Substrate Specificity of CDK2-Cyclin A

WHAT IS OPTIMAL?*

Received for publication, June 20, 2003, and in revised form, September 23, 2003
Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M306546200

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The optimal amino acid sequence of substrates for recognition by the cyclin-dependent kinases is well established as -Ser/Thr-Pro, Lys-Ser-Lys, Pro, and 150-fold, when Pro, Lys, or Lys, respectively, is substituted with Ala in a short synthetic peptide substrate. Yet, in physiological substrates of both CDK2-cyclin A and CDK2-cyclin E, it is found that Lys, Lys, and, occasionally, both Lys and Lys together are replaced with suboptimal determinants. Such suboptimal phosphorylation site motifs are invariably associated with a distinct cyclin-binding (Cy) motif, which has been shown to compensate for otherwise poor catalysis. Here we have investigated the kinetic basis for substrate recognition by CDK2-cyclin A. In the optimal motif, Pro serves to dramatically enhance both substrate binding affinity as well as the rate of chemical phosphotransfer, whereas Lys and Lys both serve to enhance mainly substrate binding. When linked to a suboptimal phosphorylation site sequence (Lys → Pro) the Cy motif increases catalytic efficiency (kcat/Km) by increasing affinity without affecting turnover (kcat). When fused to the optimal sequence, however, catalytic efficiency is only minimally enhanced, because the resulting high substrate affinity impedes the rate of the phosphotransfer reaction. Our results provide kinetic insight into the basis for selecting suboptimal specificity determinants for the phosphorylation of cellular substrates.

Cyclin-dependent kinases (CDKs) and their cyclin partners form heterodimeric complexes that drive progression of the cell cycle. The action of different CDK-cyclin complexes at different times during the cell cycle results in the ordered movement of cells through the complex cellular changes that accompany cell division. Although CDK4 and -6 in combination with cyclin D bring quiescent cells into the cell cycle, CDK2 in combination with cyclins E and A are important for initiation and progression through DNA replication phase, respectively. Similarly, CDK1-cyclin B controls transition across the G2/M-phase boundary, regulating entry into mitosis.

Putative substrates for CDK-cyclin complexes have historically been proposed on the basis of a defined consensus recognition motif established for CDK1-cyclin B (1, 2). Early work showed that the minimal sequence for substrate phosphorylation by this enzyme was X(S/T)P(X(K/R)3), depicting an absolute requirement for proline at position P, and a positively charged residue being favored at position P, (P is the phosphorylation site) (3). Recently it has become clear that the amino acid substrate specificity determinants recognized by CDK1-cyclin B, CDK2-cyclin A, CDK2-cyclin E (4), and CDK5-p25 (5) are virtually identical. Importantly, short synthetic peptides that contain these determinants serve as highly efficient substrates for these enzymes, demonstrating that the primary sequence alone is sufficient for high catalytic activity (6). Further, more comprehensive studies on the specificity of CDKs have employed two independent approaches to define the optimal structural determinants recognized by these enzymes. Classically, systematic amino acid substitution analysis of model peptide substrates allowed the contribution of each amino acid to overall catalytic efficiency to be deduced (4). In addition, the preferred substrate recognition sequence has been demonstrated independently by random screening of combinatorial peptide substrate libraries (7). Both approaches have corroborated the optimal recognition motif for various CDK-cyclin complexes to be X(S/T)PX(K/R)3.

In all cases, the specificity of CDKs has been defined with respect to the phosphorylation of model peptide substrates. Recently, knowledge of in vivo protein substrates for a number of CDK family members has emerged (8). The best characterized include the retinoblastoma protein, Rb, and the transcription factor E2F, in which phosphorylation by CDK2-cyclin E and A are required to drive cells through the G1/S-phase border and S-phase of the cell division cycle (9–12). However, a dilemma regarding the recognition of these substrates is that their sites of phosphorylation invariably do not conform to the optimal consensus sequence motif as defined by model substrate peptides. For example, a comprehensive study shows that Lys or Arg at position P, or P, is optimal (4), yet these amino acids invariably are not employed at position P, nor, in many cases, P, in in vivo substrates (Table I). In some cases, Pro, the least favored determinant at P, is often found at this position.

In conjunction with such suboptimal phosphorylation site sequences, a distinct cyclin-binding motif termed the Cy motif, with consensus sequence RXL, has recently been identified in several physiological substrates of CDK2-cyclin A and E (8). This motif, located C-terminally to the site of phosphorylation, has been shown to be critical for substrate targeting (13, 14) and to confer high catalytic efficiency (15), and it has been postulated to serve as a specificity determinant toward different cyclins (16). Notably, the Cy motif appears to be linked invariably to suboptimal phosphorylation motifs, whereas sites...
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displaying X(S/T)P(K/R)(K/R) in proteins apparently do not require and are not associated with a Cy motif.

In this study, we have examined the mechanistic basis for optimizing high catalytic efficiency and specificity in physiological substrates of CDK2-cyclin A by investigating the kinetic role of each determinant within the optimal CDK consensus sequence motif with respect to substrate binding versus phosphoryl transfer. We find that the Lys, Thr, and Tyr substrate determinants and the distally located Cy motif both enhance substrate binding, with little effect on phosphoryl transfer. Thus, these determinants are compensatory for each other in terms of thermodynamic binding. Interestingly, the increase in affinity attributable to the Cy motif impedes the rate of chemical phosphoryl transfer when fused to the optimal phosphorylation sequence. Thus, lowering the binding energy at the phosphorylation site favors catalysis. Our results prompt renewed investigation into the issue of CDK substrate specificity, and revisiting the question, "What is optimal?"

MATERIALS AND METHODS

CDK2-cyclin A—Human CDK2 (phospho-Thr\textsuperscript{160}) in complex with bovine cyclin A3 (corresponding to residues 171–432 of human cyclin A; expressed with C-terminal His\textsubscript{6} tag) was produced in bovine cyclin A3 (corresponding to residues 171–432 of human cyclin A; expressed with C-terminal His\textsubscript{6} tag) was produced in E. coli (BL21 DE3) followed by co-lysis with bacteria expressing cyclin A. Active CDK2-cyclin A was purified by glutathione-agarose chromatography followed by thrombin cleavage and then anion exchange chromatography on Uno Q (Bio-Rad). Peptides—peptides were synthesized at the University of Calgary (Alberta, Canada), by Peptigenic Research (Livermore, CA), or by Genemed Synthesis (San Francisco, CA). Peptides were 95% pure estimated by high pressure liquid chromatography.

Kinase Assays—Kinase assays and viscosometric studies were carried out as described previously (18). In brief, peptide substrate of varying concentrations (0.5–5 K\textsubscript{m}) was phosphorylated with CDK2-cyclin A in the presence of saturating (γ\textsuperscript{32}P)ATP (1 mm, 300 cpm/pmol) (K\textsubscript{ATP} = 50 μM (17)) in 20 mM MOPS, pH 7.4, 50 mM KC\textsubscript{l}, 10 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.1 mM EDTA. Saturating conditions for ATP were confirmed by doubling the ATP concentration and observing no change in initial velocity. The concentration of CDK2-cyclin A (10–500 nm) was adjusted to achieve a reasonable time course for phosphorylation. In all assays, less than 10% of substrate was turned over. Under these conditions, substrate phosphorylation varied linearly with time (not shown) verifying initial rate conditions. Reactions were stopped with 25% acetic acid and then subjected to ascending paper chromatography on Ph\textsubscript{3}I phosphocellulose (Whatman) using 20 mM H\textsubscript{3}PO\textsubscript{4} water as solvent (18). Radiolabel corresponding to phosphopeptide product was quantified by Cerenkov counting.

Viscosometric Studies and Data Analysis—Solvent viscosometric assays were conducted by carrying out traditional substrate dependence studies in saturating ATP and varied sucrose concentrations (0–40% sucrose) (19, 20). The relative solvent viscosities of buffers containing sucrose were measured using an Oswal viscometer (see Ref. 18). Initial velocities were measured in buffer containing 4–5 different sucrose concentrations (0–40% sucrose) and plotted as a function of peptide substrate concentration. The result is a family of Michaelis-Menten plots, each individual point corresponding to a different relative solvent viscosity value. The following equation describes the theoretical relationship among initial velocity, substrate concentration, and relative solvent viscosity.

\[
u = \frac{k_\text{cat}}{K_{\text{m}}} - \frac{k_{\text{cat}}}{K_{\text{m}}} \cdot \frac{v}{(k_\text{cat} + k_{\text{cat}})} = k_{\text{cat}}/K_{\text{m}} \cdot \frac{1}{(k_\text{cat} + k_{\text{cat}})} (\text{Eq. 1})\]

where E is the enzyme concentration, \(\eta\) is the relative solvent viscosity, and \(v\) is the initial velocity. The derivation of this equation is provided in Ref. 21 in the supporting information section (available via the Internet). A global fit of Equation 1 to the initial velocity data provided a family of best-fit curves through the raw data. The Michaelis-Menten equation was then separately fitted to each individual curve, and the apparent values for \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{m}}\) at each relative solvent viscosity were plotted as a function of log [sucrose] and plotted as a function of the relative solvent viscosity as a linear relationship in which slope values give the viscosity effect on these parameters (designated \(k_{\text{cat}}/K_{\text{m}}\) or \(k_{\text{cat}}/K_{\text{m}}\), respectively). The viscosity effects for each peptide substrate are given as footnotes in Tables II and III. The viscosity effects and the intrinsic steady-state constants relate to the individual kinetic constants as follows.

\[
k_{\text{cat}}/K_{\text{m}} = k_{\text{cat}}/K_{\text{m}} (\text{Eq. 2})\]

\[
k_{\text{cat}} = k_{\text{cat}} (\text{Eq. 3})\]

\[
k_{\text{cat}}/K_{\text{m}} = k_{\text{cat}}/K_{\text{m}} (\text{Eq. 4})\]

\[
k_{\text{cat}} = k_{\text{cat}} (\text{Eq. 5})\]

The simultaneous solution of Equations 2–5 allows values for \(k_{\text{cat}}/K_{\text{m}}\), \(k_{\text{cat}}\), and \(k_{\text{cat}}/K_{\text{m}}\) to be solved. \(K_{\text{m}}\) values reported in Tables II and III are taken as the ratio of \(k_{\text{cat}}/K_{\text{m}}\). The program Scientist (Micromath, Salt Lake City, UT) was used to carry out all nonlinear regression analysis. The theoretical, methodological, and technical aspects of solvent viscosometric kinetic techniques are reviewed in Ref. 21, in the supporting information section (available via the Internet).

Solvent viscosometric analyses require proof that a given viscosogen affects only the rate of molecular diffusion and does not erroneously perturb the structures of the free enzyme, free substrate, or enzyme-substrate complex. We have previously demonstrated that poor peptide substrates of CDK2 display no viscosity effect on \(k_{\text{cat}}/K_{\text{m}}\) in sucrose (6). Thus the structure of the enzyme in the enzyme-substrate complex is not perturbed by this viscosogen. Similarly, we observed little to no viscosity effect on \(k_{\text{cat}}/K_{\text{m}}\) for any of the peptides tested (Peptide 9 was not tested), suggesting that the structures of the free peptides and free enzyme were similarly unperturbed.

RESULTS AND DISCUSSION

Kinetic Analysis of TPKK—The phosphorylation site sequences targeted by CDK2-cyclin A within physiological protein substrates invariably do not display the optimal recognition determinants established by analysis of model peptide substrates. Such determinants include Lys/Arg at position P\textsubscript{2}, which enhance catalysis by 5–6-fold over Ala. Lys/Arg are also favored at position P\textsubscript{3}, but in this case by nearly 100-fold over most other amino acids (4). By comparison, physiological substrates invariably display suboptimal amino acids at position P\textsubscript{2} and, less frequently, P\textsubscript{3}, generating motifs predicted to exhibit dramatically poorer catalytic properties. In some cases, the P\textsubscript{3} amino acid is Pro, the worst determinant at this position (4). Table I shows representative sequences surrounding the sites of phosphorylation of several known physiologically relevant targets of CDK2-cyclin A. To understand the rationale for incorporating suboptimal recognition determinants into these substrates, we have investigated the kinetic role of each amino acid within the optimal motif.

The synthetic peptide PKTTPKKAKKL (Peptide 1) contains the optimal motif (underlined) for phosphorylation by CDK2-cyclin A (4) and consequently has been shown to be a highly efficient substrate (\(k_{\text{cat}}/K_{\text{m}} = 10^{-8} \text{ M}^{-1} \text{ s}^{-1}\)) for this enzyme.
The detailed kinetic mechanism for phosphorylation of this peptide has been elucidated using steady-state solvent viscosometric techniques (6, 17). These experiments were repeated here to investigate the kinetic basis for substrate specificity. Peptide 1 binds to CDK2-cyclin A with a $K_p$ value of 40 μM and is turned over at a maximum rate of $4.5 \text{s}^{-1}$. The turnover rate is limited by both the rate of the phosphoryl transfer reaction (11 $\text{s}^{-1}$) and the net dissociation of products from the active site (7.6 $\text{s}^{-1}$). These numbers vary slightly from those that we have previously reported (6, 17), a variation that we attribute to experimental error.

We have synthesized a number of derivatives of Peptide 1 that incorporate substitutions at each of the critical amino acid positions. Employing steady-state solvent viscosometric techniques (21–23), we have investigated the effects of such substitutions on either substrate binding affinity ($K_D = k_{-2}/k_2$) or the rate of chemical phosphoryl transfer (Scheme 1). The results are summarized in Table II.

Substitution of all residues in the minimal motif was found to profoundly affect substrate binding affinity ($K_D$) (Table II), including substitution of the phosphorylation site itself with Ala (not shown). Although such nonphosphorylatable substrate analogues often retain high binding affinity and thus display potent kinase inhibitory activity (24), the nonphosphorylatable analogue of Peptide 1 displayed little to no inhibitory activity (at 10 μM Peptide 1). Thus, the minimal $K_D$ value for the nonphosphorylatable peptide is in the low millimolar range. The role of Pro$_{10}$ in binding affinity may relate to the energetic cost of desolvating any other residue at this position upon binding, which the crystal structures of extracellular signal-regulated kinase-2 (25) and CDK2-cyclin A (26) reveal would not be compensated by hydrogen bonding. The role of Lys$_{10}$ in substrate binding is consistent with the crystal structure of CDK2-cyclin A, which shows hydrogen bonding between the enzyme phosphoryl group at Thr$^{160}$ and the side chain of Lys$_{10}$ in a peptide substrate (26). However, the role of Lys$_{10}$ in substrate binding is less clear. In the crystal structure its side chain shows no interaction with the enzyme but instead points into the bulk solvent (26).

Although replacement of Lys$_{10}$ in Peptide 1 with Glu, or Pro profoundly affected substrate binding, the same substitutions had little to no effect on the rates of either phosphoryl transfer ($k_2$) or overall turnover ($k_{\text{cat}}$). Only the substitution of Pro$_{10}$ (for Ala or Gly) had significant effect on reducing the rate of the chemical transfer step along the reaction pathway (Table II).

Our kinetic results are similar to steady-state kinetic parameters reported previously for CDK5-p25 (5). Substitution of Lys$_{10}$ to Ala in Peptide 1 shows dramatic effects on $K_m$, with little effect on $k_{\text{cat}}$ for this enzyme (5). The specificity of CDK5-p25 has been shown to be virtually identical to that of CDK1-cyclin B (5), which, in turn, has been demonstrated to be identical to that of CDK2-cyclin A (4). Other peptide sequences have also been used previously to analyze the specificity of CDK1-cyclin B (27, 28). These include sequences based on the known phosphorylation sites in SV40 large T antigen (ADAQHATPPEKKRKKVDPEK) and the mammalian tumor suppressor, p53 (RAALPNNTSSPQKPPKPLDGEY) (28). In these cases (27, 28), less dramatic effects were observed on $K_m$.

However, in all cases (5, 27, 28) it was apparent that $k_{\text{cat}}$ remained virtually unchanged in response to substitution at the substrate P$_{1-2}$ or P$_{3-3}$ positions. In only one study were dramatic effects on turnover rate ($k_{\text{cat}}$) in response to substitution of Lys$_{1-3}$ reported (29). In this study (29), the specificity of CDK2-cyclin A was tested using short peptide sequences fused to GST. Beyond this distinction, reasons for the kinetic disparity are not clear.

The majority of evidence shows that the substrate P$_{1-2}$ and P$_{3-3}$ residues are dispensable for catalytic turnover but are crucial for substrate binding. Interestingly, these are frequently substituted with non-basic residues in physiological substrates of CDKs, rendering them suboptimal in comparison with the classical consensus motif. By comparison, Pro$_{1-1}$ is always conserved, presumably because it is essential for efficient chemical phosphoryl transfer.

Role of the Cy Motif—Cellular substrates that display suboptimal catalytic determinants at their phosphorylation sites invariably display a specific cyclin-binding (Cy) motif. This motif displays the consensus sequence RXL and is located at a distance greater than 16 residues C-terminal to the TPXX motif (8, 15). An eight-amino acid peptide corresponding to the Cy motif of E2F1 is sufficient for binding to CDK2-cyclin A at low μM concentration (14). The crystal structure of CDK2-cyclin A bound to the Cy motif sequence of p107 has been solved (26).

Although yet to be proven, it has been proposed that the Cy motif may serve as a specificity determinant allowing substrates to be targeted to specific CDK complexes through the Cy motif (16). Regardless, the Cy motif is invariably present in all substrates that display suboptimal phosphorylation sequences, suggesting a crucial role in cellular function. In the case of CDK4-cyclin D1, the Cy motif in p107 negates the effects of suboptimal substitution at the P$_{3-3}$ position (30).

Furthermore, in the case of the substrate CDC6, a functional Cy motif confers a 100-fold enhancement in catalytic efficiency toward both CDK2-cyclin A and CDK2-cyclin E (15).

Catalytic rate enhancement classically is accounted for by stabilization of the transition-state relative to the ground-state enzyme-substrate structures (31). Stabilization of these structures may occur either uniformly or differentially, giving rise to energetic effects predominantly on substrate binding or chemical reactivity, respectively (31). To define the kinetic mechanism by which the Cy motif contributes to catalytic efficiency, we engineered a peptide in which a classical Cy motif sequence was fused to a poor phosphorylation site sequence, PKTPP-KAKKLAGPRAGLPVVRKRDLL. Such a peptide emulates a typical CDK2-cyclin A phosphorylation site within a cellular target. In this substrate, domain A (underlined) corresponds to the sequence of Peptide 6, whose catalytic efficiency ($k_{\text{cat}}/K_m$) is more than two orders of magnitude below that of the optimal Peptide 1. Domain B (double underlined) corresponds to the Cy motif of the transcriptional activator E2F1 (14), a bona fide cellular substrate of CDK2-cyclin A. A spacer, AGR, was inserted to obtain the minimal required spacing of 16 amino acids between the TPPK motif and RRL (15). As expected, we found that fusion of the E2F1 Cy motif sequence to that of Peptide 6 resulted in significantly increased catalytic efficiency (Table III), attributable entirely to a decrease in the $K_m$ value. The maximal turnover rate ($k_{\text{cat}}$) was unchanged. These results are consistent with studies on a peptide substrate derived from the CDC6 protein, a recently identified cellular target of CDK2-cyclin A (15).

Steady-state kinetic parameters do not provide direct information on the individual reaction steps of an enzymatic pathway. This is because $k_{\text{cat}}$ is a composite of both the phosphoryl transfer reaction and the net release of both products (see Scheme 1), whereas $k_{\text{cat}}/K_m$ is composed of the substrate association, dissociation, and phosphoryl transfer steps. Furthermore, all steps along the entire reaction pathway influence

**Scheme 1**

$$
\begin{align*}
\text{E}^{\text{ATP}} + \text{Pept} & \quad k_2 \quad \text{E}^{\text{ATP-Pept}} \quad k_3 \quad \text{E}^{\text{ADP-Pept}} \quad k_4 \quad \text{E} + \text{P-Pept} + \text{ADP}
\end{align*}
$$
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Table II

| Peptide | $k_{cat}$ | $K_m$ | $K_p$ | $k_{cat}$ |
|---------|----------|-------|-------|---------|
|         | s$^{-1}$ | $\mu M$ | $\mu M$ | s$^{-1}$ |
| 1 PKTPKKAKKL | $4.5 \pm 1.5$ | $20 \pm 5$ | $40 \pm 8$ | $11 \pm 2$ |
| 2 PKTAKKAKKL | $0.45 \pm 0.15$ | $4300 \pm 215$ | $4300 \pm 108$ | $0.5 \pm 0.3$ |
| 3 PKGKKAKKL | $0.8 \pm 0.2$ | $2500 \pm 125$ | $2500 \pm 66$ | $0.9 \pm 0.2$ |
| 4 PTKPAAKKL | $3.6 \pm 1.8$ | $223 \pm 55$ | $>2100 \pm 50$ | $>36 \pm 3$ |
| 5 PTKPEEAKKL | $3.3 \pm 1.5$ | $225 \pm 180$ | $>2250 \pm 50$ | $>33 \pm 3$ |
| 6 PKTPPKAKKL | $2.7 \pm 0.4$ | $2000 \pm 100$ | $3000 \pm 75$ | $4.3 \pm 2.1$ |
| 7 PKTPKAAKKL | $3.2 \pm 1.1$ | $2500 \pm 120$ | $3600 \pm 90$ | $4.5 \pm 0.6$ |

$^a$ Inferred from viscosity effects on $k_{cat}/K_m$ and $k_{cat}$, respectively, which for each peptide were determined as: 1, 0.2 $\pm$ 0.1; 2, 0.05 $\pm$ 0.2; 3, 200 $\pm$ 4; 4, 0.04 $\pm$ 0.04; 5, 0.01 $\pm$ 0.03; 6, 0.06 $\pm$ 0.1, 0.35 $\pm$ 0.1; 7, 0.01 $\pm$ 0.04, 0.32 $\pm$ 0.03.

$^b$ Numbers in parentheses represent -fold changes relative to the parent peptide, PKTPKKAKKL.

Table III

| Peptide | $k_{cat}$ | $K_m$ | $K_p$ | $k_{cat}$ |
|---------|----------|-------|-------|---------|
|         | s$^{-1}$ | $\mu M$ | $\mu M$ | s$^{-1}$ |
| 6 PKTPKKAKKL | $2.7 \pm 0.4$ | $2000$ | $3000$ | $4.3 \pm 2.1$ |
| 8 PKTAKKAKKL | $3.3 \pm 0.4$ | $139 \pm 30$ | $230 \pm 70$ | $5.6 \pm 2.3$ |
| PALGRPPVPKRRLDLE | $1.7 \pm 0.3$ | $2.4 \pm 0.5$ | ND | $2.4 \pm 0.7$ |

| Peptide | $k_{cat}$ | $K_m$ | $K_p$ | $k_{cat}$ |
|---------|----------|-------|-------|---------|
|         | s$^{-1}$ | $\mu M$ | $\mu M$ | s$^{-1}$ |
| 9 PKTPKKAKKL | $4.5 \pm 1.5$ | $20 \pm 5$ | $40 \pm 8$ | $11 \pm 2$ |
| PALGRPPVPKRRLDLE | $1.7 \pm 0.3$ | $2.4 \pm 0.5$ | ND | $2.4 \pm 0.7$ |

$^a$ Inferred from viscosity effects on $k_{cat}/K_m$ and $k_{cat}$, respectively, which for the following peptides were found to be: 8, 0.06 $\pm$ 0.03, 0.47 $\pm$ 0.2; 9, not determined (ND), 0.3 $\pm$ 0.1.

$K_m$ Depending on the kinetic reaction mechanism, a lower $K_m$ value theoretically can result from either an increase or decrease in rate of the phosphoryl transfer step, without altering $k_{cat}$. We therefore wished to determine which individual reaction steps of the overall pathway were influenced by the Cy motif sequence.

Steady-state solvent viscometric methods revealed that the Cy motif did not appreciably affect the rate of phosphoryl transfer ($k_4$), or net product release ($k_3$). However, the $K_p$ value was decreased by more than an order of magnitude (Table III). Thus, the Cy motif compensates precisely for substrate binding affinity that is lost upon substitution of Lys at the phosphorylation site.

Role of Suboptimal Specificity Determinants—Because the first hypothesis of a defined consensus sequence motif for in vitro phosphorylation by p34cdc2, the optimal amino acid sequence for CDK substrate recognition has been universally accepted as (S/T)P (23). This is supported by the high catalytic efficiency displayed both by proteins and short peptides that contain this motif (5, 6, 27), the specific selection of this sequence from combinatorial peptide substrate libraries (7), and biochemical analysis of peptide substrates by extensive systematic amino acid substitution (4). It is somewhat surprising then that bona fide cellular substrates of CDKs invariably do not display the optimal motif for recognition, even in the presence of a functional Cy motif. A survey of several cellular substrates of CDK2 shows that Lys (or Arg) at P2 is in fact selected against and, in some cases, is replaced with Pro (Table I), the worst determinant (4). In p53, a physiological target of CDK2-cyclin A, both the P2 and P3 residues are Gin and Pro, respectively, as opposed to Lys/Arg.

We therefore asked whether specific destabilization of the phosphorylation site motif might be favorable for catalysis by CDK2-cyclin A. This would be expected if the additional binding energy from the Cy motif caused increased stabilization of the ground-state relative to the transition-state enzyme-substrate complex and, consequently, an increase in the activation energy for chemical phosphoryl transfer (31). Thus we hypothesized that in the process of evolving a specific cyclin binding determinant, a compensatory decrease in binding affinity at the phosphorylation site is desirable to maintain efficient chemical transfer and catalytic turnover.

To test this hypothesis, we synthesized a peptide (Peptide 9) that contains the optimal phosphorylation site motif (domain A (underlined)) linked to the E2F1 Cy motif (domain B (double underlined)): PKTPKKAKKLGRPALGRPPVPKRRLDLE. The kinetic parameters for this peptide were then measured and compared with those of Peptide 8 to determine the functional relationship between the phosphorylation site and the Cy motif. The results are shown as a thermodynamic cycle in Fig. 1. In this figure, we report the effects on both $k_{cat}/K_m$ and $k_{cat}$ which are the rate parameters that govern an enzymatic reaction at low versus saturating substrate concentration, respectively, assuming steady-state turnover. If the reaction is not assumed to reach steady state in cells, then the microscopic rate constants are instead the relevant parameters. Thus, we
also report the kinetic effects on the phosphoryl transfer reaction step \( (k_\text{cat}) \).

Fusion of the Cy motif to a poor substrate sequence (Peptide 6) increases catalytic efficiency \( (k_\text{cat}/K_m) \) because of increased binding affinity (Table III). However, when fused to the optimal sequence (Peptide 1) the Cy motif only marginally enhances this parameter. This is because there is an approximate 5-fold decrease in the rate of the phosphoryl transfer reaction in comparison to Peptide 1. This decrease in the rate of chemistry is expected based on our original hypothesis that stabilization of the ground-state enzyme-substrate complex relative to the transition-state structure occurs, raising the energetic barrier to phosphoryl transfer. Thus, with respect to the phosphoryl transfer reaction, determinants that are optimal in a short peptide substrate may in fact be suboptimal when fused to a Cy motif.

In summary, we have described in kinetic detail the role of key recognition determinants in the phosphorylation of substrates by CDK2-cyclin A, and have presented the most extensive kinetic analysis of substrate specificity of this class of enzymes available to date (Table II). Further, we describe how the Cy motif, which has been shown to compensate for suboptimal residues at the phosphorylation site (15, 30), interacts with determinants at the phosphorylation site on a kinetic level. It is apparent that the positive effect of the Cy motif on catalytic efficiency diminishes as the phosphorylation site becomes optimized. In fact, the Cy motif impedes both phosphoryl transfer and the overall rate of turnover, by moderate but significant extents, when fused to the optimal peptide sequence. In support of this finding, the tighter binding of protein substrates to CDK2-cyclin A via adaptor proteins has been shown to be less favorable for substrate phosphorylation (32).

The relevance of our kinetic data depends on the cellular conditions of both substrate and enzyme concentrations. Three possible scenarios may exist in cells. First, if the concentration of substrate is low relative to \( K_m \), the rate of phosphorylation is dictated principally by \( k_\text{cat}/K_m \), and the configuration of substrate determinants in Peptide 9 is favorable. Second, if the substrate concentration is high, \( k_\text{cat} \) is the governing rate parameter, and the configuration corresponding to either Peptide 8 or 9 will suffice because the difference in \( k_\text{cat} \) between these configurations is only 2-fold. Finally, it has been found that the amount of enzyme may be similar to or even greater than the amount of substrate in many signal transduction systems, as seen through quantification of the cellular amounts of the mitogen-activated protein kinase signaling components (33). Also, in the case of CDK2, in vitro substrates have been isolated through affinity precipitation methods (34–36), demonstrating a stable interaction. Under such conditions, only a single pass through the active site is required for complete substrate phosphorylation. Consequently, steady-state catalysis is not reached, and the rate of substrate phosphorylation will be dictated by the rate of the phosphoryl transfer step. If such conditions apply, substitution of Lys \(_{1,2}\) to a poorer determinant, as in Peptide 8, is clearly optimal. This scenario also favors conservation of the substrate \( \text{Pro}_1,1 \), which we have shown is essential for the phosphoryl transfer step (Table II).

CDK2-cyclin A is a clear example in which the specificity of a protein kinase toward artificial substrates can differ from that of physiological targets even though catalytic efficiency toward either can be high. We have investigated this through a rigorous analysis of the kinetic role of specificity determinants in substrates of this enzyme. Our work sets the stage for further examination of the general design of CDK substrates, to determine what is truly optimal for this family of enzymes under physiologically relevant conditions.

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