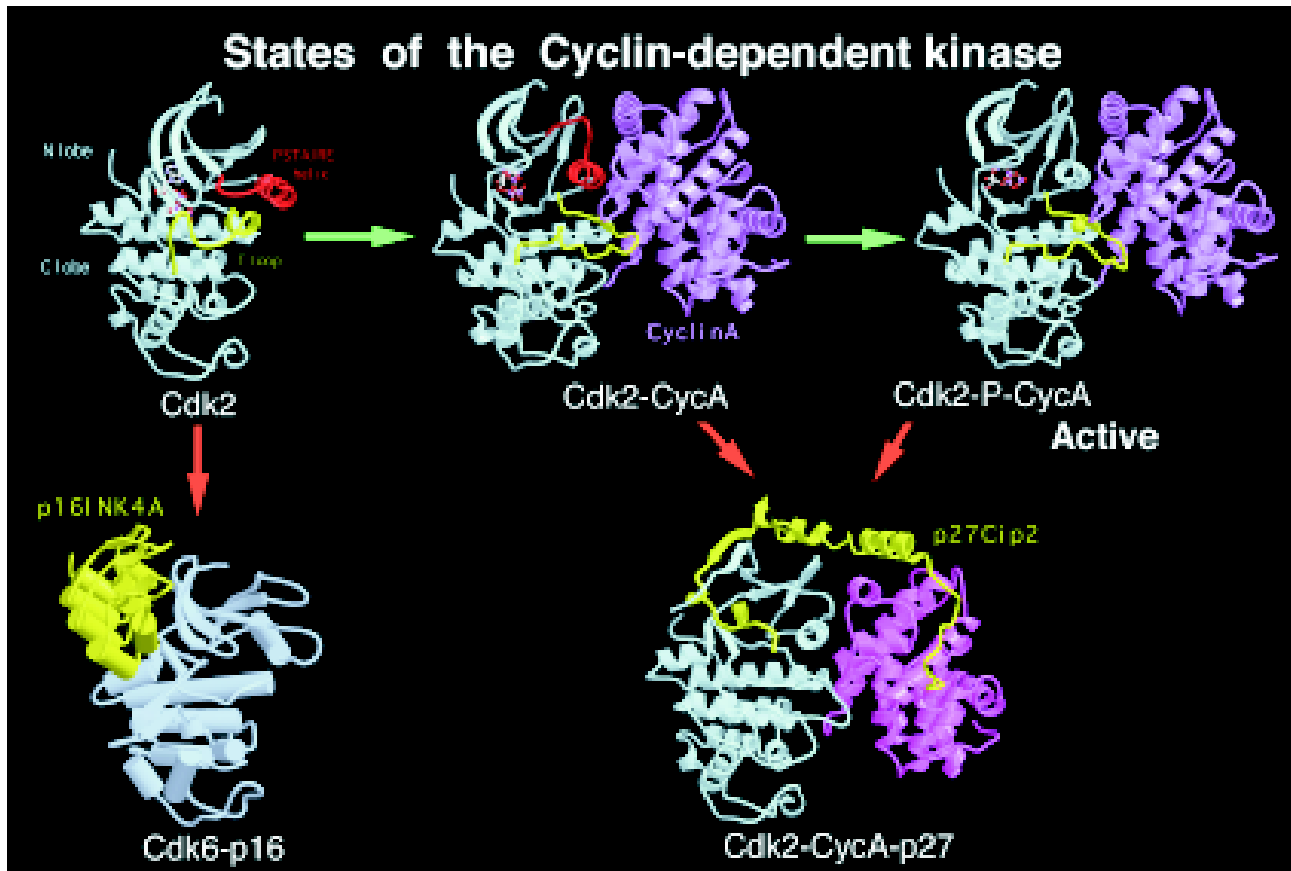


Visualising the Cell-Cycle: Structural Studies on the Cyclin-Dependent Kinases

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Regulation of CDK activity illustrated by protein crystallography. The structures of the CDK complexes are as described in this work. The structure of monomeric CDK2 is taken from ref. 7. CDK2 and CDK6 are shown in pale blue, cyclinA in magenta, p27^{Cip2} and p16^{INK4a} in yellow.

Cells from higher organisms expend a great deal of energy and time ensuring that cell growth and division (the cell-cycle) completes successfully. Failure to do so courts disaster, with possible corruption of the genome and loss of cell viability. Consequently the cell cycle is regulated by complex and multiple mechanisms involving hundreds of proteins [1]. Ultimately, however, much of these mechanisms act via regulation of a single family of proteins - the Cyclin-Dependent Kinases (CDKs). The CDKs are Serine/Threonine protein kinases which transfer the γ -phosphate of ATP onto a Ser or Thr side-chain of target substrates. Such phosphorylation is a common regulatory event in the cell.

CDKs are minimal kinases - they consist of little more than the catalytic core fold - and are inactive in their monomeric state. They are activated by the non-covalent binding of the cyclin subunit (hence the name CDK). The simple binary complex between CDK2 and cyclinA is active *in vitro*, having an activity 10,000x greater than monomeric CDK2. Effective activity *in vivo* requires the phosphorylation of Thr160 on CDK2 - by yet another cyclin-CDK complex (the CDK-activating kinase, CAK, which contains CDK7 and cyclinH). This active binary complex can be inhibited by specialized CDK inhibitors – members of the INK4 and Cip/Kip inhibitor families.

One of the features of cancer cells is their uncontrolled proliferation, which includes evasion of regulatory signals that control entry into the cell cycle. The importance of CDKs in cancer is emphasized by the observation that many CDK regulators are altered in tumor cells. The CDK inhibitor p16^{INK4a}, a specific inhibitor of CDK4 and CDK6, is a major tumor suppressor and it is found mutated or deleted in about one third of all cancer cases. Another CDK inhibitor, p27^{Cip2}, is only present at low levels in several types of cancer and this correlates with poor clinical prognosis. A CDK activator, cyclinD, is often found amplified in breast cancer. Even one of the CDKs, CDK4, is found mutated in melanoma in such a way that it evades inhibition by INK4 family inhibitors.

Cell-cycle proteins are notoriously intransigent - their existence is often fleeting, and they are not necessarily designed to survive preparation in large amounts and the indignities of crystallization. We are not alone in finding the majority of cyclins and CDKs intractable to large-scale preparation, and we've been lucky to find a pair of cooperative proteins in CDK2 and cyclinA, which we can make sufficient quantities of, and crystallize relatively readily. CDK2 is made in insect cells using a baculovirus vector, and cyclinA in E.coli. Our best data on crystals of the CDK2-cyclinA complex within the lab was at a very modest 3.0 Å resolution, but at the CHESS A-1 beamline with the CCD detector we managed to get 2.3 Å data. The better CHESS data made building the structure more straightforward, and the final structure much more accurate [2].

The structure gave us the first insights into how CDK2 is activated when cyclinA binds to it. CyclinA provokes two major structural changes within CDK2 that bring about conversion from the inactive (monomeric) kinase to the active cyclin-bound form. These two structural elements are the PSTAIRE helix, bearing the sequence motif that is characteristic of the CDK family, and the neighboring T-loop (also called the activation loop). Movement of the PSTAIRE helix (rotation of 90 degrees and translation by 5 Å) brings residues within the active site into their proper alignment for catalysis by comparison with structures of other eukaryotic protein kinases, such as protein kinase A [3], and also provokes a major structural rearrangement of the T-loop. This rearrangement involves the melting of one secondary structure element (changing from an α -helix to β -sheet) and movement of some residues in the loop by as much as 12 Å. The rearrangement also helps align active site residues, and also removes the obstruction that would have prevented transfer of the γ -phosphate group of ATP onto the substrate.

The next structure in the series was that of the CDK2-cyclinA complex phosphorylated at Thr160 on CDK2,

corresponding to the fully active kinase [4]. In common with the original complex, synchrotron radiation was again needed to extract the most out of this structure, and we obtained 2.6Å data at the CHESS A-1 beamline. The structure revealed still more rearrangements of the kinase, with the activation loop locally refolding to accommodate the phosphothreonine group. Three positively-charged residues from other regions of the kinase group around the phosphate moiety to neutralize its negative charge and stabilize the new folded conformation of the activation loop. Although the ATP-binding site on the kinase is not altered during this refolding, the putative substrate binding site is located precisely at this region of the structure, and the activation loop conformation in the phosphorylated form of CDK2 is presumed to offer a better substrate binding.

Having gone to the trouble of making a fully active CDK2-cyclinA complex, we then went ahead and inhibited it by adding the CDK inhibitor p27^{Cip2}. The CDK2-cyclinA-p27 complex is completely inactive (basically the same as monomeric CDK2). We had some problems building this complex since we didn't appreciate beforehand what a radical structure p27 has. Once completed, the structure revealed that N-terminus of p27 was not folded up like a typical globular protein - it extended over the surface of the CDK2-cyclinA complex, forming hydrophobic regions between itself and regions on the cyclin and the kinase [5]. The inhibitory mechanism of p27 is multi-faceted: it occupies a secondary substrate recruitment site on the cyclin; it binds to the N-terminal lobe of CDK2, flattening it out and disrupting the active site; it inserts itself into the ATP binding site, blocking ATP binding to the CDK2 structure. With p27 bound, there is no prospect of the kinase being able to bind either substrate or ATP.

Satisfied that we understood the mechanism CDK2 activation and inhibition, we sought to expand our horizons to CDK6 - one of the two CDKs that control the cell's commitment to DNA replication (restriction point). Working with CDK6 makes CDK2 preps look straightforward, and we needed to use p16^{INK4a} and p19^{INK4c} affinity columns to overcome CDK6's reluctance to stay in solution. With perseverance we have been able to produce CDK6 in complex with two members of the INK4 inhibitor family - p16^{INK4a} and p19^{INK4c}. In contrast to the Cip/Kip family of inhibitors, which bind to and inhibit many different CDKs, the INK4 inhibitors act only against CDK6 and the closely-related CDK4. INK4-bound CDK6 and INK4-bound CDK6-cyclin complexes are inactive, as is monomeric CDK6.

Both p16-CDK6 and p19-CDK6 structures [6] presented considerable technical challenges from a data collection standpoint. p16-CDK6 diffracts relatively poorly - to about 3.8 Å in the lab, and we needed to expose the crystals

to a great deal of radiation at the CHESS A-1 beamline to extract the most amount of data from them. We processed the p16-CDK6 dataset frame-by-frame as we collected it, in order to obtain the maximum amount of data in the minimum amount of time before radiation damage reduced the resolution limit of the crystals. The p16-CDK6 structure was solved by a combination of multiple isomorphous replacement and molecular replacement techniques.

The p19-CDK6 structure presented a different set of challenges - the crystals would usually crack the moment the hanging drop was opened! This proved a formidable obstacle, and over one hundred crystals were mounted on the X-ray machines before the final optimal strategy was determined (cross-linking the crystals with glutaraldehyde while they were still in the hanging drop). Ultimately, the best p19-CDK6 data extended to 2.8 Å resolution, but were very anisotropic (the anisotropic correction differs in two directions by $>60 \text{ Å}^2$). The p19-CDK6 complex gave us more detail and further insights to CDK regulation, but was qualitatively very similar to the p16-CDK6 complex.

The two INK4-CDK6 complexes gave us a look at a completely different mechanism of CDK inhibition. INK4 inhibitors are unrelated to the p21/p27/p57 family of inhibitors in all except function, and use different binding sites and mechanisms. The INK4 mode of inhibition also uses more than one method: it disrupts the edge of the ATP binding site near where the adenosine ring of ATP binds, eliminating productive ATP binding, and it alters the relative position of the N- and C-lobes of the kinase (CDK6), further disrupting the alignment of active site residues that would be required for ATP phosphate transfer.

The result of many years of intensive study of the CDKs, and their associated proteins, has given us considerable insight into how the cell regulates the cell-cycle machinery at the atomic level. CDKs emerge as being very flexible molecules whose conformations can be manipulated by the binding of other proteins, to alter their activity for substrates. Access to the intense X-ray source at CHESS has enabled us to witness these events in much greater detail than we could have ever hoped to using a lab source.

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