Different Mechanisms of CDK5 and CDK2 Activation as Revealed by CDK5/p25 and CDK2/Cyclin A Dynamics*

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A detailed analysis is presented of the dynamics of human CDK5 in complexes with the protein activator p25 and the purine-like inhibitor roscovitine. These and other findings related to the activation of CDK5 are critically reviewed from a molecular perspective. In addition, the results obtained on the behavior of CDK5 are compared with data on CDK2 to assess the differences and similarities between the two kinases in terms of (i) roscovitine binding, (ii) regulatory subunit association, (iii) conformational changes in the T-loop following CDK/regulatory subunit complex formation, and (iv) specificity in CDK/regulatory subunit recognition. An energy decomposition analysis, used for these purposes, revealed why the binding of p25 alone is sufficient to stabilize the extended active T-loop conformation of CDK5, whereas the equivalent conformational change in CDK2 requires both the binding of cyclin A and phosphorylation of the Thr160 residue. The interaction energy of the CDK5 T-loop with p25 is about 26 kcal-mol−1 greater than that of the CDK2 T-loop with cyclin A. The binding pattern between CDK5 and p25 was compared with that of CDK2/cyclin A to find specific regions involved in CDK/regulatory subunit recognition. The analyses performed revealed that the αNT-helix of cyclin A interacts with the α6–α7 loop and the α7 helix of CDK2, but these regions do not interact in the CDK5/p25 complex. Further differences between the CDK5/p25 and CDK2/cyclin A systems studied are discussed with respect to their specific functionality.

Cyclin-dependent kinases (CDKs) control the progression of the cell cycle (1) and participate in a subset of apoptosis programs (1–3). CDKs consist of two subunits, one catalytic and the other regulatory. The catalytic subunit is a Ser/Thr kinase from the CMGC kinase family (4); the associated regulatory proteins are called cyclins (1). Cyclins are highly specific for individual kinases, resulting in the formation of distinct complexes, e.g. CDK1/cyclin B1 and CDK2/cyclin E1. The primary biological function of these complexes is related to regulation of the cell cycle. However, certain CDKs do not participate in cell cycle control, instead they are involved in controlling cell differentiation in neuronal and muscle cells. This class of CDKs is exemplified by CDK5, which plays a critical role during neuronal development (5–8).

Several CDks (CDK1–4 and CDK6) show a dual mechanism of activation based on the binding of the cyclin box fold (CBF) region of the regulatory subunit to the kinase and phosphorylation of the activation loop (also known as the T-loop) of the kinase (1). However, this mode of activation is not observed in CDK5, despite sequence identities of almost 60% for CDK2-CDK5 pairs in different species (Scheme 1). CDK5 is a unique member of the CDK family, because it is not activated by a cyclin. It binds to cyclins D and E, but they fail to induce its kinase activity (5, 6). Instead, CDK5 activity is triggered by p35NCCKA and p39NCKAI (henceforth referred to as p35 and p39, respectively; see Scheme 2), proteins expressed only in neurons and a few other cell types (6, 9). Neither p35 nor p39 exhibit any detectable similarity to cyclins. The association of CDK5 with p35 or p39 is sufficient to induce full activation of CDK5. Furthermore, CDK5 is not activated by phosphorylation of the activation loop, although the loop contains a residue (Ser159, equivalent to Thr160 of CDK2) that could potentially be phosphorylated (5, 10–12).

Although CDK5 is expressed in a number of tissues, its activity is restricted to neurons by the localization of its activators. Unregulated CDK5 activity has been implicated in the pathology of many neurodegenerative diseases. Deregulation of CDK5 activity is induced by the proteolytic cleavage of p35 by calpain, forming the active fragment p25. The p25 fragment induces hyperactivation of CDK5. Furthermore, unlike p35, it is not localized in the cell membrane. The CDK5/p25 complex can thus translocate from the plasma membrane to the cytosol and nucleus, where it hyperphosphorylates a number of substrates, leading to neuronal cell death (8, 9, 13). Because the deregulation of CDK5 has been implicated in neurodegenerative diseases, there is significant interest in the development of new chemical inhibitors of CDK5 to treat these serious brain illnesses (6, 14).

Structural aspects of CDK5 activation by p25 have been revealed by analysis of the crystal structure of the CDK5/p25 complex (5). The structure of the unphosphorylated complex confirms that the cyclin box fold (CBF) is a structural feature of p25. p25 has eight α-helices, five of which (α1 to α5) adopt a topology similar to that of the CBF (Fig. 1A). The p25 CBF binds to the activation loop and the C-a helix of CDK5, the latter of which contains the PSAALRE sequence motif (residues 45–51). Overall, the structural features of the CDK5/p25 complex are similar to those of the CDK2/cyclin A complex (Fig. 1B) (5, 15). However, there are also some noteworthy structural and regulatory differences between the two complexes. One such difference is that even in the absence of a phosphate group on Ser159, the CDK5 activation loop adopts the correct
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**SCHEME 1.** A dendrogram of selected human cyclin-dependent kinases showing the high similarity between CDK5 and CDK2. CDKs belong to class of the CMGC group of protein kinases (4); the complete human kinome has been published previously (36).

**SCHEME 2.** The activation of CDK2 involves a two-step process, i.e. cyclin binding and then phosphorylation of the activation T-loop (at Thr^{160}). By contrast, association with either the p35 or p39 activator proteins alone is sufficient to fully induce CDK5 activity.

CDK5 induces in a single step the conformational changes that CDK2 undergoes only after two independent regulatory events: cyclin A binding and the phosphorylation of Thr^{160} (6, 8) (see Scheme 2).

The Thr^{160} (phosphothreonine) of CDK2 acts as an organizing center that interacts with three neighboring arginine residues (Arg^{126}, Arg^{126}, and Arg^{150}), stabilizing the active, extended conformation of the T-loop (16, 17). In the CDK5/p25 complex, the structural requirement for phosphorylation is bypassed by an extensive network of interactions between p25 and the CDK5 activation loop, a feature that is not observed in the phospho-CDK2/cyclin A complex. The phosphorylation of the activation loop (Ser^{159}) may even have an inhibitory effect on the CDK5/p35 interaction. This is suggested by the fact that substituting Ser^{159} with glutamic acid impairs the interaction of CDK5 with p35, and even the apparently conservative substitution of Ser^{159} by threonine causes a significant decrease in p35 binding ability (5), whereas the Ser^{159} → Ala-CDK5/activator complex is fully active.

The phosphorylation of Thr^{14} and Tyr^{15} in the glycine-rich loop (G-loop; residues 11–16) that forms the “ceiling” of the ATP-binding site is important in the regulation of CDK activity (18). Certain structural aspects of CDK2 were recently proposed on the basis of molecular dynamics simulations (19, 20). Inhibitory phosphorylation of specific residues (Thr^{14} and Tyr^{15}) in CDK2 results in ATP misalignment and a shift of ~5 Å in the position of the G-loop, “opening” the substrate-binding site. Phosphorylation of Thr^{14} of CDK5 by an unidentified kinase results in similar inhibition. However, this has only been observed *in vitro*, and the significance of Thr^{14} phosphorylation *in vivo* remains unclear. The phosphorylation of Tyr^{15} of CDK5, on the other hand, occurs both *in vitro* and *in vivo*. The Tyr^{15} is phosphorylated by Abl tyrosine kinase, and this increases CDK5 activity to some extent (5, 21, 22). By contrast, the phosphorylation of Tyr^{15} and Thr^{14} by kinases of the Wee1 family inhibits CDK1 and CDK2 (18). It is not yet known why the phosphorylation of Tyr^{15} in CDK5 has an opposite effect to that of phosphorylation of the adjacent Thr^{14}.

As the key regulatory enzymes of the cell cycle, CDKs have become important pharmaceutical targets for inhibitor design. The purine derivative roscovitine (2-(1-ethyl-2-hydroxy-ethylamino)-6-benzylaminono-9-isopropylpurine; Scheme 3) has been shown to be a potent inhibitor of CDKs, with IC_{50} values of 0.16 μM for CDK5 (23) and 0.70 μM for CDK2 (24). The crystal structure of the CDK2/roscovitine complex indicates that the inhibitor binds in the ATP-binding pocket of CDK2 but that its mode of binding is different from that of ATP (24–26). Recently, Mapelli *et al.* (27) described the crystal structure of CDK5 (144N)^{1044}/p25/roscovitine. The (R)-enantiomer (CYC202) of the
inhibitor roscovitine, which is discussed in this paper, is particularly interesting because of its importance in medicinal chemistry; it is now entering phase II cancer clinical trials and phase I clinical tests against glomerulonephritis, following encouraging results from preclinical tests (28).

MATERIALS AND METHODS

Molecular dynamics simulations of CDK5/p25 and CDK5/p25/roscovitine complexes and of versions of these complexes phosphorylated "in silico" on the Tyr(15) residue of the G-loop were carried out using the program PMEMD-3.01 (29) with the parm99 force field (30). The CDK5/p25 starting structure was taken from the Protein Data Bank (code 1H4L), and the starting structure for CDK5/p25/roscovitine was created by superposition of the CDK5/p25 and CDK2/roscovitine complexes. The residues not observed (residues 1, 11–14, and 39–42) in the crystal structure (Protein Data Bank code 1H4L) were added to the simulated proteins using Insight II (using the CDK2/roscovitine x-ray crystal structure as a template structure (24)) and annealed and minimized before running the MD simulation protocol with constraints on those residues whose position was specified in the crystal. The missing terminal residues of CDK5 (residues 288–292) were not included in the simulations. The MD simulation protocol applied was as follows. First, the protonation states of all histidines were checked using WHATIF (31) to create an optimal H-bond network. All hydrogens were added using the Xleap program from the AMBER 6.0 package (32). The structures were neutralized by adding 6 Cl− counter ions to the unphosphorylated systems and 4 Cl− counter ions to the systems phosphorylated at Tyr(15). Each system was solvated in a rectangular water box with a layer of water molecules 10 Å thick. The energy of each system was then minimized as follows prior to the main molecular dynamics simulation run. The protein was frozen, and the solvent molecules and counterions were allowed to move during a 1000-step minimization and a 2-ps long molecular dynamics run under NpT conditions. The side chains were then relaxed using several sequential minimizations with the force constants applied to the backbone atoms being decreased in each run. Following this relaxation, the system was heated to 250 K for 10 ps and then to 298.15 K for 40 ps. The production phases were run for 10 ns for both unphosphorylated systems and for 5 ns for the Tyr(15)-phosphorylated systems. Each individual system studied consisted of ~55,000 atoms. The simulation period was chosen as a compromise between the quality of configuration space sampling and the calculation length. Time integration steps of 2 fs were used, together with particle-mesh Ewald methods for electrostatic interactions. All simulations were run under periodic boundary conditions in the NpT ensemble at 298.16 K and at a constant pressure of 1 atm. The SHAKE algorithm with a tolerance of 10−5 Å was applied to fix all bonds containing hydrogen atoms. Non-bonding interactions were subject to a 10.0-Å cutoff. Coordinates were stored every 2 ps. All analyses of the MD simulations were carried out using the CARNAL, ANAL, and PTRAJ modules of AMBER-6.0 (32), and with GROMACS (33). The method of Cornell et al. (34) was used for the parameterization of roscovitine and the phosphorylated tyrosine (19) and threonine residues.

The results obtained were compared with previously obtained data on the fully active form of CDK2 (Thr(P)160-CDK2/cyclin A/ATP) and the fully active form of CDK2 complexed with the peptide substrate HHASPRK (Thr(P)160/CDK2/cyclin A/ATP/HHASPRK) (19, 20). The results of an MD simulation of the CDK2/roscovitine complex (25) were used to compare the interactions of roscovitine with CDK5 to those with CDK2.

RESULTS

Table 1 shows global characteristics of the MD trajectories. The CDK5/p25 and CDK5/p25/roscovitine complexes were stable during the production runs of the molecular dynamics simulations, as confirmed by analysis of the root mean square deviation from the x-ray crystal structure, radius of gyration ($R_g$), secondary structure elements (data not shown), and total energy.

The overall protein fold was stable throughout the simulations, except for the G-loop, which relaxed in both the CDK5/p25 and CDK5/p25/roscovitine complexes at an early stage of the simulations. In both cases, the simulated relaxed G-loop conformation agreed well with that in the recently published x-ray crystal structure of the CDK5(1449/p25/roscovitine complex (27)). The position and conformation of the G-loop were not affected by the presence of roscovitine in the active site, but the flexibility of the G-loop was reduced somewhat in the presence of the inhibitor; this was confirmed by analysis of the temperature B-factors and visual inspection.

Interactions of Roscovitine with CDK5—Four hydrogen bonds (H-bonds) were observed between CDK5 and roscovitine in the CDK5/p25/roscovitine complex (Fig. 2). The network of H-bonds involves the residues Cys83 (which is involved in two H-bonds), Ile16, Glu12, and Gln130. The Cys83 amide N–H forms an H-bond with the G-loop (Ile10 and Glu12) and the hinge region (Cys83) (H-bonds) were observed between CDK5 and roscovitine in the CDK5/p25/roscovitine complex. The Cys83 amide N–H interacts with the roscovitine N-7 atom, whereas the Cys83 carbonyl C=O forms an H-bond with the roscovitine N-6 atom. In the CDK2/roscovitine complex, Leu83 also forms H-bonds with the N-6 and N-7 atoms of roscovitine (see Scheme 3 for atom numbering). The hydroxyl group of the chiral hydroxyethyl substituent (R)-roscovitine is H-bonded to the Glu12 carbonyl oxygen at the beginning of the CDK5/p25/roscovitine sim-

| Trajectory                      | t (ns) | r.m.s. (CDK5) | r.m.s. (G-loop) | $R_g$ (Å) | $E$ (kcal mol$^{-1}$) |
|--------------------------------|-------|---------------|----------------|----------|----------------------|
| Cdk5/p25                       | 10    | 1.50 ± 0.15   | 1.26 ± 0.16    | 23.60 ± 0.08 | −1.308 ± 0.001       |
| Cdk5/p25/roscovitine           | 10    | 1.35 ± 0.13   | 1.23 ± 0.16    | 23.84 ± 0.08 | −1.320 ± 0.001       |
| Tyr(P)15-Cdk5/p25/roscovitine  | 8     | 1.56 ± 0.14   | 1.34 ± 0.10    | 23.94 ± 0.15 | −1.310 ± 0.001       |

*a r.m.s. is root mean square deviations of backbone atoms compared with initial x-ray crystal structures.

*b $R_g$ is the mean radius of gyration. $R_g$ for Cdk5/p25 x-ray structure (Protein Data Bank code 1H4L) and Cdk5/p25/roscovitine equals 23.47 Å.

$c$ $E$ is the mean total energy.

*d Tyr(P)15-Cdk5/p25 trajectory was also produced for comparison.
ulation but shifts to the Gln$^{130}$ carbonyl oxygen during the course of the simulation. By contrast, in the CDK2/roscovitine simulation, the roscovitine hydroxyl group alternately formed bonds with the Ile$^{10}$ carbonyl oxygen, the Asp$^{86}$ O-$\delta$ atom, and Gln$^{131}$ carbonyl oxygen at various points during the molecular dynamics run. The amino group containing the N-2 atom of (R)-roscovitine (i.e. the amino group of the C-2 substituent) forms an H-bond with the Ile$^{10}$ carbonyl oxygen of CDK5 (Table 2).

To quantify the interactions between roscovitine and CDK5/CDK2, the energies of interaction between the inhibitor and the respective kinase were calculated using the AMBER force field (parm99) and averaged over the MD trajectories (Table 3). The AMBER force field appears, on average, to reproduce satisfactorily the interaction energies (see Fig. 3). The interaction patterns of roscovitine with CDK5 and CDK2 are almost identical except for two minor differences in the interactions formed by N-7 and C-8 (Fig. 3A). The differences in the interactions of roscovitine with CDK5 or CDK2 arise from the facts that the position of Cys$^{83}$ in CDK5 is occupied by Leu$^{85}$ in CDK2, and where the inhibitor interacts with Leu$^{133}$ in CDK5, it interacts with Leu$^{134}$ in CDK2 (Fig. 3B).

**Conformational Behavior and Energetics of Tyr(P)$^{15}$-CDK5—Phosphorylation of the Tyr$^{15}$ residue of the G-loop of the CDK5/p25/roscovitine complex causes the position of the G-loop to shift relative to that in the unphosphorylated complex and also increases the flexibility of the loop. The loop moves away from the roscovitine-binding site, but the Tyr(P)$^{15}$ (phosphotyrosine) side chain still points toward the C2 group of roscovitine. Tyr$^{15}$ phosphorylation shifts the position of the G-loop by about $-8$ Å compared with that in the unphosphorylated CDK5/p25/roscovitine complex (the distance quoted is that between the relative positions of the C-$\alpha$ atoms of Thr$^{14}$ in the two complexes) (Fig. 4A). However, Tyr$^{15}$ phosphorylation in the CDK5/p25 and CDK5/p25/roscovitine complexes does not affect the secondary structure elements of CDK5 (results not shown).

The interaction energies of roscovitine with the unphosphorylated and phosphorylated CDK5/p25 complexes are equal to $-54.4 \pm 0.1$ and $-51.4 \pm 0.1 \text{ kcal mol}^{-1}$, respectively. The mean interaction energies differ by about 5.5%, reflecting the experimental finding that Tyr$^{15}$-CDK5 phosphorylation does not significantly influence roscovitine binding to CDK5 (27). The H-bond network formed between CDK5 and roscovitine in the unphosphorylated complex is similar to that formed after Tyr$^{15}$ phosphorylation.

**Conformational Behavior and Energetics of the Activation Segment—**

Like the x-ray structural analysis (5), analysis of the interaction energy strongly suggests that interactions with p25 effectively stabilize the active conformation of the T-loop in the CDK5/p25 complex (Fig. 5A). The mean interaction energy between T-loop residues (residues 150–165) and p25 is $-86 \text{ kcal mol}^{-1}$, whereas the equivalent interaction energy between CDK2 and cyclin A is lower, at $-69$ and $-60 \text{ kcal mol}^{-1}$ for CDK2/cyclin A/ATP and Thr(P)$^{160}$-CDK2/cyclin A/ATP, respectively. The lower interaction energy between CDK2 and cyclin A in the fully active CDK2 complex reflects known aspects of the stabilization of the active T-loop conformation in CDK2. The effect of the phosphorylation of the Thr$^{160}$ side chain is not to increase the strength of the interaction between CDK2 and cyclin A, but rather to increase the strength of the interactions between T-loop residues and specific CDK2 arginine residues (Arg$^{50}$, Arg$^{150}$, and Arg$^{126}$). The interaction energy patterns of Thr$^{160}$, Thr(P)$^{160}$ (CDK2/cyclin A, Thr(P)$^{160}$-CDK2/cyclin A), and Ser$^{159}$ (CDK5/p25) were further analyzed to further elucidate the mechanism of the conformational change in the T-loop. The CDK5 Ser$^{159}$ forms favorable interactions with other CDK5/p25 residues, namely Ala$^{190}$, Tyr$^{156}$, Glu$^{240}$, Glu$^{161}$, Arg$^{125}$, Tyr$^{179}$, Val$^{162}$, Leu$^{177}$, Asn$^{239}$, Cys$^{197}$, Ile$^{241}$, Arg$^{149}$, and Asp$^{192}$ (the underlined residues come

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5 P. Dobes, M. Otyepka, M. Strnad, and P. Hobza, submitted for publication.

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6 The CDK5 activation loop corresponding to the T-loop of CDK2 is referred to as the T-loop in this article even though it contains Ser instead of Thr at the phosphorylation site.

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**TABLE 2**

Hydrogen bonds between roscovitine (ROC) and CDK5 (see Scheme 3 for atom labeling)

| Trajectory                  | Donor          | Acceptor | Duration | Mean distance | Mean angle |
|-----------------------------|----------------|----------|----------|---------------|------------|
| CDK5/p25/roscovitine        | Cys$^{83}$ N   | ROC N-7  | 50.4     | $3.18 \pm 0.15$ | 19.78 $\pm$ 0.74 |
|                             | ROC N-6        | Cys$^{83}$ O | 88.6    | $2.92 \pm 0.13$ | 20.20 $\pm$ 5.78 |
|                             | ROC N-2        | Ile$^{10}$ O | 39.0     | $3.17 \pm 0.18$ | 14.62 $\pm$ 7.04 |
|                             | ROC O-1        | Glu$^{17}$ O | 15.3     | $2.90 \pm 0.20$ | 16.04 $\pm$ 7.13 |
|                             | ROC O-1        | Glu$^{130}$ O | 5.6      | $2.91 \pm 0.20$ | 15.11 $\pm$ 6.72 |
| Tyr(P)$^{15}$-CDK5/p25/roscovitine | Cys$^{83}$ N  | ROC N-7  | 43.7     | $3.24 \pm 0.14$ | 20.02 $\pm$ 7.01 |
|                             | ROC N-6        | Cys$^{83}$ O | 85.5    | $2.95 \pm 0.15$ | 20.32 $\pm$ 5.72 |
|                             | ROC O-1        | Asp-86 O-D1 | 7.4      | $2.78 \pm 0.18$ | 11.46 $\pm$ 6.34 |
|                             | ROC O-1        | Glu$^{130}$ O | 9.3      | $2.79 \pm 0.15$ | 14.25 $\pm$ 6.83 |
TABLE 3
The total interaction energy $E_{\text{tot}}$ in kcal mol$^{-1}$ and its electrostatic ($E_{\text{el}}$) and van der Waals ($E_{\text{vdw}}$) components between roscovitine and CDK5/CDK2

| Trajectory | $E_{\text{el}}$ | $E_{\text{vdw}}$ | $E_{\text{tot}}$ | IC50 |
|------------|----------------|-----------------|-----------------|------|
| CDK5/p25/roscovitine | $-4.0 \pm 0.1$ | $-50.4 \pm 0.1$ | $-54.4 \pm 0.1$ | 0.16 |
| CDK2/roscovitine | $-2.8 \pm 0.1$ | $-47.5 \pm 0.1$ | $-50.3 \pm 0.1$ | 0.70 |

$^a$ Data are from Ref. 25.
$^b$ Data are from Ref. 36; MP2/aug-cc-pVDZ calculation.

from p25. The total (nonbonded) interaction energies of Ser$^{159}$ with CDK5 and p25 are $-16$ and $-7$ kcal mol$^{-1}$, respectively. The interaction energy of Thr$^{160}$ with CDK2 residues is equal to $-17$ kcal mol$^{-1}$, whereas that of Thr$^{160}$ with cyclin A is negligible. The strength of the interaction between Thr$^{160}$-CDK2 is $-151$ kcal mol$^{-1}$, but the phosphorylation of Thr$^{160}$ also gives rise to repulsive interactions with cyclin A that sum to $+28$ kcal mol$^{-1}$. Thus, an energetic price is paid in converting the T-loop of CDK2 to its active conformation. The Thr$^{160}$ residue also forms stabilizing interactions with the substrate peptide (HHASPRK) that sum to $-36$ kcal mol$^{-1}$. Specifically, there is a favorable interaction involving the Lys residue at the P$_{\text{A}}$ position of the peptide (numbered from the site of phosphorylation, i.e. Ser is at position zero, $P_0$) equal to $-24$ kcal mol$^{-1}$ (Fig. 5B) (20).

In the x-ray structure, no H-bond between Ser$^{159}$ and p25 was observed. The CDK5 Ser$^{159}$-O$_{\text{Y}}$ atom remained H-bonded to the Glu$^{240}$ O$_{\text{Y}}$-1/2 for the entire MD simulation of the CDK5/p25 complex (Table 4). The Ile$^{153}$ backbone amide, at the bending tip of the activation loop, forms an H-bond with the Asn$^{270}$ O$_{\text{Y}}$ atom in both CDK5/p25 and CDK5/p25/roscovitine complexes. The Cys$^{157}$ S$_{\text{Y}}$-atom is H-bonded to the Asn$^{239}$ backbone carbonyl oxygen in the CDK5/p25 complex (Fig. 6).

The Val$^{163}$ residue adopts a left-handed conformation with $\phi = 53.3^\circ \pm 14.9^\circ$, $\psi = 155.9^\circ \pm 38.2^\circ$, and $\omega = 130.3^\circ \pm 11.6^\circ$ in the CDK5/p25 and CDK5/p25/roscovitine complexes, respectively. The unusual Val conformation is stabilized by the H-bond network, in which the Val$^{163}$ backbone carbonyl oxygen forms an H-bond with the guanidine group of the Arg$^{168}$ side chain (N-H1 group), whereas the Val$^{163}$ backbone amide forms an H-bond with the Arg$^{125}$ backbone carbonyl oxygen in the CDK5/p25 complex. The left-handed Val$^{163}$ conformation is also stabilized by two H-bonds with the Arg$^{125}$ and Arg$^{168}$ side chains in the CDK5/p25/roscovitine complex. The left-handed Val$^{163}$-CDK2 conformation is one of the two characteristic structural features of the active conformation of the activation loop (16). The conformation of Val$^{163}$ in the CDK5/p25 complex parallels that of the Val$^{164}$ conformation in Thr$^{160}$-CDK2/cyclin A, i.e. it adopts the conformation of the fully activated complex (Fig. 1C).

The Ser$^{159}$ residue in the activation loop of CDK5 is a potential phosphorylation site, comparable with Thr$^{160}$ in the T-loop of CDK2. Differences in the positioning of the Thr$^{160}$ residues of the various CDK2 complexes relative to that of Ser$^{159}$ in CDK5/p25 were assessed by superimposing the averaged structure of each protein obtained at the end of their respective MD runs and then calculating the distance between the C-$\alpha$ atoms of the relevant residues in each complex. The separations thus obtained were 7.5, 2.9, and 1.9 Å, respectively, for

FIGURE 4. A, a stereoview of the CDK5 G-loop shift (gray tube representation) after Tyr$^{15}$ phosphorylation (thin black tube). Tyr$^{15}$ and Tyr(P)$^{15}$ are shown in stick representation. B, comparison of G-loop positions at the end of MD simulations of Thr(P)$^{160}$-CDK2/cyclin A (thick black) and Thr(P)$^{160}$-CDK2/cyclin A/HHASPRK (thick gray), where the presence of the substrate peptide causes reorientation of the Tyr$^{15}$ (stick) side chain. Positions of the Tyr(P)$^{15}$ side chain do not differ in the Thr(P)$^{160}$-CDK2/cyclin A (thin black), and TyP(P)$^{160}$-CDK2/cyclin A/HHASPRK (thin gray) complexes.

FIGURE 3. A plot representing contributions of different roscovitine (Rosc) moieties to the interaction energy between CDK5/CDK2 and the inhibitor. A plot of the interaction energies of CDK5/CDK2 residues with roscovitine showing that only 10 residues contribute significantly to inhibitor binding ($E_{\text{tot}} < -1.5$ kcal mol$^{-1}$). The phosphorylation of Tyr$^{15}$ (CDK5) does not significantly affect the interaction of roscovitine with CDK5. IC50 values are taken from the literature (see text for detailed discussion). Asterisks denote residues at which the primary sequences of CDK5 and CDK2 differ.
CDK5/p25 Interactions: Kinase/Cyclin Recognition

Energy decomposition analysis was also used to determine the extent to which different regions of the complex contributed to the interaction energy between CDK5 and p25, and, for comparative purposes, to that between CDK2 and cyclin A (see Supplemental Material). Such detailed analysis of interaction patterns can provide useful insights into the origins of the specificity of the binding between the CDK and the regulatory subunit, which remains a mystery of cell biology. The key structural elements of CDK5 in terms of p25 binding were found to be the C-α helix (the PSTAIRE helix) and the following loops: C-α-1–C-α-2 (residues 50–60), C-α-2–C-α-3 (residues 71–75), C-α-3–C-α-4 (residues 116–123), the T-loop (residues 150–161), and the loop after the short C-α-4 helix (residues 177–181). The key binding elements of p25 were found to be the C-α-1–C-α-2 and C-α-2–C-α-3 loops and the C-α-3 (C terminus), C-α-4, C-α-5, and C-α-6 helices. Similar regions were identified in the CDK2/cyclin A complex. In general, the regions of CDK2 that interact with cyclin A are similar to those of CDK5 that interact with p25 (cf. Fig. 5B); in addition to these, it was found that the C-α-5–C-α-6 loop and C-α-6 helix of CDK2 forms significant interactions with the αNT-helix of cyclin A. As expected, more significant differences were observed between cyclin A and p25. The αNT, α2 (N terminus), α3, α5, and α6 helices of cyclin A contribute significantly to its interactions with CDK2, as do the α2–α3, α3–α4, and α5–α6

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FIGURE 5. A, a plot of the interaction energies of the CDK5 activation segment (T-loop) residues with the whole p25 (light gray), and the T-loop residues of Thr(P)160-CDK2 (black) or CDK2 (dark gray) with cyclin A (cA). B, the most significant interactions between CDK5 residues and p25 and between CDK2 residues and cyclin A (cA).

TABLE 4

Hydrogen bonds between the CDK5 activation segment and p25

| Trajectory               | Donor     | Acceptor       | Duration | Mean distance | Mean angle |
|--------------------------|-----------|----------------|----------|---------------|------------|
| CDK5/p25                 | Ile153 N  | Asn276 O–61   | 58.8     | 3.13 ± 0.19   | 11.95 ± 6.37 |
|                          | Cys157 S–γ| Asn239 O      | 19.0     | 3.25 ± 0.13   | 16.29 ± 7.03 |
|                          | Ser159 O–γ| Glu240 O–61   | 40.1     | 2.71 ± 0.14   | 10.96 ± 5.83 |
|                          | Glu240 O–2|               | 42.9     | 2.69 ± 0.14   | 10.54 ± 5.45 |
|                          | Asn239 O–2| Cys157 O      | 49.6     | 2.88 ± 0.13   | 11.61 ± 6.10 |
|                          | Ser159 N–82|            | 52.8     | 3.00 ± 0.17   | 16.02 ± 7.08 |
|                          | Ile153 N  | Asn276 O–61   | 58.9     | 3.15 ± 0.19   | 13.11 ± 6.81 |
| CDK5/p25/roscovitine     | Asn239 N–2| Cys157 O      | 49.6     | 2.88 ± 0.13   | 11.61 ± 6.10 |
|                          | Ser159 O  | Asn239 O      | 52.8     | 3.00 ± 0.17   | 16.02 ± 7.08 |
|                          | Ile153 N  | Asn276 O–61   | 58.9     | 3.15 ± 0.19   | 13.11 ± 6.81 |
loops (Fig. 7). These findings broaden and quantify previously published experimental data suggesting that residues 150–200 of p25 are responsible for CDK5 binding, and in addition, residues 279–291 are required for CDK5 activation (10).

Fig. 5B highlights several significant differences in the interactions between residues of CDK5 or CDK2 and those of p25 and cyclin A, respectively. It was found that residues Glu<sup>57</sup>, His<sup>71</sup>, and Lys<sup>177</sup> of CDK5 formed more favorable interactions with p25 than the corresponding residues of CDK2 did with cyclin A. On the other hand, the Asp<sup>73</sup> and Asn<sup>121</sup> residues of CDK5 interacted more favorably with one another than did the equivalent residues in the CDK2/cyclin A complex. Glu<sup>57</sup> (CDK5) makes a favorable contact with the close α6 helix of p25, whereas in CDK2 Glu<sup>57</sup> binds to Arg<sup>122</sup> (mutation in CDK5 has replaced this residue with Asn<sup>122</sup>), but its interaction with cyclin A is weak. His<sup>71</sup> (CDK5) forms a salt bridge with the residues Asp<sup>259</sup> or Glu<sup>255</sup> in the N-terminal part of the α5 helix of p25, but there is no equivalent salt bridge acceptor in the CDK2/cyclin A complex. The Asp<sup>73</sup> (Glu<sup>73</sup> in CDK2) repels the acidic residues of the N-terminal part of α5 of p25. In contrast, the corresponding part of α5 (CDK2) contains basic residues (KKQVLRMEHLVLK). The Lys<sup>177</sup> side chain interacts with the T-loop backbone in CDK5 but not in CDK2. The strong attraction between Arg<sup>122</sup> (CDK2) and cyclin A is probably caused by favorable electrostatic interactions with acidic residues of the αNT-helix. Such interactions are not observed in CDK5 because of the Arg/Asn mutation in its primary sequence. A difference in the interactions of CDK5 with p25 and of CDK2 with cyclin A centers on the Arg<sup>50</sup> residue, which is discussed in some detail by Barrett and Noble (38). In the fully activated CDK2 complex, Arg<sup>50</sup> (together with Arg<sup>126</sup> and Arg<sup>150</sup>) “anchors” Thr(P)<sup>160</sup>. In the
CDK2/cyclin A complex, it interacts with the α3-α4 loop of cyclin A; the magnitude of this interaction is reduced in the Thr(P)160-CDK2/cyclin A complex relative to that in unphosphorylated CDK2/cyclin A (cf. Fig. 5B). It should be noted that although our investigations did not suggest that there were any differences between the interaction energies of the three arginine residues (50, 126, 150) with Thr(P)160, such differences are discussed by Barrett and Noble (38). The interaction energies between Thr(P)160 (Thr160 in the unphosphorylated complex) and Arg50, Arg126, and Arg150 were, respectively, −41.5, −40.9, and −42.7 kcal mol\(^{-1}\) in Thr(P)160-CDK2/cyclin A; −40.8, −41.3, and −42.8 kcal mol\(^{-1}\) in Thr(P)160-CDK2/cyclin A/HHASPRK; and 0.0, −0.2, and −0.1 kcal mol\(^{-1}\) in CDK2/cyclin A.

**DISCUSSION**

**CDK5/CDK2-Roscovitine Interactions**—Roscovitine belongs to the class of very effective, selective, and therapeutically potent purine-like inhibitors of CDKs (25, 27, 28) and competes with ATP for occupation of the CDK active site. Roscovitine binds in the same position and orientation to both CDK5 and CDK2, and its position during MD simulations does not significantly differ from that implied by the crystallographic data. The mean CDK5/roscovitine and CDK2/roscovitine interaction energies are equal to −54.4 ± 0.1 and −50.3 ± 0.1 kcal mol\(^{-1}\), respectively. These values are consistent with roscovitine’s IC\(_{50}\) values of 0.16 μM for CDK5 and 0.70 μM for CDK2. Energy decomposition analysis was used to quantify differences between the interaction footprints of the inhibitor with CDK5 and CDK2. The mean interaction energies differ significantly only for Cys83 and Leu133 (which correspond to Leu134 and Leu134, respectively, in CDK2). The difference between Cys83/Leu133 and Cys83/Leu133 is attributed to the different electrostatic properties of these residues, whereas the difference between interactions with Leu133 (CDK5) and Leu134 (CDK2) arises from the van der Waals term. The interaction energies for these residues with roscovitine are −4.2 and −3.3 kcal mol\(^{-1}\) for Cys83 (CDK5) and Leu133 (CDK2), respectively, whereas those for Leu133 (CDK5) and Leu134 (CDK2) are −5.4 and −4.2 kcal mol\(^{-1}\), respectively. In both cases, the energies of interaction between roscovitine and CDK2 are lower than those with CDK5 because of the differences in side chain conformations between the two proteins; in CDK2, the side chains are directed away from the bound roscovitine. The van der Waals contributions to the interaction energy suggest that roscovitine fits into the active site of CDK5 better than it does in that of CDK2 because the van der Waals interaction energy between CDK5 and roscovitine is 2.9 kcal mol\(^{-1}\) lower than that between CDK2 and roscovitine (Table 3). In addition, as also shown in Table 3, van der Waals (i.e. dispersion) interactions are responsible for about 94% of the total interaction energy of roscovitine with CDK2/CDK5. This finding illustrates the importance of the dispersion energy in the binding of purine-like inhibitors to CDKs and is in agreement with recent correlated ab initio calculations.5

An understanding of the interaction energy pattern is essential in rational drug design and is useful in the design of new, more selective inhibitors. In this context, it is surprising that only 10 residues (Ile10, Val18, Phe40, Phe82, Leu/Cys83, His/Asp84, Glu85, Asp86, Glu131/130 and Leu133/134) contribute significantly to the total interaction energy of roscovitine with CDK2/CDK5 and that contributions from other residues are negligible. This finding suggests that it may be necessary to force new inhibitors to interact with these “unimportant” residues to obtain stronger and more selective binding. Such forcing may be achieved by synthesizing roscovitine derivatives with extended (longer and bulkier) substituents. From another perspective, the interaction energy patterns for roscovitine with CDK5 are not significantly different from those with CDK2, implying that the inhibitor will exhibit very limited selectivity between the two CDKs. This opens up the possibility of designing purine derivatives with longer substituents capable of binding to the kinases via different binding modes or even using different structures to increase their selectivity for CDK5 or CDK2. An excellent study describing the use of approaches such as those discussed above for the rational design of potent and selective (non-purine) compounds targeting CDK5 has been published recently (39).

**G-loop Dynamics and Interactions**—A GXGXXG consensus sequence pattern has been identified in proteins with a preference for dinucleotide binding (40, 41), and this motif is one of the most highly conserved in protein kinases (4). The functionality of the motif in protein kinases has been investigated in several studies (20, 42, 43). Phosphorylation of the Thr and/or Tyr residues of the G-loop depresses CDK1 and CDK2 functionality; by contrast, Tyr15 phosphorylation increases CDK5/p35 activity (21). The structural mechanism of activation by Tyr15 has been considered by Mapelli et al. (27) in the context of roscovitine binding. Simulations of the Tyr(P)2/Cdk5/p25/roscovitine complex suggest that Tyr15 phosphorylation leads to a G-loop shift which results in the side chain of Tyr(P)15 being directed toward the bound roscovitine (Fig. 4A). Analysis of interaction energies between roscovitine and CDK5/Tyr(P)15-CDK5 reveals that the influence of Tyr15 phosphorylation on roscovitine binding is almost negligible (Fig. 4B), a finding supported by experimental observations (27). Tyr15 phosphorylation in CDK2 results in the phosphate group being exposed to the solvent, along with a shift of the G-loop (20). The binding of the substrate peptide also prompts changes in the conformations of the Tyr15 side chain (cf. Fig. 4B). However, these results do not explain why phosphorylation is inhibitory in CDK2 but stimulatory in CDK5. Because molecular dynamics simulations of the protein alone have not provided any clear explanations of this phenomenon, it has been suggested that it may be necessary to consider the effects of phosphorylation in the presence of the substrate. CDK2 prefers basic substrates at the P1–P4 position ([S/T]PRK is the favored substrate motif (44)); the origins of this preference were revealed with the publication of the crystal structure of the Thr(P)160-CDK2/cyclin A/HHASPRK complex. The structure provided evidence of a direct interaction between Lys3 and Thr(P)160 (17), the magnitude of which was found to be −24 kcal mol\(^{-1}\) (20). CDK5 also exhibits a preference for basic residues at the P1–P4 positions despite the lack of phosphorylation on Ser159 (45–47). It has been proposed that the Glu240 residue of p25 interacts with basic P1–P4 substrate residues, which fulfil the role of Thr(P)160 in CDK2 (6), but analysis of a superposition of CDK5/p25 and Thr(P)160-CDK2/cyclin A/HHASPRK suggested that interactions with two additional acidic residues, Glu161 (the Glu161 → Ala mutation leads to a loss of CDK5 activity toward H1-derived peptide (47)), and Glu182 (CDK5), should also be taken into account. Consequently, Tyr(P)15 may serve as an ideal partner interacting with the basic substrate residues. This hypothesis is also supported by the fact that a direct interaction between R12 and Tyr(P)15 has been observed in simulations of Tyr(P)15, Thr(P)160, CDK2/cyclin A/HHASPRK complex (20), and the presence of peptide substrates results in changes in the conformation of the Tyr15 side chain, as observed in x-ray structures (17) (Fig. 4B).

The experimental results suggest that phosphorylation of Thr14 is inhibitory in CDK5, whereas the phosphorylation of Tyr15 is stimulatory (21). Activation by Tyr15 phosphorylation is discussed above. By contrast, the mechanism of inhibition by Thr14 phosphorylation may be similar to the mechanism of CDK2 inhibition by Thr15 phosphorylation, which results in significant misalignment of ATP in the active site because of electrostatic repulsion between two adjacent negatively
charged groups, i.e. the phosphate moiety of ATP and that of Thr(19, 20). Consequently, the misaligned ATP γ-phosphate cannot be transferred to the substrate Ser/Thr.

**T-loop Dynamics and Interactions**—Despite the absence of a phosphate group on Ser159, the CDK5 activation loop (T-loop) adopts an extended conformation typical of active proline-directed kinases. This conformation is almost identical to that observed in the fully active CDK2 (Thr(160)-CDK2/cyclin A/HHASPRK) complex (Fig. 1D). Superposition of the averaged structures of the fully activated CDK2/cyclin A/HHASPRK complex and the CDK5/p25 complex obtained during MD simulations reveals that the C-α atom of Ser159 is only 1.9 Å away from the C-α atom of Thr(160) in the Thr(160)-CDK2/cyclin A/HHASPRK complex. The Val163 residue of CDK5/p25 adopts a left-handed orientation typical of the active loop conformation (Fig. 1C). These structural aspects imply that CDK5 may be activated without phosphorylation of the activation loop, although its activation loop contains a potential phosphorylation site in Ser159.

The binding patterns of Thr160, Thr(160) (CDK2), and Ser159 (CDK5) were analyzed to determine why T-loop phosphorylation is necessary for CDK2 to adopt the active T-loop conformation. The interaction energy between Ser159 and other residues of CDK5 and p25 is equal to −16 and −7 kcal·mol−1, respectively, and that between Thr160 and other CDK2 residues is −17 kcal·mol−1, but the interaction energy of Thr(160) with cyclin A residues is 0 kcal·mol−1. Phosphorylation of Thr160 increases the Thr160/CDK2 interaction energy to −151 kcal·mol−1 but also results in a repulsive interaction between Thr160 and cyclin A of +28 kcal·mol−1. The interaction energy between the whole T-loop (residues 150–165) of CDK5 and p25 is −86 kcal·mol−1, whereas the equivalent interaction energy between CDK2 and cyclin A is −69 and −60 kcal·mol−1 in the cases of CDK2/cyclin A/ATP and Thr(160)-CDK2/cyclin A/ATP, respectively. These results support the conclusion drawn from studies of the complexes of the x-ray structures, namely that Thr(160) (CDK2) acts as an organizing center, interacting with three neighboring Arg residues (Arg50, Arg126, and Arg150), and stabilizes the active, extended conformation of the T-loop (16, 17). Furthermore, the results reported in this study enable these interactions to be quantified. It is also observed that Thr(160) forms stabilizing interactions with the substrate peptide (HHASPRK) and that the magnitude of these interactions is equal to −36 kcal·mol−1. The interaction with the P3 residue is particularly noteworthy, contributing −24 kcal·mol−1 (20). The −17 kcal·mol−1 difference in interaction energy between CDK5/p25 and CDK2/cyclin A is sufficient to stabilize the active conformation of the T-loop in the former complex.
CDK5/p25 Binding and Dynamics

This fact illustrates the complex interplay between CDK/regulatory subunit recognition and regulation of CDK activity. Analysis of the interaction energy between CDKs and regulatory subunits reveals considerable differences between CDK5 and CDK2, with Ile153, Cys157, and Ser159 forming H-bonds to p25 in the former case (Fig. 6). However, in general, it is not easy to relate differences in interaction patterns to one or a few residues, and consequently it seems that the processes and interactions underpinning CDK/regulatory subunit recognition and specificity are complex and cooperative.

Insofar as we are aware, there is no evidence suggesting that CDK5 activity requires the phosphorylation of Ser159. However, a simulation of the Ser(P)159-CDK5/p25 complex was performed to investigate the extent (if any) to which phosphorylation affects the structure of the activation segment. Ser159 was phosphorylated in silico, using the conformation of Thr(P)160-CDK2 as a starting point for the Ser(P)159-CDK5 complex, with the phosphate group directed toward three anchoring arginines (Arg50, Arg127, and Arg151).

CDK5/p25 and CDK2/Cyclin A Interaction Patterns—The energy decomposition analysis revealed some important differences in the interactions between CDK5 and p25 and between CDK2 and cyclin A. These differences may underpin the preferential binding of CDK5 to p25 and that of CDK2 to cyclins A or E. The surface of p25 interacting with CDK5 is smaller than the surface of cyclin A interacting with CDK2, because of the different positions of the αNT-helix in p25 and cyclin A (Fig. 8, A–E). The αNT-helix of cyclin A contributes significantly to its interaction energy with CDK2, and thus plays a significant role in CDK/cyclin recognition. By contrast, the surface of p25 in contact with the T-loop adopts a different shape to that of cyclin A and seems to be more negatively charged (Fig. 8, D and E). Certain specific interactions with p25 that stabilize the T-loop of CDK5 in its active conformation are discussed above, but in general adoption of the active T-loop conformation appears to be a consequence of cooperative interactions of all of the T-loop residues with the p25 surface, which provides a good geometric match for the active conformation of the T-loop (Fig. 5A).

These differences may contribute to substrate discrimination and give rise to CDK5/cyclin specific functionality, i.e., to the different biological roles of specific complexes (51).

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