Crystal Structure of a Human IκB Kinase β Asymmetric Dimer

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Background: IκB kinase β is a key regulator in the NκB signaling pathway.

Results: Crystal structure of a human IKKβ asymmetric dimer shows one kinase active site phosphorylated and in the active conformation and the other unphosphorylated and inactive.

Conclusion: Depending on the phosphorylation state, IKKβ can adopt distinct dimeric geometry.

Significance: High resolution structure of hIKKβ provides structural basis for its activation and potential use of inhibitor design.

Phosphorylation of inhibitor of nuclear transcription factor κB (IκB) by IκB kinase (IKK) triggers the degradation of IκB and migration of cytoplasmic κB to the nucleus where it promotes the transcription of its target genes. Activation of IKK is achieved by phosphorylation of its main subunit, IKKβ, at the activation loop sites. Here, we report the 2.8 Å resolution crystal structure of human IKKβ (hIKKβ), which is partially phosphorylated and bound to the staurosporine analog K252a. The hIKKβ protomer adopts a trimeric structure that closely resembles that from Xenopus laevis (xIKKβ): an N-terminal kinase domain (KD), a central ubiquitin-like domain (ULD), and a C-terminal scaffold/dimerization domain (SDD). Although hIKKβ and xIKKβ utilize a similar dimerization mode, their overall geometries are distinct. In contrast to the structure resembling closed shears reported previously for xIKKβ, hIKKβ exists as an open asymmetric dimer in which the two KDs are further apart, with one in an active and the other in an inactive conformation. Dimer interactions are limited to the C-terminal six-helix bundle that acts as a hinge between the two subunits. The observed domain movements in the structures of IKKβ may represent trans-phosphorylation steps that accompany IKKβ activation.

The immune, inflammatory, and acute phase responses of vertebrates depend on exquisitely controlled and marshaled patterns of gene expression. Among the central regulators of these coordinated events are members of the NF-κB family of nuclear transcription factors (1, 2). Dysregulation of their function contributes to numerous diseases, including atherosclerosis, arthritis, cancer, cachexia, diabetes, euthyroid sick syndrome, acquired immune deficiency syndrome, inflammatory bowel disease, and stroke (3, 4).

In their resting or inactive state, NF-κB factors exist as cytoplasmic complexes with members of the NF-κB inhibitor protein family, IκBα, IκBβ, and IκBε. These interactions prevent translocation of NF-κB proteins to the nucleus and restrain them from binding to cognate sites on DNA and stimulating the transcription of target genes.

The multisubunit protein kinase IKK regulates NF-κB activation (5). Multiple stimuli such as inflammatory cytokines, bacterial, or viral products, or various types of stress lead to IKK-catalyzed phosphorylation of IκB inhibitors, an event that activates the canonical NF-κB pathway. Phosphorylation permits IκB proteins to be polyubiquitinated and then catalyzed by the proteasome. Liberation from their inhibitors leaves NF-κB factors free to enter the nucleus and activate transcription of genes encoding proteins that participate in the immune and inflammatory response, cell adhesion, growth control, and protection against apoptosis. A subset of inducers can also stimulate the non-canonical NF-κB pathway, in which IKK-mediated phosphorylation of the IκB-like domain in the NF-κB2 protein leads to activation of that transcription factor (6).

IKK is a multiprotein complex that contains two possible kinase subunits, IKKα and IKKβ, and the regulatory subunit, NEMO (NF-κB essential modulator) (7, 8). NEMO is essential for the activation and substrate specificity of IKK (9, 10). Both IKKα and IKKβ contain an N-terminal kinase domain (KD, residues 15–308 in hIKKβ), a leucine zipper region (residues 458–479), and a helix-loop-helix region (residues 603–642) (11). Each kinase has a NEMO binding domain at its carboxyl terminus (residues 737–742). IKKβ also has an additional ULD domain after the KD, which is absent in IKKα. The novel ULD domain is required for the functional activity of IKKβ and important for its substrate specificity (12, 13).

This article contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 4KKH) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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5 The abbreviations used are: IKK, IκB kinase; hIKKβ, human IKKβ; KD, kinase domain; ULD, ubiquitin-like domain; SDD, scaffold/dimerization domain; xIKKβ, Xenopus laevis IKKβ.
Two IKK-related kinases, IKKe (or IKK-I) and TBK1 (TANK-binding kinase) (14) also contribute to immune responses but mediate different signal pathways (15). Of the principal kinases, IKKα and IKKβ seem to have very distinct functions: IKKβ is a more potent NF-κB activator and plays a major role in the canonical NF-κB pathway responsible for immune responses, whereas IKKα is more important in the non-canonical pathway required for developmental processes (16, 17).

Because of its importance in many human diseases, hIKKβ has been viewed widely as a potential therapeutic target (4, 18–20). The first crystal structures of an IKKβ, reported for the phosphomimetic mutant of *Xenopus laevis* IKKβ, xIKKβ(S177E/S181E), have greatly advanced our understanding of IKK (13). Similar structural elucidation of IKKβ in an active phosphorylated state has been hampered by the inherent kinase heterogeneity due to phosphorylation. We have produced and isolated a near full-length human IKKβ wild type protein, which was phosphorylated at the activation loop and retained kinase activity. We then co-crystallized this protein with the staurosporine analog K252a. Here, we report the resulting 2.8 Å resolution crystal structure of a phosphorylated hIKKβ dimer and delineate its mechanistic similarities and distinctions from that of the unphosphorylated xIKKβ dimer. In addition, compared with the reported structures of xIKKβ determined at 3.6 and 4.0 Å resolution, respectively (13), the present hIKKβ structure resolves many new additional details at a sufficiently high resolution to permit structure based drug design. We complement our data with biochemical analysis of the dimer formation and mass spectrometric analysis of the phosphorylation state.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification*—A construct encoding a hexahistidine tag followed by residues 1–664 of hIKKβ was cloned into a pBacPAK vector (Clontech) to enable its expression in baculovirus-infected insect cells. As a result of cloning, the hIKKβ(1–664) sequence was extended with the vector-derived residues SPGRPLN at its C terminus. The vector was then transfected into Sf9 insect cells according to the manufacturer’s instructions (Clontech), and a clonal isolate was amplified in running buffer (25 mM HEPES, 250 mM NaCl, 5% glycerol, 2 mM β-glycerophosphate, 5 mM DTT) and loaded onto a heparin affinity column. The column was washed extensively with the lysis buffer, after which hIKKβ(1–664) was step-eluted with lysis buffer containing 200 mM imidazole. The nickel column eluate was diluted 4-fold with buffer A (25 mM HEPES, pH 7.5, 10 mM NaCl, 5% glycerol, 2 mM EDTA, 5 mM β-glycerophosphate, 5 mM DTT) and loaded onto a heparin affinity column. The column was washed extensively with buffer A, and the column was developed with a NaCl gradient to 1.0 M, and hIKKβ-containing fractions were combined and diluted 3-fold with buffer A before being loaded onto a MonoQ HR10/10 column (Pharmacia). The MonoQ column was washed extensively with buffer A, and the column was developed with a NaCl gradient to 1.0 M. Fractions containing pure hIKKβ were identified by a SDS-PAGE 4–12% gel and concentrated before loading onto a TSK G3000 gel filtration column equilibrated with the SEC running buffer (25 mM HEPES, 250 mM NaCl, 5% glycerol, 2 mM tris(2-carboxyethyl)phosphine). The hIKKβ peak was collected and used for subsequent studies.

**Crystallization and Data Collection**—The His-tagged hIKKβ (1–664) protein was crystallized using the hanging drop method. Crystallization drops were formed by mixing protein solution of 22% PEG 3350 and 120 mM trilithium citrate. Crystallization was carried out using program suite CCP4 (22). The crystallographic data set was collected on a single crystal which diffracted to 2.8 Å at the Advanced Light Source (Berkeley, California) and was processed with program HKL2000 (21). Statistics for data collection are listed in Table 1. Further processing was carried out using program suite CCP4 (22).

**Structure Determination and Refinement**—The hIKKβ structure was solved by molecular replacement using the published xIKKβ structure (Protein Data Bank code 3QA8) as model with the program Phaser (23). Extensive model rebuilding was carried out using Coot (24), and refinements were carried out using program Buster with TLS options (25). The refinement statistics are listed in Table 1.

### TABLE 1

| Data collection and refinement statistics |
|------------------------------------------|
| **Space group** | *P*2<sub>1</sub> |
| **Cell dimensions** (Å) | *a*, *b*, *c* |
| | 110.954, 68.689, and 107.401 |
| **α, β, γ** | 90, 107.401, and 90° |
| **Resolution (Å)** | 47.2–2.8 |
| **R<sub>merge</sub>** | 0.103 (0.574) |
| **I/σ** | 10.0 (1.5) |
| **Completeness (%)** | 95.3 (80.8) |
| **Redundancy** | 2.9 (2.0) |

| **Refinement** |
|----------------|
| **Resolution (Å)** | 47.2–2.8 |
| **No. reflections** | 34,922 |
| **R<sub>free</sub>** | 0.239 (0.35) |
| **No. atoms and B** |
| **Protein** | 10,282, 53.0 Å<sup>2</sup> |
| **Ligands** | 112, 34.0 Å<sup>2</sup> |
| **Water** | 253, 36.5 Å<sup>2</sup> |
| **r.m.s.d.<sup>a</sup>** |
| **Bond lengths (Å)** | 0.010 |
| **Bond angles** | 1.14° |

**Ramachandran (%) (favored, outliers)** | 96.9, 0.2 |

<sup>a</sup> Statistics in the highest resolution shell are shown in parentheses.

<sup>b</sup> r.m.s.d., root mean square deviation.
Human IKKβ Crystal Structure

![Crystal structure diagram](image)

**FIGURE 1.** Characterizations of N-terminal His-tagged hIKKβ(1–664) used in crystallization. A, mass spectrum of protein indicated that the protein has one to three sites phosphorylated (top). These phosphate groups could be removed by dephosphorylation with alkaline phosphatase treatment (bottom). The theoretical molecular weight of N-terminal acetylated hIKKβ(1–664) is 78,112 Da (mass discrepancy of 128 ppm). B, analytical size-exclusion chromatography indicated that hIKKβ was a dimer in solution. Elution times of molecular weight standards are marked arrows.

**Dynamic Light Scattering**—Dynamic light scattering experiments were conducted using a DynaPro DLS instrument for each sample to determine the hydrodynamic radius of the protein. Protein samples were centrifuged for 10 min at 14,000 rpm in a desktop microfuge at 4°C prior to the reading at 18°C. Measurements were based on the average of triplicate runs, with each run involving a minimum of 20 readings.

**RESULTS**

**Analysis of Protein Constructs and Crystallization**—Two protein constructs of hIKKβ were prepared and analyzed for crystallization trials: full-length N-FLAG-IKKβ (data not shown) and N-His$_6$-hIKKβ (1–664). Full-length hIKKβ was heterogeneously phosphorylated at 6–19 sites owing to inclusion of the C-terminal NEMO binding domain region, which is prone to heavy phosphorylation (data not shown). With the same region absent, N-His$_6$-hIKKβ(1–664) was phosphorylated at only one to three sites (Fig. 1A). These two recombinant forms of the kinase were shown to have similar specific activities (data not shown). In addition, several commercially available kinase inhibitors exhibited similar IC$_{50}$ values against the full-length hIKKβ and hIKKβ(1–664). Among these inhibitors, the staurosporine analog K252a showed the best potency. Furthermore, analytical size-exclusion chromatography (Fig. 1B) and dynamic light scattering (data not shown) indicated that both forms of the enzyme existed as dimers in solution.

Crystallization screens produced crystal hits for both protein forms, the full-length hIKKβ (1–742) and the truncated form N-His$_6$-hIKKβ(1–664). However, only the truncated form of the protein, consistent with its protein heterogeneity profile, yielded crystals that were suitable for structure determination and that diffracted to 2.8 Å resolution. We refer to this construct as hIKKβ throughout the text, except when otherwise noted.

**Overall Structure of hIKKβ**—There are two hIKKβ molecules in the crystal asymmetric unit, and consistent with the biochemical studies, they form a dimer around a quasi-2-fold rotational axis (Fig. 2A). Similar to its xIKKβ homolog, each hIKKβ monomer has a tridimensional linear architecture: the N-terminal kinase domain, KD(1–309), the central ubiquitin-like domain, ULD(310–404), and the C-terminal dimerization domain, SDD(408–664). The KD has a typical bilobal kinase fold, the ULD has the ubiquitin fold (20), and the SDD has a helical blade structure consisting of six α-helices, α1s–α6s (Fig. 2B).

The overall interdomain architecture of xIKKβ is largely maintained in hIKKβ, with the first two domains, KD and ULD, tightly aligned along the N-terminal end of the third SDD domain (Fig. 3). This arrangement is stabilized by van der Waals and hydrogen bond contacts between all three domains, with a major contribution from the KD–ULD interactions (Fig. 3). Most of the residues involved in interdomain interactions are conserved among different IKKβ species (supplemental Fig. S1), highlighting the biological significance of this arrangement.

In contrast to the xIKKβ crystal structure, in which eight independent representations of the IKKβ molecule are similar (13), the two hIKKβ protomers are less alike. In particular, protomer A exhibits a higher level of structural disorder compared with protomer B (Fig. 2C) and differs in a way that appears functionally significant, as discussed in detail below.

Pairwise comparison of the two hIKKβ protomers based on superimposition of their KDs gives the root mean square deviation of 0.58 Å for 262 Cα pairs and an root mean square deviation value of 1.22 Å for 541 Cα pairs of the whole protomer structure (Fig. 2C). The observed differences result mainly from variations in orientation of the C-terminal part of the SDD relative to the rest of the molecule, which is reflected in a slightly more bent (~6°) structure of protomer B (Fig. 2C). Thus, the two hIKKβ protomers on the whole assume slightly different conformations, one more extended (and more similar to xIKKβ) and the other bent.

**Structures of the Active and Inactive Kinase Domains in the hIKKβ Dimer**—hIKKβ in our crystal form presents two versions of the kinase domain, one with the activation loop phosphorylated (protomer B) and the other not (protomer A). When the two KD structures are compared, the most significant differences occur in the N-terminal lobe and at the activation loop. The ATP-binding pockets in both KDs are occupied by the staurosporine analog inhibitor K252a. It binds in a similar fashion to staurosporine, making hydrogen bond contacts from the lactam ring to the hinge backbone and an additional hydrogen bond from the hydroxyl to the carbonyl oxygen of Glu-149 (Fig. 4A). The presence of this additional hydrogen bond explains the higher affinity of K252a compared with staurosporine (data...
not shown). The glycine-rich loop in protomer B is well structured with the tip of the loop protruding from the ATP site. This loop in the structure of protomer A is partially disordered (Fig. 4B). The entire N-terminal lobe of protomer A has weaker electron density in general compared with protomer B and some regions of completely missing density.

The activation loop in protomer B (residues 166–194) assumes a conformation characteristic of an active kinase (Fig. 4C), with three residues missing (residues 174–176) due to disorder. The corresponding region in protomer A is less well defined. It has 10 residues disordered (residues 170–179) and adopts a conformation that is not compatible with protein substrate binding (Fig. 4B). Because the inhibitor binding mode is identical in both domains, we conclude that the apparent differences in kinase flexibility and conformation are solely due to the differences in phosphorylation state between the two domains. These differences are unlikely to be caused by crystal packing effects, as both protomers, A and B, make wide ranging contacts with crystal symmetry mates and pack equally tightly.

Phosphorylation of the activation loop at two sites, Ser-177 and Ser-181, was reported to be required for the activation of IKKβ (8, 11, 26, 27). In protomer B, both sites are phosphorylated. The electron density for the phosphate group at Ser-181 is strong, and at Ser-177, it is clearly observed but weaker. All three phosphate oxygen atoms of phospho-Ser-181 make direct ionic interactions with the cationic cluster formed by Arg-144 from the catalytic loop and Lys-171 from the activation loop. Additionally, one of the phosphate oxygen hydrogen bonds to the main chain amide nitrogens of phospho-Ser-181 and the preceding Thr-180 (Fig. 4, B and C). As in many active kinase structures, these phosphate-mediated interactions provide a direct link to the catalytic center, helping to stabilize the correct orientation of the catalytic base Asp-145 (Asp-135 in TBK1) and allow for the formation of the polypeptide substrate pocket (Fig. 4C). Ser-177, although phosphorylated, is completely exposed to solvent with no apparent direct role in the stabilization of the observed active conformation. Although phosphorylation at Ser-177 may play a role in long range electrostatic interactions, differences in the dispositions of phospho-Ser-181 and phospho-Ser-177 suggest that phosphorylation of Ser-181 alone may be sufficient for hIKKβ activation. Indeed, the IKKβ S177A mutant was shown to be fully active, whereas the S177A/S181A double mutant was inactive (28). This is akin to the observation that monophosphorylation at Ser-172 in TBK1, a residue equivalent to Ser-181 in hIKKβ (supplemental Fig. S1), is necessary and sufficient for the stabilization of the active conformation of TBK1 (29, 30).

In contrast, in protomer A both Ser-177 and Ser-181 are more mobile, with fewer intramolecular interactions, and appear to be unphosphorylated. The activation loop segment on which Ser-181 resides rearranges into a short α-helical segment (residues 180–188) and relocates to a new position (Fig. 4B). Here, Ser-181 is ordered, but displaced by ∼19 Å from its phosphorylated counterpart position. There is clearly no density for the phosphate group at Ser-181 and residue Ser-177 is completely disordered. When compared with those in the pep-
for hydrogen bonding with the catalytic lysine 44 (Lys-38 in TBK1), a bond required for catalysis (Fig. 4B).

The hIKKB and TBK1 crystal structures (29, 30) both demonstrate that the activation loop, when unphosphorylated, adopts conformations that are incompatible with protein substrate binding, highlighting the essential role of phosphate-mediated interactions for the activities of these kinases. The structure of xIKKB is no exception. The xIKKB activation loop harboring the S177E/S181E double mutation appears in yet another off conformation (13). In the structures of phosphorylated hIKKB and TBK1 (29, 30), all three oxygens of the essential phosphate phospho-Ser-181 (phospho-Ser-172 in TBK1) interact with the cationic subsite of the kinase active site. This indicates that glutamates at these positions would serve as only a partial mimic of phosphoserines and could result, as in the cases of the TBK1 S172E and the hIKKB S177E/S181E double mutant, in much weaker activity than the phosphorylated kinases (33, 34).

Altogether, these observations provide the structural basis for the lack of kinase activity of IKKβ (S177A/S181A) (26) and TBK1 (S172A) mutants (34).

In this regard, the message from the xIKKB S177E/S181E crystal structure is somewhat surprising. On one hand, the reported glutamate substitutions render the enzyme constitutively active (35), albeit with a lower kinase activity compared with the phosphorylated wild type kinase (33). On the other hand, these mutations seem to destabilize the correct folding of the activation loop. The kinase mutant adopts a conformation in which the phosphomimetic loop and the glycine-rich loop touch each other so that the protein substrate is excluded from binding. The high similarity in the conformations of this loop seen in all crystalline copies of xIKKB (13) suggests that this conformation also exists in solution rather than being the result of crystal lattice contacts only. It is possible that the S177E/S181E mutant could sample the more productive, active conformations only when an ATP molecule is bound.

Interestingly, there is at least one more phosphorylation site, phospho-Ser-257, that appears to be occupied, albeit partially, as judged by electron density and phosphate tetrahedral geometry. This site is occupied in protomer A but not in protomer B. It is positioned toward the C-terminal lobe end and far away from the activation loop and has no apparent impact on the inactive KD conformation of protomer A. Hence, the double phosphorylation of one hIKKB protomer as opposed to monophosphorylation of the other, as visualized in the two independently crystallized kinase copies, is in accord with a one to three site occupancy profile identified by mass spectrometry (Fig. 1A).

**Structural Implications for hIKKB Inhibitor Design**—A number of studies have shown that IKKβ has unusually high affinity (K_m 100–600 nM) for ATP (26, 33, 35), posing significant challenges for ATP binding site inhibitors in view of the high levels of cellular ATP (36, 37). The binding mode of K252a to hIKKB is virtually identical regardless of the phosphorylation state of the KD, suggesting that such an inhibitor would not differentiate between the active and inactive kinase conformations. However, outside of the ATP binding pocket, the protein conformations are distinct, and this could potentially provide some advantages in designing selective and non-ATP competitive
inhibitors. One of the potential binding sites that is remote from the ATP-binding pocket and that is part of the substrate binding site is seen at the KD-SDD interface (Fig. 5). It features a 12-Å-long and 10-Å-deep channel, which is wide open in protomer B (Fig. 5B) but partially closed in protomer A (Fig. 5A). Inhibitors designed to bind to these differently folded pockets would selectively stabilize either active (protomer B) or inactive kinase form (protomer A) and can be tested in substrate competitive inhibition studies. Interestingly, studies have shown that there exist potent IKKβ inhibitors that are competitive with substrate IκB, but allosterically inhibit IKKβ (38).

**Dimerization Interface of hIKKβ**—It has been demonstrated that IKKα and IKKβ have strong tendencies to form homo- or heterodimers (13, 33, 35). Our results from dynamic light scattering and analytical size exclusion chromatography experiments indicated that our hIKKβ proteins existed as dimers in solution. Furthermore, similar C-terminal SDD-mediated dimers can be identified in the crystals of both xIKKβ and hIKKβ, in which the KDs are kept apart by the N-terminal ends of the SDDs, and two kinase active sites face opposite from each other (Fig. 6A). Although the crystal structures illustrate that the hIKKβ and xIKKβ dimers are related, they differ in one respect: hIKKβ adopts an open configuration and the xIKKβ dimer a closed one (Fig. 6A). In the dimer of xIKKβ, the kinase active sites are separated by a mere 67 Å (measured by the distance between two Ca atoms of the catalytic Asp-145 residues), with SDDs stretching parallel to each other almost their entire length (Fig. 6A). In the dimer of hIKKβ, the two SDDs bend away from each other at an angle of 45°, similar to an open pair of scissors. The two pairs of KD move further apart, increasing the separation between the two active sites by an additional 35 Å. In this open arrangement, the SDDs contact each other only at the blade ends through intertwined helices (Fig. 6A).

Each SDD contributes its α2, α3, and α6 helices to the interface in a similar, but not equivalent way to xIKKβ (Fig. 6B). The resulting helical bundle buries 1570 Å² surface area per monomer, with a minor contribution from α3. Comparison of hIKKβ and xIKKβ structures shows that, with a few exceptions, the same residues are involved. The buried surface is largely hydrophobic but utilizes also an extensive hydrogen bonding network that is not seen in the structure of xIKKβ. This hydrogen bond network is formed by residues Lys-482, Trp-655, and Gln-651 from one protomer and Ala-481, Asp-493, and Ser-489 from another (Fig. 6B). In total, there are 10 interchain hydrogen bonds between these residues. In turn, Gln-651, Asp-493, and
Ser-489 form intramolecular hydrogen bonds with Gln-647 and Arg-650 (Fig. 6B). Hydrophobic side chains of Phe-485, Leu-654, and Leu-658 also contribute to maintaining this interface in both the hIKKβ and xIKKβ structures. The central role of this interface in IKKβ dimerization was previously underlined by the failure of three hIKKβ mutants L654D/W655D, W655D/L658D, and L654D/W655D/L658D to dimerize (13).

Given that the backbone positions of the interfacing residues are comparable in the two structures with just their side chain rotamers varying, the absence of the hydrogen bond network at the xIKKβ dimer interface could simply be due to the limited resolution at which the xIKKβ model was built; alternatively, it may also be due to the plasticity of the interface. Structural analysis indicates that the former most probably is the case. In both the hIKKβ and xIKKβ structures, the hydrophilic side chains of Ser-489, Asp-493, Gln-647, and Gln-651 are partially or completely buried at the interface. Formation of the observed hydrogen bonds in the hIKKβ interface can compensate for the desolvation penalty associated with the burial of polar atoms. Because of the directionality of hydrogen bonds, the observed network can also increase the complementarity and the rigidity of the dimerization interface. It is notable that the residues at the dimer interface are among the most conserved in different species of IKKβ, and in hIKKα as well, but not in IKK-1 and TBK1 (supplemental Fig. S1). This observation may underlie the ability of IKKβ and IKKα to form homo- or heterodimer complexes (33) and the significant difference in dimerization interfaces between IKKβ and TBK1 (6, 30, 39).

**DISCUSSION**

Dimerization is a necessary precondition for the phosphorylation and activation of both IKKα and IKKβ (11, 33, 40, 41). More recent studies have shown that dimerization of IKKβ is necessary for its activation but not essential for its kinase activity once the activation loop is phosphorylated (13). The mechanism through which the SDD-dependent dimerization promotes activation loop transphosphorylation is not well understood, and little is known regarding the interkinase interactions once the activation loop is phosphorylated. To better understand the interplay of these interactions, we have determined the crystal structure of hIKKβ in a phosphorylated state.

In the structure described herein, we observe an SDD-mediated hIKKβ parallel dimer in which one KD is phosphorylated and adopts an active conformation, whereas the other is unphosphorylated and in an inactive conformation. This arrangement is distinct from that seen in the structure of xIKKβ (13). First, asymmetry in the phosphorylation pattern breaks the 2-fold symmetry of the hIKKβ dimer, so that the two IKKβ protomers are no longer equivalent. Second, although in both structures, the IKKβ dimer forms through reciprocal SDD-SDD interactions, the open geometry of hIKKβ minimizes this interaction to the C-terminal helix bundle only. Superimpositions of different hIKKβ protomers (Fig. 2C) and comparison of xIKKβ and hIKKβ dimers (Fig. 6A) indicate that IKKβ protomers have certain degrees of plasticity and that the change from closed to open state occurs solely through the flexion of three helices (n2, α5, and α6) at the N-terminal end of the SDD. Hence, the six-helix bundle acts like a hinge so that the two chains can swing away or toward each other.

IKKα and IKKβ could be activated either by an upstream kinase or auto-phosphorylation (28, 33). The main feature that is common to the hIKKβ, xIKKβ, and TBK1 structures is that autophosphorylation within the dimer is precluded by steric factors (Fig. 6A). This apparently fulfills the requirement for tight control of IKKβ activation. On the other hand, it also implies that dimerization by itself is necessary but not sufficient for their autophosphorylation, making trans-autophosphorylation through oligomerization a likely mechanism for activation (13, 30). In the crystals of xIKKβ, the IKKβ dimers are structured into higher order tetramers with a symmetry that can be described as a dimer of dimmers (Fig. 6A) (13). The second dimer is an anti-parallel, face-to-face dimer, in which the active sites of two KDS face each other in proximity (Fig. 6C). It was suggested that this proximity might allow the activation loop of one protomer to reach into the active site of the other for transphosphorylation (13).

Inspection of our crystal structure reveals that, along with a different dimer geometry, the tetrameric xIKKβ crystal symmetry is also no longer maintained (Fig. 6A). However, an anti-parallel, face-to-face KD-KD* interface exists in hIKKβ. This interface resembles that in xIKKβ but is not the same (Fig. 6C). First, the hIKKβ face-to-face dimer is asymmetric because it too involves an unphosphorylated-phosphorylated kinase pair (protomer A and crystal symmetry related protomer B*). Next, it exhibits different contact geometry. In hIKKβ, the two protomers readjust their positions, enhancing the interface and bringing the two active sites closer together (Fig. 6C). In this orientation, the KD N-terminal lobes dock onto the N-terminal ends of the SDD of the dimeric partner and the KD C-terminal lobes into each other. This interaction allows for a better prox-
iminity of the fully formed active site of one KD toward the unphosphorylated activation loop of the other and hence a more proper positioning for transphosphorylation. Interestingly, although the binding interface of the second KD-KD* dimer is more extensive compared with the SDD-SDD dimer (total buried surface area of 1760 Å² versus 1570 Å²), the binding free energy, calculated with the protein interface analysis program PISA (43), is less favorable for the second dimer than for the primary one (−2 kcal/mol versus −24 kcal/mol). This high value indicates that the face-to-face dimer is less stable, consistent with the transient nature of this association for transphosphorylation.

The differences in these face-to-face dimer interfaces in hIKKβ and xIKKβ could well be due to crystal packing variations, and the differences in the oligomeric organization of two proteins could also be due to species difference. However, although the biological relevance of this anti-parallel dimer interface is unclear, we can speculate that the observed conformational differences between the two forms of IKKβ are functionally relevant. Based on our modeling analysis, it is apparent that realignment of the activation loop upon phosphorylation would impose steric strain into the face-to-face dimer of xIKKβ, forcing its rearrangement, regardless crystal packing or protein species.

The results discussed above raise the possibility that change in the overall geometry of IKKβ, similar to the loss of 2-fold symmetry, is due to the differences in activation loop status of hIKKβ and xIKKβ, and hence in the active versus inactive state conformations that they adopt. We suggest that the structure of xIKKβ describes IKKβ in its early “closed off” state that pre-

![Diagram of similarities and differences in oligomeric structures of hIKKβ and xIKKβ.](image-url)
cedes the intermediate active, open state represented by our structure of phosphorylated hIKKβ. By analogy with other protein kinases that get activated by dimerization (44, 45), this change in geometry could be required to properly orient the kinase domains once an activated fraction is in play. Further studies of these enzymes will be required to confirm or dispute these findings.

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Addendum—This activation mechanism based on a change in geometry does not seem to apply to the IKK-related kinase TBK1. In two independent studies that were published while our manuscript was in preparation, Larabi et al. (30) and Tu et al. (46) ruled out the possibility for dimer opening in TBK1 because the active and inactive TBK1 crystal structures both maintain compact symmetric dimer configurations, with much more extensive dimerization interfaces than observed in both xIKKβ and hIKKβ.

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