

The Active Conformation of the PAK1 Kinase Domain

Ming Lei,^{1,4} Michael A. Robinson,³
and Stephen C. Harrison^{1,2,*}

¹Laboratory of Molecular Medicine

²Howard Hughes Medical Institute

Children's Hospital

320 Longwood Avenue

Boston, Massachusetts 02115

³Department of Biological Chemistry and Molecular
Pharmacology

Harvard Medical School

240 Longwood Avenue

Boston, Massachusetts 02115

Summary

The p21-activated kinases (PAKs) participate in cytoskeletal control networks, downstream of Rho-family GTPases. A structure of PAK1 in an autoregulated, “off” state showed that a regulatory region, N-terminal to the kinase domain, forces the latter into an inactive conformation, prevents phosphorylation of Thr423 in the activation loop, and promotes dimerization. We have now determined structures at 1.8 Å resolution for the free PAK1 kinase domain, with a mutation in the active site that blocks enzymatic activity, and for the same domain with a “phosphomimetic” mutation in the activation loop. The two very similar structures show that even in the absence of a phosphorylated Thr423, the kinase has an essentially active conformation. When Cdc42 binds the regulatory region and dissociates the dimer, PAK1 will be in an “intermediate-active” state, with a capacity to phosphorylate itself or other substrates even prior to modification of its activation loop.

Introduction

Protein kinases are sensitive regulatory switches. They respond in various, well defined ways to multiple inputs, which include phosphorylation of residues on the kinase itself and association with activators or derepressors. Good examples of such control elements are the p21-activated kinases (PAKs), a family of serine-threonine kinases represented in all eukaryotes (Bagrodia and Cerione, 1999; Bokoch, 2003; Sells and Chernoff, 1997). They participate in a wide range of cellular control processes, including regulation of transcription in response to pheromone signaling in yeast and modulation of cytoskeletal dynamics in multicellular eukaryotes. Rho-family p21 GTPases, specifically Cdc42 and Rac in their GTP-bound forms, bind and stimulate PAKs, both in vitro and in vivo (Knaus and Bokoch, 1998; Lim et al., 1996; Manser et al., 1994, 1997; Martin

et al., 1995). This common activation trigger gives the family its name.

Like most protein kinases, PAKs have a phosphorylatable residue in their “activation loop”, a stretch of about 30 residues in the large lobe of the kinase domain. This loop can adopt multiple conformations, and modification promotes rearrangement to its “on” or “active” state. In PAK1, the best studied of the mammalian PAKs, the modifiable residue is Thr423. PAKs also have regulatory segments that lie outside the kinase domain. In PAK1, the key autoregulatory region (residues 67–150; Figure 1A) maintains an “off” state by forcing the kinase domain (residues 249–545) into an inactive conformation and by preventing phosphorylation of Thr 423 (Frost et al., 1998; Lei et al., 2000). GTP-bound Cdc42 or Rac can interact with part of the autoregulatory region, thereby prying it away from the kinase, enabling phosphorylation of Thr423, and allowing the kinase domain to adopt an active structure (Hoffman and Cerione, 2000; Lei et al., 2000; Tu and Wigler, 1999; Zenke et al., 1999; Zhao et al., 1998). The autoregulatory region inhibits PAK1 even when supplied *in trans* as an independent fragment.

The foregoing outline of activation is derived in part from the structure of an autoinhibited PAK1 complex, which we described in a previous article (Lei et al., 2000). That complex (Figure 1B) included an N-terminal regulatory fragment (residues 70–149, with elements defined in Figure 1A) and the C-terminal kinase domain (residues 249–545). In the structure, residues of the kinase inhibitor (KI) segment block the cleft between the two lobes of the kinase domain. Association of the inhibitory switch (IS) domain with the large lobe of the kinase presents the KI segment appropriately. The latter pushes the activation loop of the kinase domain into the active site, thereby preventing entry of ATP. NMR structures of Cdc42 complexes with fragments of PAK and WASP show that GTP-bound Cdc42 associates with the p21 binding domain (PBD) segment, which partly overlaps the IS domain (Abdul-Manan et al., 1999; Morreale et al., 2000). This interaction causes the IS domain to unfold, releasing the KI segment from the cleft of the kinase. The kinase domain, no longer restrained, changes conformation. Thr423 also becomes susceptible to phosphorylation, and its modification stabilizes the active state. PAK1 can then autophosphorylate at several other sites to fix the active conformation even more firmly by preventing refolding of the IS domain even after Cdc42 dissociates.

Autoinhibited PAK1 is a dimer, in the crystal structure, in solution, and in cells (Lei et al., 2000; Parrini et al., 2002). Part of the dimer contact is provided by a segment N-terminal to the IS domain, but because the crystals contained separate regulatory and kinase fragments, the structure by itself left open the issue of *cis*- or *trans*-autoinhibition. The effects of defined mutations on intracellular homo- and heterodimerization of coexpressed forms of PAK1 resolved the question and showed that the autoinhibitory interaction is in *trans*—in other words, that the IS domain and KI segment as-

*Correspondence: harrison@crystal.harvard.edu

⁴Present address: Department of Biological Chemistry, University of Michigan Medical School, 5413 Medical Science I, 1301 Catherine Road, Ann Arbor, Michigan 48109.

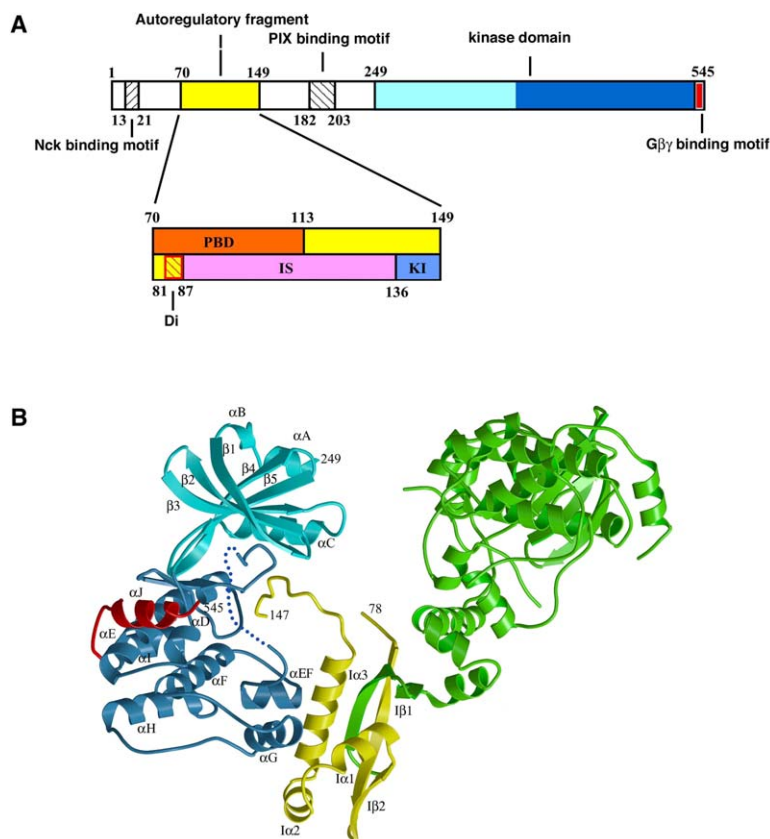


Figure 1. Autoregulation of PAK1

(A) Organization of the PAK1 polypeptide chain, with segments present in the crystal structure of the inactive dimer (Lei et al., 2000) shown in color (autoregulatory fragment, yellow; kinase small lobe, light blue; kinase large lobe, dark blue). The detailed substructure of the autoregulatory region is shown in the expanded lower part of the diagram. The divisions of the upper bar correspond to the p21 bound state; those in the lower bar correspond to the autoinhibited state. The autoregulatory fragment, N-terminal to the kinase domain, adopts different conformations in the inactive PAK1 dimer (in which the dimerization segment [DI], the inhibitory switch domain [IS], and the kinase-inhibiting segment [KI], have defined structural roles) and in complex with the activator, Cdc42 (in which only the "PBD" segment has a defined structure).

(B) Ribbon diagram of the inactive PAK1 dimer, illustrating how the autoregulatory fragment enforces an inactive conformation of the kinase domain and blocks (through the position of the KI segment) the catalytic site. All figures were prepared using MolScript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

sociated with an inhibited kinase domain belong to the polypeptide chain of the *other* member of the dimer (Parrini et al., 2002).

The structure of PAK1 suggests that the phosphorylation state of Thr423 determines whether the kinase will be susceptible to Cdc42-sensitive autoinhibition, because the activation loop in its "on" conformation excludes association of the autoregulatory region with the kinase domain. Replacement of Thr423 by alanine partly inactivates PAK1 (Zenke et al., 1999), but the autoregulatory fragment (residues 67–150), supplied in *trans* can still inhibit the residual Cdc42-stimulated activity. Thus, the T423A mutation destabilizes the active state, but does not significantly affect the inactive conformation. Moreover, the H83L PAK1(67–150) autoregulatory fragment, which cannot bind Cdc42, inhibits PAK1 T423A whether or not Cdc42 is present. By contrast, the constitutively active PAK1 T423E mutant is completely insensitive to inhibition by the wild-type autoregulatory fragment, which has no effect on the capacity of the mutant kinase to phosphorylate other residues or heterologous substrates (Zenke et al., 1999).

To visualize the "on" conformation of the kinase domain of PAK1, we have determined two structures (Figure 2). One has the wild-type sequence, except for a change from Lys to Arg at position 299 (equivalent, for example, to Lys72 in protein kinase A [PKA]) (Knighton et al., 1991). This "kinase-dead" mutation inactivates the enzyme, which would otherwise be toxic to the expressing cells. Evidence from other kinase structures

leads us to believe that the mutation does not produce any large-scale conformational changes (Robinson et al., 1996). Comparison of our structure with one recently reported for the active kinase domain of TAO2, a MAP3K closely related to PAKs, supports this assumption (Zhou et al., 2004). The second structure we report contains, in addition to the K299R substitution, a glutamic-acid residue at position 423 (T423E). When the wild-type lysine is present at 299, T423E generates a constitutively active enzyme (Sells and Chernoff, 1997; Zenke et al., 1999). That is, the glutamic acid substitution can, in this case, adequately mimic a phosphothreonine. The structures described here are actually quite similar (Figure 2). That is, even in the absence of a phosphorylated threonine (or its glutamic acid surrogate), the kinase adopts an essentially active conformation. This result implies that once Cdc42 enters and dissociates the dimer, the enzyme is in an intermediate active state, with significant capacity to modify other parts of its own polypeptide chain or to phosphorylate heterologous substrates.

Results and Discussion

Overview

We expressed in *Escherichia coli* two variants of the human PAK1 kinase domain, residues 249–545. One had the K299R "kinase-dead" mutation; the other had, in addition, the mutation T432E. The crystal structures were determined by molecular replacement using the

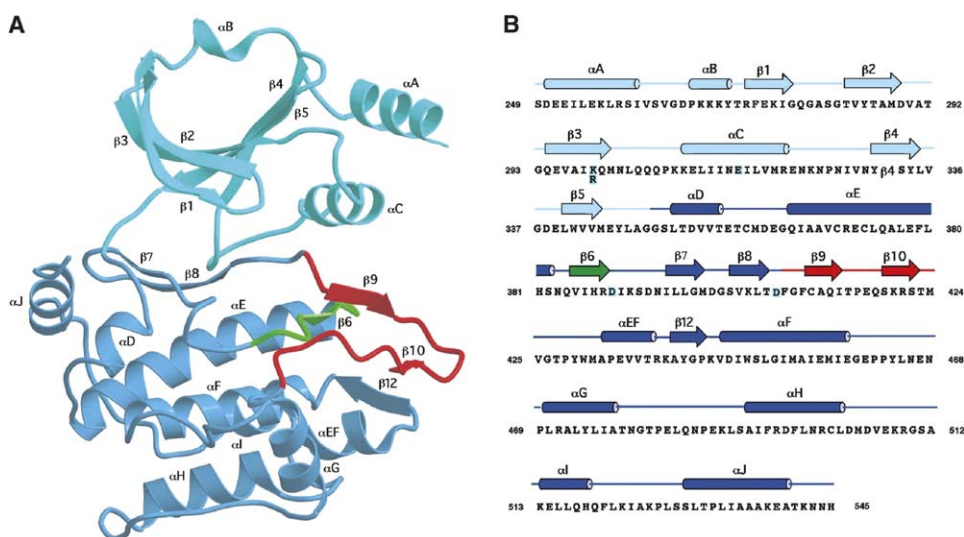


Figure 2. The Active PAK1 Kinase Domain

(A) Ribbon diagram showing conformation of the domain in the PAK1(K299R) and PAK1(K299R,T432E) structures (small lobe in light blue; large lobe in dark blue; activation loop in red). Secondary-structure elements are labeled using previously established nomenclature for the kinase domain (Knighton et al., 1991). In the autoinhibited PAK1 structure, four short β strands (β 6 in the catalytic loop, β 9 and β 10 in the activation loop, and β 12 between helices EF and F), conserved in all the active kinase structures, are either disordered (β 9 and β 10) or folded differently (β 6 and β 12). The activation loop (residues 405–430), partly disordered in the autoinhibited structure, is ordered in the active conformation, with an average thermal parameter of $40 \text{ \AA}^2$ (compared with $36 \text{ \AA}^2$ for the entire kinase domain).

(B) Amino acid sequence of PAK1 kinase domain. Secondary structure assignments are shown above the sequence as cylinders (α helices) and arrows (β strands), and colored as in (A). Residues in blue blocks are important for catalysis. The green and red blocks show the catalytic loop and the activation loop, respectively.

large lobe of the kinase domain of autoinhibited PAK1 as a search model. The X-ray diffraction data extended to $1.8 \text{ \AA}$ resolution for both, and we refined the structures to overall R_{free} values of 24.3 and 24.5% (Table 1). Details of expression and structure determination are reported in Experimental Procedures.

Both models contain residues 249–541, with 196 waters for K229R and 232 for the double mutant (Figure 2). The two structures are almost identical. The $C\alpha$ atoms of the small lobe and large lobe superpose well on those of autoinhibited PAK1, with root-mean-squared deviations of 1.09 and $0.94 \text{ \AA}$, respectively. The lobes are rotated toward one another by about 15° with respect to their relative orientations in the autoinhibited conformation. The activation loop is ordered in both structures, with an average thermal parameter of $40 \text{ \AA}^2$, essentially the same as the average of $36 \text{ \AA}^2$ for the whole kinase domain.

Conformation of the Activation Loop

The activation loop in both structures, residues 327–428, has the conformation typical of all active protein kinases. In the double mutant (K229R, T432E), there is a network of electrostatic side-chain interactions (Figure 3A). Glu423 forms salt bridges with Arg388 and Arg421, thus helping to position the catalytic base, Asp389, as well as to anchor the activation loop. Arg388, conserved in both serine/threonine and tyrosine kinases (Narayana et al., 1997), has additional hydrogen bonds with the main-chain carbonyl of Phe410 and with the phenolic oxygen of Tyr441. The latter interaction,

thought to be characteristic of the active structures of serine/threonine kinases dependent on phosphorylation of their activation loops (Zhou et al., 2004), is seen here even in the K299R form, which lacks the “phosphomimetic” E432 (and, of course, lacks a phosphothreonine). Arg421 hydrogen bonds with the main-chain carbonyl of Gln413. A contact between the side chain of the latter residue and that of Asn314 (in helix C) is the only contact between the activation loop and the small lobe. Two short β strands (β 9, residues 411–414, and β 10, residues 419–422), present in all active kinase structures so far determined, guide the activation loop so that it passes toward the rear of the kinase domain and then back forward and anchor the charge network just described.

The conformation of the mutated PAK1 activation loop closely resembles those of the phosphorylated loops in PKA (Knighton et al., 1991) and Cdk2 (Russo et al., 1996) and of the nonphosphorylatable loop in phosphorylase kinase (PHK), which has a naturally occurring glutamic acid at the position homologous to PAK1 423 (Lowe et al., 1997). Indeed, PAK1 Glu423 (Figure 3A) appears to interact in the same way as PHK Glu182 (Figure 3B), pThr197 in PKA (Figure 3C), and pThr160 in Cdk2, and the equivalents of Arg388 and Tyr441 are conserved in all these other kinases. There are limits to the extent to which Glu423 adequately mimics a pThr, however (Figure 3). In PKA, pT197 has three hydrogen bonds, including one with Lys189 at the N terminus of the activation loop (Figure 3C). In PAK1 (K299R,T432E), we instead observe a network that in-

Table 1. Statistics for Data Collection, Data Processing, Model Refinement, and Final Geometry of PAK1 Kinase Domain Structures

	PAK1(K299R,T432E)	PAK1(K299R)
Data collection and Processing Statistics		
Resolution (Å)	50–1.8	50–1.8
$R_{\text{sym}}^{\text{a,b}}$ (%)	7.8	7.3
Total observations	307,639	123,637
Unique observations	30,537	28,485
Completeness (%)	98.6	92.4
$\langle I/\sigma(I) \rangle$	9.5	11.3
Refinement Statistics		
Total number of reflections (50–1.8 Å)	30,174	28,120
Working set: no. of reflections	28,681	26,751
R factor (%)	22.3	22.6
Test set: no. of reflections	1493	1369
$R_{\text{free}}^{\text{c}}$ (%)	24.5	24.3
Total no. of residues (protein)	297	297
Total no. of water molecules	232	196
Geometry Statistics		
Rmsd ^d (bond distance) (Å)	0.011	0.007
Rmsd (bond angle) (°)	1.63	1.31
Rmsd (B factor)		
Main chain atoms (Å ²)	1.08	0.79
Side chain atoms (Å ²)	2.00	1.02
All atoms (Å ²)	1.64	0.93
Average B factor		
All protein atoms (Å ²)	35.8	37.13
Main chain atoms (Å ²)	34.4	39.65
Side chain atoms (Å ²)	37.4	37.87
Water molecules (Å ²)	46.2	43.6
χ^2 imperfection (°) ^d	18.5	15.3
Ramachandran plot ^e		
Residues in most favored region (%)	89.8	88.6
Residues in additional allowed region (%)	9.1	10.1
Residues in generously allowed region (%)	1.1	1.3
Residues in disallowed region (%)	0	0

Rmsd, root-mean-square deviation from ideal geometry.

^a $R_{\text{sym}} = \sum |I| - \langle I \rangle / \sum |I|$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of multiple observations of symmetry-related reflections.

^b $R = \sum |F_o| - |F_c| / \sum |F_o|$, where F_o and F_c are observed and calculated structure factor amplitudes, respectively.

^c R_{free} is calculated for a randomly chosen 5% of reflections; the R factor is calculated for the remaining 95% of reflections used for structure refinement.

^d χ^2 imperfection value is the rms difference between observed χ^2 values and the nearest preferred values as defined by a database of well refined structures.

^eResidue regions are defined by PROCHECK (Laskowski et al., 1993).

cludes Glu423, Arg421, and Gln413. Because these last residues are on opposite sides of the activation loop, the hydrogen bond between the side chain of Arg421 and the main chain of Gln413 probably helps stabilize the active conformation, even without Glu423 (as in our singly mutated K299R structure).

The activation loops in our two structures superpose well, and all side-chain interactions expected to stabilize the active conformation are present in both, except for the two salt-bridge interactions of Glu423. The structure of PAK1(K299R) presumably represents one possible conformation in the sequence of changes between release of the KI segment from the catalytic cleft (after derepression by Cdc42) and phosphorylation of Thr423. We refer to the unmodified but derepressed kinase as an “intermediate-active” state. The presence of the Arg388/Tyr441 interaction is additional evidence that this form would have enzymatic activity were it not for the K299R mutation. To accept a phosphate, the

loop must breathe open again, and we presume that this state is an equilibrium between the structure seen in the crystal and an open, inactive conformation. The observed activity of the kinase will be an appropriate average over the various structures present in solution. Crystallization appears to have trapped the active conformer. We suggest that the intermediate-active kinase may catalyze phosphorylation of Thr423 in other PAK1 molecules, leading to a cascade of activation events.

Structures of kinases with unmodified activation loops fall into two classes. Cdk2 and IRK are illustrations of enzymes that adopt inactive conformations in their resting states. The activation loop has an inhibitory fold, blocking the sites for ATP and substrate (De Bondt et al., 1993; Hubbard et al., 1994). PAK1 and PKA, when freed of a negative regulator such as the IS domain/KI segment (Figure 4A) or the R subunit, respectively (Knighton et al., 1991; Lei et al., 2000), appear to relax into an active conformation.

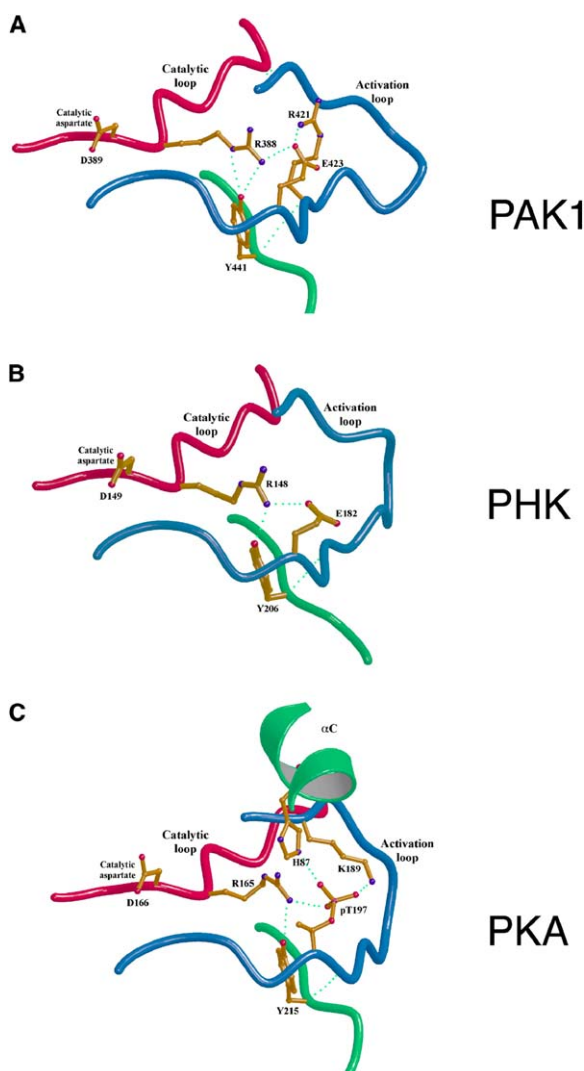


Figure 3. The Activation Loop of PAK1 Compared with Those of Phosphorylase Kinase and Protein Kinase A in Their Active States (A) PAK1 (from the double-mutant structure), with side chains that participate in an electrostatic network as described in the text. (B) Phosphorylase kinase (PHK) (Lowe et al., 1997 [PDB accession code 2PHK]), which has a glutamate in the activation loop at the position corresponding to Thr423 of PAK1 (and mutated to Glu in our double mutant). (C) Protein kinase A (PKA) (Narayana et al., 1997 [PDB accession code 1BKX]). The view in all three panels is as if looking from about 45° to the right in Figure 2A. Colors as in Figure 2.

ATP Binding Site

The structure of the ATP binding pocket in the double mutant structure very closely resembles those of the ATP bound states of PHK (Lowe et al., 1997; Figures 4B and 4C) and TAO2 (Zhou et al., 2004). The positions of the tips of the two side chains in the invariant ion pair are interchanged, however, with respect to the nucleotide. Thus, unlike Lys48, Arg299 in kinase-dead PAK1 cannot contact the α and β phosphates, and the affinity for ATP is probably low. Nearly precise superposition

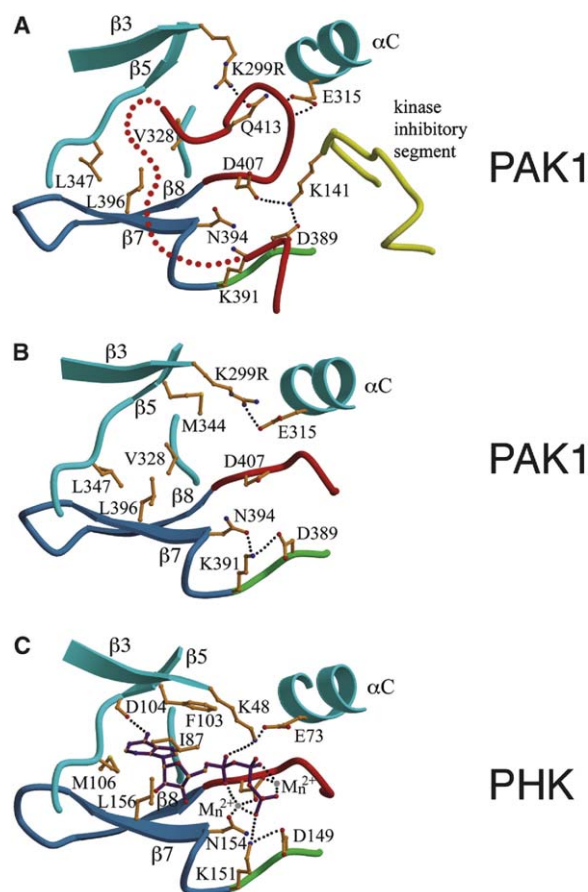


Figure 4. The ATP Binding Site of PAK1 Compared with That of PHK (A) Inactive state of PAK1 (see Lei et al., 2000). The segment of the inhibitory region that inserts into the outer part of the active site is shown in yellow. Other colors are as in Figure 2. (B) Active state of PAK1 (from the structures reported here). (C) ATP bound form of PHK. The view in all three panels is the same as the view in Figure 2A. Although there is no ATP or analog in the PAK1(K299R,T432E) crystals, the overall architecture closely resembles that of PHK complexed with ATP. Moreover, except for mutation at position 299 from Lys to Arg, the residues important for phosphoryltransfer occupy similar positions and adopt similar orientations as they do in PHK.

of all other residues important for phosphoryl transfer indicates that, except for Arg299, the PAK1(K299R, T432E) structure represents an active conformation.

The P+1 Pocket

Apart from the activation loop, the only part of the large lobe that differs in conformation from its counterpart in autoinhibited PAK1 is the pocket between the helices α EF and α G. This is the site for the substrate residue that follows the targeted serine or threonine. In autoinhibited PAK1, it is occupied by hydrophobic side chains from one face of the third helix in the inhibitory switch domain. In the present structures, side-chain orientations and interactions, conserved in the active forms of other kinases but disturbed in autoinhibited PAK1, are restored. For example, the side chains of Tyr429,

Trp430, and Met431 all have their canonical active-state contacts. The likelihood of these rearrangements has been suggested previously, based on the structure of the active kinase domain of TAO2 (see Figure 5 in the article by Zhou et al. [2004]).

“Extra” Helices and the Conformation of the Small Lobe

A core domain ($\beta 1$ - αI) is conserved among essentially all protein kinases, with important variability only in the activation loop (between $\beta 9$ and αF), in its phosphorylatable residue, and in the presence of an insert between the two lobes in certain receptor tyrosine kinases (Hanks and Hunter, 1995; Hanks and Quinn, 1991). Most crystallographically determined kinase-domain structures reveal N- or C-terminal extensions, however, often packed tightly against the core and contributing to its compact fold. These extensions have no sequence relationship to each other, and they adopt quite different conformations. In the kinase domain of PAK1, for example, there are three “extra” α helices, two (αA and αB) at its N terminus and one (αJ) at its C terminus.

The N-terminal helices of the PAK1 kinase domain, αA and αB , augment the fold of the small lobe. Helix A, which contains a sequence motif conserved in the PAK family from yeast to mammals (Figure 5), packs against the surface formed by αC and $\beta 4/\beta 5$. It connects across the top of the small lobe to $\beta 1$, the first element of the kinase core, through the single-turn αB . Several other kinases have α helices roughly coincident with PAK1 αA . In extracellular signal-regulated kinase (ERK) 2, the C-terminal extension (residues 315–358) contains a helix known as αL_{16} (residues 338–353). When the small lobes of ERK2 and PAK1 are superposed on each other, αL_{16} and αA coincide. Their N-to-C directions are the same, and they both run parallel to αC . The hydrophobic residues important for forming the binding interface of these helices are conserved (Figure 5), despite widespread differences between the sequences of PAK and ERK2, which belong to different kinase families.

The structural relationship between the small lobes of PAK1 and ERK2 has not, of course, been evident from primary-structure alignment because the augmenting helices come from opposite termini of their kinase domains. Alignment of small-lobe sequences from various protein kinases (Hanks and Hunter, 1995) shows that the residues in αC , $\beta 4$, and $\beta 5$ that create the hydrophobic groove for αA in PAK1 and αL_{16} in ERK2 are quite broadly conserved. Thus, we anticipate that there will be a hydrophobic groove on the surface of the small lobe of most protein kinases and that elements from elsewhere in the kinase polypeptide chain, or from other regulatory proteins, will interact there (Griffith et al., 2004; Huse et al., 1999; Wybenga-Groot et al., 2001). The same small-lobe surface is also the interaction site for cyclins and cyclin-like proteins in their complexes with Cdks (Brotherton et al., 1998; Russo et al., 1996), although no single helix from the cyclin domain appears to insert quite like the ones we have just described.

At the C terminus of the kinase domain, αJ packs against the interdomain hinge (Lei et al., 2000). In Ste20, the yeast homolog of PAK1, the corresponding

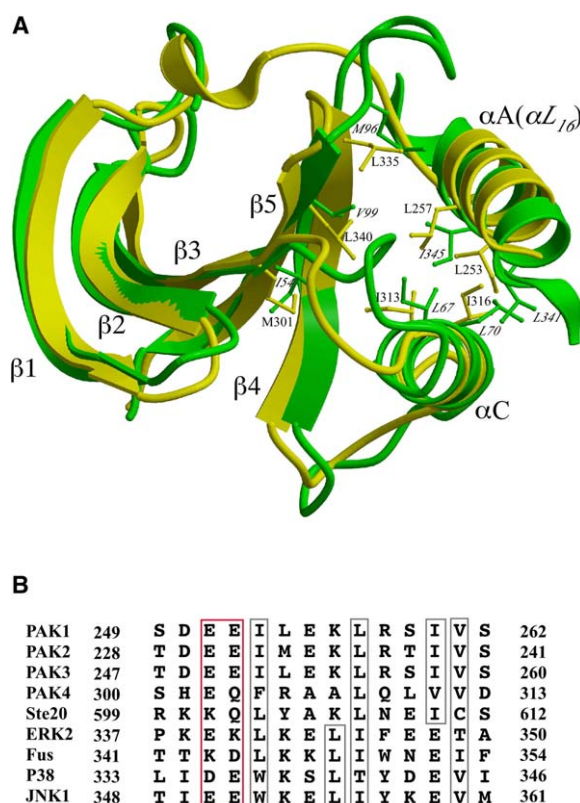


Figure 5. The “Extra” Helices in the Small Lobes of Various Kinases (A) Comparison of the PAK1 small lobe (yellow) with that of ERK2 (green) (Zhang et al., 1994 [PDB accession code 1ERK]). The extra helices superimpose closely, although αA in PAK1 comes from a segment of polypeptide chain just N-terminal to the small lobe, whereas αL_{16} in ERK2 comes from a segment just C-terminal to the large lobe. The orientations of αA and αL_{16} are the same, so that they pair with the C helix in antiparallel fashion. Side chains of the residues shown in the figure are largely conserved. Labels for residues in ERK2 are in italics, to differentiate them from residues in PAK1.

(B) Sequences of “extra” helices in members of the PAK family, including Ste20 (*S. cerevisiae*), and various MAP kinases. The alignment with the MAP kinase family is based on the crystal structure of ERK2 (Zhang et al., 1994). The gray boxes show the conserved hydrophobic residues important for interactions between αA , αC , and the β sheet; the red box shows polar residues in αA .

C-terminal sequence is a target for regulation by $G_{\beta\gamma}$ in signaling from the yeast pheromone receptor (Leberer et al., 2000; Leeuw et al., 1998). Pheromone signaling might influence Ste20 activity by influencing the relative mobility of the two lobes, just as assembly of the Src regulatory apparatus clamps the kinase into an inactive conformation (Xu et al., 1999).

Rotation of the Small Lobe

When PAK1 is activated, release of the KI segment from the catalytic cleft and phosphorylation of Thr423 (mimicked by glutamic acid in PAK1[K299R,T432E]) induce a reorientation of the small and large lobes of the kinase domain with respect to each other. By superposing the large lobes in the structures of autoinhibited PAK1 and PAK1(K299R,T432E), we find that the small lobe rotates

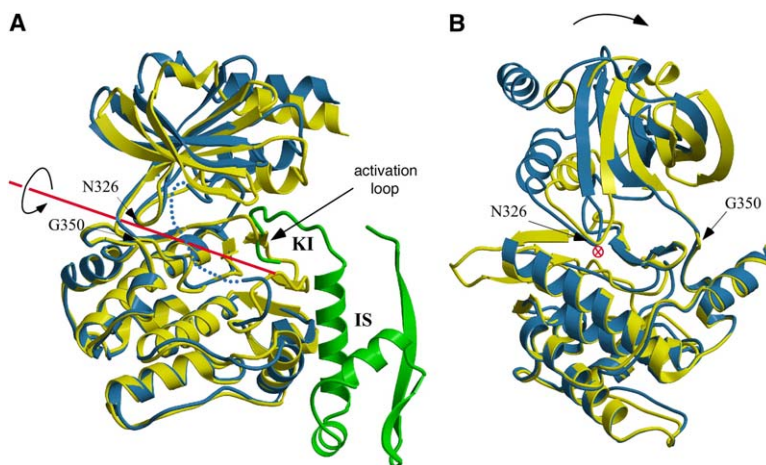


Figure 6. Transition from Inactive to Active State

(A) Superposition of the large lobes of autoinhibited PAK1 (blue) (Lei et al., 2000) and PAK1(K299R,T432E) (yellow). The autoregulatory fragment is in green. The small lobe moves as a rigid body with two pivot points (N326 and G350). The rotation axis (red) passes through residue N326. The black arrow specifies the rotation required to align the small lobe of the autoinhibited kinase domain with that of the double-mutant kinase domain. The C terminus of the KI segment in the autoinhibited structure partially overlaps the N terminus of the activation loop of PAK1(K299R,T432E) and prevents it from adopting an active conformation.

(B) As in (A), but viewed from the left. The rotation axis (red circle and cross) is perpendicular to the paper and passes through residue N326.

about 15° toward the large lobe, about an axis passing through Asn326 in the loop between α C and β 4, and Gly350 in the connection between the two lobes (Figure 6). Helix C moves about 5 Å toward the active site, creating contacts that stabilize the catalytic center (Figure 3B).

The shift of the small lobe in PAK1 is a rigid-body motion; the small-lobe coordinates from the two structures superpose well on each other (Figure 7A). The same is true of ERK2, for which structures of active and inactive states are known (Figure 7B) (Canagarajah et al., 1997; Zhang et al., 1994). We believe that the extra helices, α A in PAK1 and α L₁₆ in ERK2, lock α C into a fixed position with respect to the rest of the small lobe. By contrast, when kinases, such as Cdks, Src family members, and insulin-receptor kinase (IRK), which do not have an extra small-lobe helix, undergo activation, α C can shift by as much as 10 Å relative to other small-lobe secondary-structure elements (Figures 7C and 7D) (De Bondt et al., 1993; Hubbard, 1997; Hubbard et al., 1994; Russo et al., 1996; Xu et al., 1999; Yamaguchi and Hendrickson, 1996). The shift in α C in these proteins is a combination of two rotations, about axes perpendicular to each other (Figure 7E). In the overall PAK1 activation transition, which includes both an overall rotation of the small lobe as well as the internal shift in α C, the α C- β 4 loop and the C terminus of α C are almost stationary, and the α C- β 4 loop anchors the small lobe by inserting into a pocket in the large lobe.

The correlation between the presence of α A, which locks α C in place, and the intrinsic activity of PAK1, which catalyzes phosphotransfer even when the activation loop is not phosphorylated, is striking. But not all kinases that are intrinsically active in the absence of phosphorylation have such a lock, nor is Erk2, which does have one, active without being phosphorylated. That is, α C can, in some kinases, adopt a catalytically competent position even without reinforcement, and when present, such reinforcement only fixes the structure of the small lobe and does not determine the preferences of the activation loop. The position of α C and the conformation of the activation loop are independent switching elements that contribute to the overall logical response of the kinase.

The Logic of PAK1

What sort of regulatory program does PAK1 execute? We can distinguish four distinct states of the protein: (1) the autoinhibited dimer; (2) the monomer released from inhibition (e.g., by binding Cdc42 or Rac1) but otherwise unmodified; (3) the monomer activated by phosphorylation at Thr423; and (4) the monomer further modified by phosphorylation at several serine residues, including two (144 and 149) that could prevent reestablishing the autoinhibited structure (Manser et al., 1997). State 1 corresponds to the structure previously described (Lei et al., 2000). State 2 has an intermediate activity because it is actually a mixture of two substates—one (2') with the activation loop disordered (e.g., as in state 1) or folded into an inactive conformation; the other (2'') with the activation loop folded into an active conformation (as seen here in the structure of KD). The equilibrium under crystallization conditions evidently favors the latter substate, given the structure found in the crystals, unless that equilibrium is influenced by the K299R substitution. Taking the autoinhibited dimer as the initial state of the protein, transition to state 2' will occur if Cdc42 (or Rac1) is nearby and if that molecule is "on" (GTP bound conformation). As long as Cdc42 remains bound, there will then be a (probably rapid) shift back and forth between 2' and 2'', but only 2' can make the transition to state 3 (i.e., serve as a substrate for a kinase that modifies Thr423). One plausible candidate for such a kinase is the freshly dissociated dimer partner, but results of experiments using *in vitro* autophosphorylation do not conform to this expectation (Parrini et al., 2002). In principle, Cdc42 dissociation could still allow return to the initial, inactive state, with pThr423 exposed (the entire second half of the activation loop appears to be disordered in the autoinhibited structure [Lei et al., 2000]), but further phosphorylation at Ser144 and Ser149 (inter alia), probably carried out *in cis* (Parrini et al., 2002), would prevent correct insertion of the KI loop, even if the dimer were to reform. A corresponding finite-state automaton would have three one-bit inputs ("yes" or "no" for bound Cdc42, pThr423, and pSer144+149), and could probably be assigned a single-bit output (inactive or active).

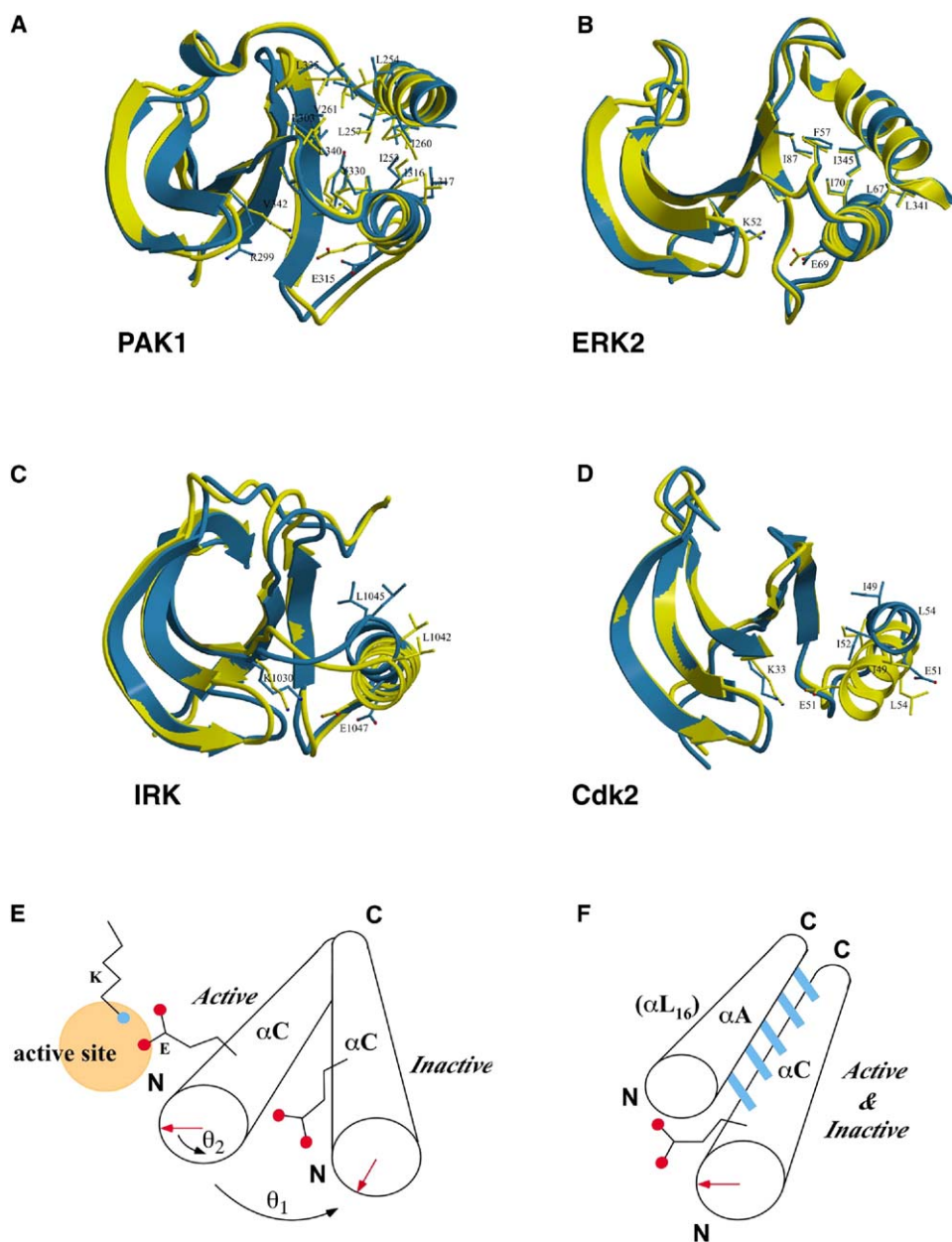


Figure 7. Correlation between the Absence or Presence of an Extra Helix in the Small Lobe and the Displacement of the C Helix during Activation

Active (yellow) and inactive (blue) conformations of four kinases have been superposed using the small-lobe β sheet.

(A) PAK1.

(B) ERK2 (Canagarajah et al., 1997 [PDB accession code 2ERK]; Zhang et al., 1994 [PDB accession code 1ERK]).

(C) IRK (Hubbard, 1997 [PDB accession code 1IR3]; Hubbard et al., 1994 [PDB accession code 1IRK]).

(D) Cdk2 (De Bondt et al., 1993 [PDB accession code 1HCK]; Russo et al., 1996 [PDB accession code 1JST]).

(E) In IRK, Cdk2, and a number of other kinases that lack an extra small-lobe helix, αC swings into the active site upon activation of the enzyme (e.g., by phosphorylation of the activation loop, as in IRK, or by binding of a cyclin, as in Cdk2). The motion of αC can be decomposed into two rotations (θ_1 and θ_2), which move the helix away from the active site and break the interaction (orange circle) between the conserved catalytic-site lysine in $\beta 3$ and the glutamic acid in αC .

(F) In PAK1 and ERK2, hydrophobic contacts (blue lines) with the extra helix prevent αC from swinging outward, even in the inactive form of the kinase.

Conclusions

The two very similar structures described in this article show that when it is relieved of specific autoinhibition,

the PAK1 kinase domain relaxes into an equilibrium that favors an active conformation, even without phosphorylation of the activation loop. Modification of Ser144

and Ser149 can then eliminate the possibility of restoring the inactive conformation, whatever the phosphorylation state of Thr423. Thus, the enzyme behaves as a relatively simple, effectively irreversible switch. Were phosphorylation of Thr423 essential for even partial activity, the regulatory properties of PAK would instead resemble those of a timer that balances the rate of Cdc42 unbinding against the rate of Thr423 modification (e.g., by another kinase recruited to the linker region between the IS and kinase domains of PAK).

Experimental Procedures

Cloning, Expression, and Purification

The kinase domain (residues 249–545) of human PAK1 was cloned into the GST-fusion vector pGEX2N (Pharmacia), with a thrombin cleavage site C-terminal to the GST moiety and with a gene for ampicillin resistance. Both constructs, PAK1(K299R) and PAK1(K299R,T432E), contained the inactivating mutation K299R because the wild-type form is toxic to bacterial cells. The T423E mutation was performed using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The proteins were expressed and purified as described before (Lei et al., 2000).

Crystallization, Data Collection, and Processing

Crystals of the single and double mutant were grown by hanging-drop vapor diffusion by equilibrating against 23% PEG4000, 1.5 M NaCl, 10 mM DTT, 100 mM PIPES, pH 6.5 (well solution), and 28% PEG4000, 1.0 M NaCl, 10 mM DTT, 100 mM PIPES, pH 6.5, respectively. Each drop contained 2 μ l protein solution in buffer C plus 2 μ l well solution. The crystals grew to 100–200 μ m in about 10 days at 4°C. For data collection at low temperature, crystals were transferred stepwise into a stabilizing solution containing 25% glycerol, 25% PEG4000, 1.5 M NaCl, 10 mM DTT, 100 mM PIPES, pH 6.5, for PAK1(K299R,T432E), or 25% glycerol, 30% PEG4000, 1.0 M NaCl, 10 mM DTT, 100 mM PIPES, pH 6.5, for PAK1(K299R). The crystals were flash-frozen by immersion in liquid nitrogen. Diffraction data were collected at APS Beamline BM 14-C using an ADSC Quantum-4 detector. Data were integrated and scaled using the HKL programs (DENZO and SCALEPAK) (Otwinowski and Minor, 1997). The crystals are in space group C22₂, $a = 51.96$ Å, $b = 103.30$ Å, $c = 122.88$ Å for crystals of the double mutant and $a = 51.76$ Å, $b = 103.05$ Å, $c = 122.64$ Å for those of PAK1(K299R). The final data-processing statistics are presented in Table 1.

Structure Determination

The initial phases for PAK1(K299R, T432E) were obtained by molecular replacement using AmoRe (Navaza, 1994). The large lobe of the kinase domain from the 2.3 Å resolution structure of autoinhibited PAK1 (Lei et al., 2000) was used as the search model; because the activation loop usually has different conformations in inactive and active states, residues 403–430 were omitted. The calculation used data between 15 and 3.5 Å resolution. The best rotation-translation solution had a correlation of 28% and an R factor of 51%. The overall position of the initial large-lobe model obtained from molecular replacement was first refined by rigid-body refinement with the program CNS (Brunger et al., 1998), using data between 50 and 1.8 Å. The R_{free} was reduced from 49% to 47%, and the R factor for the reflections in the working set decreased from 48% to 46%. Further refinement was made using the maximum likelihood approach as implemented in CNS. Model building was performed with the program "O" using the weighted electron density map (Jones et al., 1991). The density for the large lobe was very clear, and after several refinement rounds, when the R factor dropped to 41% ($R_{\text{free}} = 48\%$), the difference density maps showed the residues of the small lobe and those of the activation loop. During a further few rounds of refinement, each followed by rebuilding, a model was built that contained most of the small lobe and activation loop. The validity of each step of rebuilding the small lobe was monitored by the R_{free} . Water molecules were added gradually using CNS. A total of 232 water molecules were included in the final coordinates for the double mutant. At this stage, both

the $2F_o - F_c$ and the $F_o - F_c$ electron density maps showed clear, positive density for the side-chain of glutamic acid 423. After the final refinement cycle, the R_{free} was 24.5% and the R factor was 22.3% for data between 50 and 1.8 Å (Table 1). Because the space groups and cell dimensions of the two variants are the same, an initial model of the single mutant was obtained by applying rigid-body refinement (CNS) to the double-mutant coordinates without the activation loop (residues 405–430) using the single mutant dataset. Further refinement and model rebuilding were performed using the programs CNS and O, respectively. The electron density of the activation loop could be seen after the first-round refinement. The final structure includes residues 249–545 and 196 water molecules. The R_{free} is 24.3% and the R factor is 22.6% for data between 50 and 1.8 Å (Table 1). The quality of both models was assessed with the structure validation program PROCHECK (Table 1) (Laskowski et al., 1993). Some minor corrections were made based on the results of PROCHECK.

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