The Replacement of ATP by the Competitive Inhibitor Emodin Induces Conformational Modifications in the Catalytic Site of Protein Kinase CK2*

Received for publication, May 18, 2000, and in revised form, July 3, 2000
Published, JBC Papers in Press, July 5, 2000, DOI 10.1074/jbc.M004257200

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The structure of a complex between the catalytic subunit of Zea mays CK2 and the nucleotide binding site-directed inhibitor emodin (3-methyl-1,6,8-trihydroxyanthraquinone) was solved at 2.6-Å resolution. Emodin enters the nucleotide binding site of the enzyme, filling a hydrophobic pocket between the N-terminal and the C-terminal lobes, in the proximity of the site occupied by the base rings of the natural co-substrates. The interactions between the inhibitor and CK2α are mainly hydrophobic. Although the C-terminal domain of the enzyme is essentially identical to the ATP-bound form, the β-sheet in the N-terminal domain is altered by the presence of emodin. The structural data presented here highlight the flexibility of the kinase domain structure and provide information for the design of selective ATP competitive inhibitors of protein kinase CK2.

Protein kinase CK2 (also misnamed “casein kinase 2,” after its propensity to phosphorylate the artificial substrate casein in vitro) is a highly conserved, ubiquitous, Ser/Thr protein kinase belonging to the CMGC group (1). The CK2 holoenzyme is an heterotetramer composed of two catalytic subunits, α and/or α’, tightly associated with two regulatory β-subunits. The latter share no homology with any known protein except for the product of the stellate gene in Drosophila (2, 3). Evidence for the existence of free catalytic subunits playing autonomous role in the cell has been also reported (4, 5). Unlike the majority of Ser/Thr protein kinases, whose substrates present consensus sequences generally determined by basic and/or prolyl residues, CK2 recognizes phosphoacceptor sites that are specified by multiple negatively charged amino acids. CK2 is probably the most pleiotropic member of the protein kinase family, with more than 160 substrates known to date (6). Among these are key enzymes in different metabolic pathways and proteins playing important roles in a wide variety of cellular functions, with special reference to transcription, translation, signal transduction, and cell cycle regulation (7). Such an extraordinary pleiotropism is likely related to another unusual feature of CK2, namely its apparent lack of any mechanism of tight control. Unlike most protein kinases, the catalytic subunits of CK2 are in fact constitutively active, either alone or when incorporated into the heterotetrameric holoenzyme. Moreover, its association with the β-subunits may affect in opposite directions the catalytic activity, depending on the nature of the phosphoacceptor substrate (6). Such a high basal activity has been suggested to underlie the implication of CK2 in neoplasias and possibly also in infectious diseases. Indeed, CK2 activity is always abnormally high in transformed cell lines as well as in solid tumors (8), and transgenic mice that expressed elevated levels of CK2α in lymphoid organs displayed a stochastic propensity to develop lymphomas (9). Co-expression of c-Myc (9) or Tal-1 (10) transgenes in these mice or simultaneous suppression of p53 (11) resulted in neonatal leukemia. The oncogenic potential of CK2α and α’ is also highlighted by their ability to co-operate with Ha-ras in fibroblast transformation (12). On the other hand, the constitutive activity of host cell CK2 is exploited by many viruses for the phosphorylation of proteins that are essential to their life cycle (8).

Given these premises, the development of specific inhibitors of CK2 would be highly desirable. On one hand, they may have pharmacological applications in a number of pathological situations; on the other hand, they will help to unravel the still enigmatic role of CK2 in cell regulation and in individual signal transduction pathways. It should be preliminarily noted in this respect that staurosporine, perhaps the most potent broad specificity inhibitor of protein kinases, is poorly effective on CK2 (13). This observation, in conjunction with the ability of CK2 to use GTP besides ATP as phosphate donor (14), is symptomatic of a co-substrate binding site with unique features, which could be exploited for tailoring selective inhibitors.

The most straightforward strategy toward this aim would be to grow crystals of CK2α in complex with ATP site-directed competitive inhibitors and to solve their structure. Based on our present knowledge, CK2 inhibitors of this type may fall in three categories (Fig. 1): (i) Isoquinoline derivatives (15). The lowest Ki value, however, is about 70 μM, denoting a quite modest affinity, especially considering that another acidophilic protein kinase, CK1, is inhibited much more efficiently (Ki = 5 μM). (ii) Halogenated benzimidazole/benzotriazole derivatives, whose parent compound has to be considered 5,6-dichloro-1-(β-D-furanosyl)-benzimidazole (16), commercially available as DRB and whose inhibitory potential correlates with the num-

* This work was supported by the Italian National Research Council, Rome, Italy and from grants from European Community (Biomed-2, BMHI4-CT-0047), Armenise-Harvard Foundation, AIRC, Italian Ministry of Health (Project AIDS), Italian MURST (PRIN 1998), and Consiglio Nazionale delle Ricerche (Target Project on Biotechnology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1F0Q) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 38, Issue of September 22, pp. 29618–29622, 2000 Printed in U.S.A.
Crystal Structure of CK2 in Complex with Emodin

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The catalytic α subunit of Z. mays CK2 was expressed in E. coli and purified as described elsewhere (22).

Crystallization and Data Collection—The Z. mays CK2 α-subunit was crystallized as described in Ref. 22, with a crystallization solution composed of 30% polyethylene glycol 4000, 200 mM sodium acetate, and 100 mM Tris-Cl, pH 8.0, in absence of co-substrate, either ATP or GTP. Single crystals grew in several days at 292 K. Because of their small size, data had to be collected at the x-ray beamline of synchrotron ELETTRA in Trieste. Before mounting, crystals were extensively washed and then soaked for several hours with a solution saturated with emodin. The inhibitor, an anthraquinone derivative (3-methyl-1,6,8-trihydroxyanthraquinone) purchased from Sigma, is poorly soluble in aqueous media, and its solution presents a red color. At the beginning, sitting drops were uniformly colored red, but after some hours the color concentrated in the crystals, whereas the surrounding solution changed to pale orange, indicating the penetration of the inhibitor inside the crystals. Before mounting, the crystal was soaked for some seconds in a cryoprotectant solution, consisting of crystallization buffer and 5% glycerol. Measurements were done with a wavelength of 1.2 Å at 100 K and a crystal to detector distance of 300 mm. A data set with a completeness of 89.4% and an Rmerge of 4.0% could be collected at a resolution of 2.63 Å. Statistics on data collection are summarized in Table I. Emodin-soaked crystals are isomorphous to the ATP-bound α–CK2 space group C2, a = 143.08 Å, b = 52.08 Å, c = 44.88 Å and β = 99.33°. The Matthews coefficient Vm is 2.6 Å³ Da⁻¹ with one molecule in the asymmetric unit, for a solvent content of 53%.

Structure Determination and Refinement—The starting point for the refinement was the structure of the α-CK2 subunit, deprived of the ligand and solvent molecules (23). The automatic crystallographic refinement was carried out with CNS (24) with inspection and manual adjusting of the model performed with O (25). The presence of the inhibitor was revealed by a flat electron density in the active site of the enzyme, clearly visible both in a 2Fh – Fc map and in a Fc – Fo map. The presence of the anthraquinone derivative was corroborated by simulated annealing omit maps. During the last cycles of refinement, 58 solvent molecules were introduced in peaks of the Fourier difference map close to hydrophilic residues. The final model, whose statistics are reported in Table I, presents an overall R factor of 19.2 (Rmerge = 57.2) and a good stereochemistry: for 2747 protein atoms, the root mean square deviation of bond lengths is 1.4 Å and in bond angles is 1.0 Å.

RESULTS AND DISCUSSION

Crystals of α-CK2 obtained in the absence of co-substrate and subjected to soaking with the inhibitor emodin were isomorphous with that of the protein crystallized in the presence of ATP or GTP (14, 23). The presence of emodin inside the crystals, suggested by the red color they assumed after the

### Table I

| Data collection and final model statistics |
|-------------------------------------------|
| Numbers in parentheses refer to the highest resolution bin. |
| Data collection | Resolution (Å) | 25.7–2.63 (2.77–2.63) |
| Independent reflections | 8792 (1186) |
| Multiplicity | 2.0 (1.9) |
| (I/σI) | 12.3 (4.8) |
| Rmerge | 0.040 (0.156) |
| Completeness (%) | 89.4 (83.6) |
| Final model | Reflection used in refinement | 8751 |
| Protein atoms | 2747 |
| Solvent molecules | 58 |
| Rmerge free | 19.2/27.2 |
| r.m.s. on bonds distances (Å) | 0.009 |
| r.m.s. on bond angles (°) | 1.4 |

The abbreviations used are: r.m.s., root mean square; HCK, hematopoietic cell kinase.
The map was calculated without the inhibitor in the model. Residues with atoms within a radius of 5.5 Å from the center of the inhibitor are included. Some important residues discussed in the text are indicated.

As expected, emodin is located in the active site of the enzyme but in a position slightly different from that occupied by the natural co-substrates (Fig. 3). The binding of the inhibitor induces some significant structural changes in the N-terminal lobe of the macromolecule, although the overall architecture of the protein is similar to that observed in the binary complex with the ATP analog. Whereas the C-terminal domains can be almost perfectly superimposed (the r.m.s. deviation for 214 C\(_{\alpha}\) atoms from positions 120 to 333 is 0.40 Å), the N-terminal domain is affected by the presence of the inhibitor, and the r.m.s. deviation for 113 C\(_{\alpha}\) atoms from positions 7 to 119 increases to 1.29 Å. As shown in Fig. 4, the alterations are concentrated in few areas. The two segments from residues 58 to 63 and from residues 102 to 108 are exposed loops, far away from the position of emodin. The electron density of the second long loop is poorly defined, indicating a remarkable flexibility. More interesting are the differences in the position of amino acid Asn\(_{118}\), of the side chain of His\(_{160}\), and of the triplet Arg\(_{47}\)-Gly\(_{48}\)-Lys\(_{49}\), which is part of the Gly-rich loop. As shown in Figs. 4 and 5, the backbone segment composed of the three residues Arg\(_{47}\), Gly\(_{48}\), and Lys\(_{49}\) is shifted toward the interior of the active site, and this movement traps the emodin in an inner cavity. The inhibitor, in fact, is deeper embedded into the protein than the ATP or GTP molecules, with only the two outer six-membered rings roughly superimposing to the nucleotide base (Fig. 3). The inhibitor interacts with the protein mainly through hydrophobic contacts (Table II), filling a hydrophobic pocket between the two lobes of the protein, formed by residues Val\(_{146}\), Val\(_{153}\), Ile\(_{166}\), Leu\(_{85}\), Val\(_{165}\), Leu\(_{111}\), Phe\(_{113}\), Val\(_{116}\), Met\(_{163}\), and Ile\(_{174}\) (Fig. 2). This cavity is, at least in part, isolated from the bulk solution not only by the mentioned 46–50 segment but also by residues Asn\(_{118}\) and His\(_{160}\) (Fig. 5).

The C\(_{\alpha}\) atom of Asn\(_{118}\) moves of about 1.16 Å from its original position, and its side chain is reoriented in such a way to block the exit of the ligand. The imidazol ring of His\(_{160}\) is turned back forming an H bond with the carbonyl oxygen of Arg\(_{47}\). This hydrogen bond was not present in the ATP-bound enzyme.

Many of the nonpolar residues that make up the hydrophobic pocket surrounding emodin are conserved or conservatively replaced along the primary structure of the majority of protein kinases, this, however, does not apply to Ile\(_{166}\) and Ile\(_{174}\) (27). The former, in particular, although conserved or conservatively replaced by Val in all the \(\alpha\) and \(\alpha’\) CK2 subunits from different species, is replaced by an invariant alanine in all the other protein kinases. The presence of a bulky hydrophobic residue in position 66 could be particularly relevant in the binding of emodin and could confer unique features to the CK2 ATP-binding site.

In addition to the hydrophobic pocket, some loose polar interactions may also contribute to anchor the inhibitor into the active site. The emodin groups involved are the two hydroxyls at positions 6 and 8 (Table II). The latter is 3.99 Å away from the side chain carbonyl of Asn\(_{118}\), the former is 3.40 Å away from the backbone carbonyl of Val\(_{146}\), 3.70 Å from the amide nitrogen of Arg\(_{47}\), and 3.98 Å from the imidazol nitrogen of His\(_{160}\). These distances seem too long for justifying the formation of hydrogen bonds, but the relatively low resolution of our model does not allow an unequivocal interpretation. In any case, these polar interactions would be consistent with the requirement of these two OH for optimal inhibitory potential.
FIG. 5. Major changes in the active site of the CK2-emodin complex compared with the ATP-bound enzyme.

For the CK2-emodin complex (model A), carbon atoms of residues Asn118 and His160 are shown in green, and that of the same residues in the ATP-bound enzyme (model B, Protein Data Bank code 1A6O) are indicated in yellow. The positions of these two residues hamper the release of emodin from the cavity. There is also a shift of residues Arg47-Gly48-Arg49, whose positions in the models A and B are indicated with thick green and yellow lines, respectively. The hydrogen bond between the side chain of His160 and the backbone carbonyl of Arg47 is indicated by a dotted line. Nitrogen atoms are shown in blue, and oxygen atoms are in red.

### Table II

| Main interactions between residues of CK2α and emodin | Distance[^a] | Å |
|-----------------------------------------------------|-------------|---|
| Hydrophobic pocket                                  |             |   |
| Val[^b]                                             | C7          | 3.92 |
| Val[^b]                                             | O10         | 3.69 |
| Ile[^b]                                             | C7          | 3.78 |
| Leu[^b]                                             | CH₃         | 5.08 |
| Val[^b]                                             | O1          | 3.08 |
| Leu[^b]                                             | CH₃         | 5.29 |
| Phe[^b]                                             | C2          | 3.46 |
| Val[^b]                                             | O9          | 3.86 |
| Met[^b]                                             | C₆          | 3.00 |
| Ile[^b]                                             | C6          | 3.48 |
| Polar interactions                                  |             |   |
| Val[^b]                                             | O           | 3.40 |
| Arg[^b]                                             | O6          | 3.70 |
| Asn[^b]                                             | O8          | 3.99 |
| His[^b]                                             | Ne2         | 3.98 |

[^a] In the case of the hydrophobic pocket, it is the minimum distance between a carbon atom of residues forming the hydrophobic pocket and emodin.

(20). The cavity occupied by emodin is slightly larger than the actual volume of the inhibitor, suggesting that emodin maintains some degree of freedom once encapsulated into the pocket. The shape of the electron density and the mean value of thermal B factor of emodin, higher than that of the backbone atoms of the enzyme (58 Å² versus 50 Å²), are consistent with this hypothesis.

Despite the lack of any strong hydrogen bond or polar interaction, the relatively high affinity of emodin for CK2 could be possibly accounted for by the conformational changes promoted by the binding of the inhibitor, with special reference to the aforementioned movements of strand 46–50 and of the side chains of residues 118 and 160. These conformational changes entrap emodin inside the hydrophobic pocket, and, by hampering its release, they may increase its apparent affinity for CK2α.

Emodin is known to inhibit other protein kinases, besides CK2. For instance, its inhibitory activity against CK1 is only slightly lower than that measured for CK2, whereas that against PKA is 2 orders of magnitude lower (19). In the case of CK1, the same requirements for the binding of emodin are essentially conserved. The crucial residue His160 is replaced by Asp135, but its side chain has the possibility to rotate as the His in CK2 does. In PKA the situation is different, and it can explain the much lower activity of emodin on this kinase; a superposition of PKA and CK2 molecular models shows that the positions corresponding to Val166, Ile174, and Phe113 in the CK2 cavity are replaced by Ala70, Thr183, and Met126 in PKA, and that the latter two are closer in space (only 3.80 Å apart).

As a consequence, in the case of PKA, emodin cannot stand in the same position occupied in CK2, unless a relevant movement of the two residues Thr183 and Met126 takes place.

Emodin is also known as a tyrosine kinase inhibitor (20, 21, 28) but with IC₅₀ values higher than that of CK2. The structure of a tyrosine kinase belonging to the Src family, the hematopoietic cell kinase (HCK), in complex with ATP and different inhibitors is known. A comparison of these structures with that of the CK2-emodin complex reveals that the binding site of HCK can easily accommodate emodin, but possibly the binding is not so tight because the entrance of the cavity is not blocked after the binding of the inhibitor. In fact, residue His160 is replaced by an alanine (position 390 of HCK), which has a side chain that cannot rotate around its Cβ to form a hydrogen bond between its side chain and the residue corresponding to Arg47 in CK2. Because such a residue in CK2 contributes to lock the binding site and to trap emodin inside, its absence could influence the apparent affinity constant of the inhibitor for HCK.

### CONCLUSIONS

Emodin penetrates into the active site of CK2α, partially overlapping the position occupied by ATP; consequently, it prevents in a competitive manner the binding of the natural co-substrate. Hydrophobic interactions seem to be the driving force for the formation of the complex. The deeper position occupied by emodin in the hydrophobic pocket and the lack of a tail analogous to the triphosphate chain of ATP lead to conformational rearrangements that trap the inhibitor inside the binding site. These conformational changes involve Asn118, the side chain of His160, and three residues of the Gly-rich loop, Arg47-Gly48-Lys49. Superposition to our model of the structures of other protein kinases, whose activity is inhibited by emodin to different extents, discloses the important role played by His160 in the binding of the inhibitor. A movement of Asn118 is also significant; in the ATP bound enzyme, this residue has the function to coordinate the ribose O3′ atom, whereas in our complex it moves and prevents the departure of the inhibitor. The absence of the triphosphate tail is responsible for the inward collapse of the Gly-rich loop. Polar interactions between the backbone of residues 45 and 47 and the hydroxyl group at C6 of emodin may account for the importance of such a group to optimize the inhibitory potential. The elucidation of the molecular details of the interactions of CK2 with emodin discloses the possibility to design more potent and selective inhibitors of this ubiquitous and pleiotropic member of the protein kinase family.

Acknowledgments—We thank the CNR staff at ELETTRA (Trieste, Italy) for help during measurements at the diffraction beam line.
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