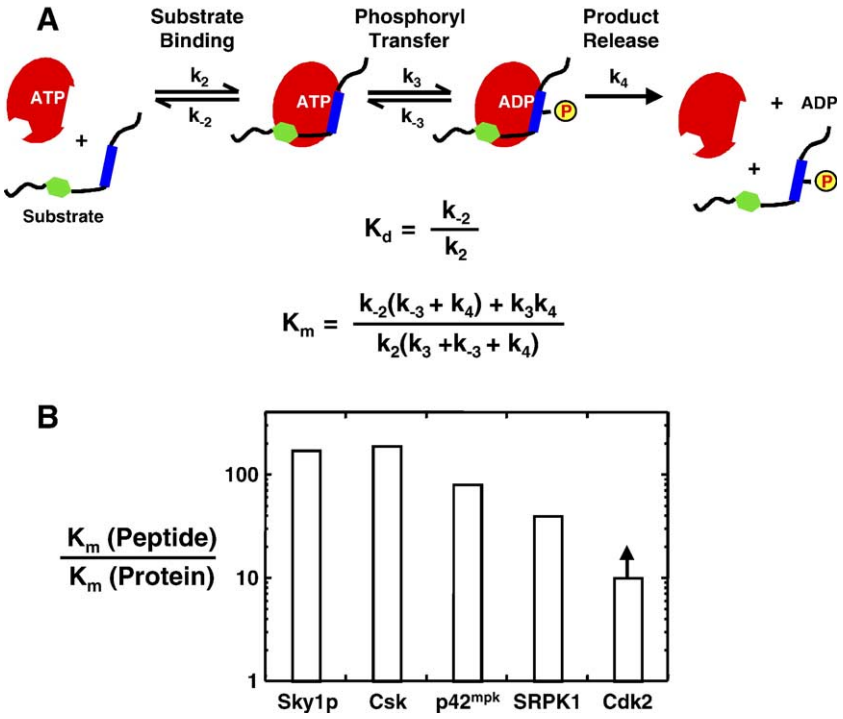

Fig. 1. Substrate phosphorylation by protein kinases. (A) Kinetic pathway for protein substrate phosphorylation by a protein kinase at high ATP concentrations. Three steps important for catalysis are included: substrate binding (k_2, k_{-2}), phosphoryl transfer (k_3, k_{-3}), and irreversible, net product release (k_4). The K_m and K_d expression for the substrate are shown below. Proximal (blue) and distal (green) binding determinants for the protein substrate are designated. (B) Comparison of K_m values for several protein and peptide substrates. The data for Sky1p compares the K_m's of Npl3 and the Npl3 peptide (Fig. 3). The data for Csk compares the K_m's of kd-Src (a kinase dead version of Src with a single mutation K295M and the first 81 amino acids deleted) and the peptide substrate AFLEDYFTSTEPQYQPGENL [7,35]. The data for $p44^{MPK}$ compares the K_m's for MBP and the peptide substrate KNIVTPRTPPPSQGK [36]. The data for SRPK1 compares the K_m's for ASF/SF2 and the peptide substrate $PRSPSYG(RS)_8$ (unpublished results). The data for cdk2 in complex with cyclin A bound compares the K_m's for Histone H1 and the peptide substrate PKTPKKAKKL [37].

eluded researchers, thus far, other structural studies have offered evidence for substrate docking sites far removed from the active site. For example, the recognition of the transcription factor MEF2A and the activating enzyme MKK3b by p38 MAP kinase requires sequences in the C-terminal lobe that are outside the active-site pocket [6]. The recognition of Src by Csk is likely to involve residues from the C-terminal lobe in helix αD [7]. In some cases, these docking sites appear to extend beyond the kinase domain and encompass neighboring subunits. For example, a small peptide sequence, important for the productive phosphorylation of p107, binds to the regulatory cyclin associated with cdk2 [8]. Such studies reveal a large exploitable surface for protein substrates that extend beyond the confines of the active site (Fig. 1A) and, in some cases, beyond the kinase domain itself.

2. Apparent and real substrate affinities

While steady-state kinetic parameters are used commonly to assess specificity (K_m), they do not provide a direct measure of the real affinity of the substrate and the kinase (K_d). These differences arise from the inherent complexity of enzyme-catalyzed reactions. As shown in Fig. 1A, the kinetic pathway for substrate phosphorylation by a protein kinase is composed of three fundamental events: substrate binding (k_2, k_{-2}), the phosphoryl transfer step (k_3, k_{-3}) and irreversible, net product release (k_4). While the dependence of substrate concentration on the reaction rate provides k_{cat} (turnover number) and K_m, the relationship between these parameters and the steps in Fig. 1A can be difficult to resolve. For substrate recognition, K_m includes all the rate constants in the reaction scheme in Fig. 1A, while the real substrate affinity term ($k_{-2}/k_2 = K_d$) remains deeply embedded and obscured. The intermingling of catalytic and product release steps with the K_d term makes it difficult to define real substrate affinity from the classic initial velocity versus substrate plots. For this reason, we commonly refer to the K_m as an apparent affinity since it can be more or less than K_d.

3. Relationship between K_m and K_d

The X-ray structure of the first protein kinase, cAMP-dependent protein kinase [PKA], revealed that the essential activating phosphorylation event for this kinase occurs in a polypeptide segment known as the activation loop [9,10]. While this structural observation provided insights into how a phosphate could stabilize the active form of a kinase, it did not provide the essential clues into how this modification acts as a reversible switch for kinase function. Insights into this problem came from the X-ray structures of the tyrosine kinase domain of the insulin receptor (InRK) which was