Activation of transcription factor NF-κB is regulated by phosphorylation and subsequent degradation of its inhibitory subunit IκB. The signal-induced phosphorylation of IκB involves two IκB kinases, IKKα and IKKβ. In the present study, we investigated the kinetic mechanisms of IKKα and IKKβ by substrate and product inhibition. For both IKKα and IKKβ, the product ADP was a competitive inhibitor versus ATP and a non-competitive inhibitor versus IκBα. An alternative peptide substrate, IκBα(21–41), was a competitive inhibitor versus IκBα and a non-competitive inhibitor versus ATP for both kinases. These results rigorously eliminate the possibility of an ordered sequential mechanism and demonstrate that both kinases have a random sequential bi bi mechanism. Two natural compounds, quercetin and staurosporine, had previously been shown to inhibit the NF-κB pathway, but the molecular target(s) of these compounds in the event had not been established. Here we demonstrate that quercetin and staurosporine potently inhibit both IKKα and IKKβ. Daidzein, a quercetin analogue that does not inhibit NF-κB activation, showed no significant inhibition of either enzyme. This suggests that the inhibitory properties of quercetin and staurosporine in the NF-κB pathway are mediated in part by their inhibition of IKKα and IKKβ. Mechanism studies reveal that staurosporine is a competitive inhibitor versus ATP, whereas quercetin serves as a mixed type inhibitor versus ATP. The strong inhibition of IKKβ by staurosporine (K_i = 172 nM) and ADP (K_i = 136 nM) provides a rationale and structural framework for designing potent ATP-site inhibitors of IKKβ, which is an attractive drug target for inflammatory diseases.

The transcription factor NF-κB is regulated by the signaling of receptors for inflammatory cytokines such as TNFα, interleukin-1, or other external stimuli (1). In resting cells, NF-κB is sequestered in the cytoplasm through its association with inhibitory proteins termed IκB. When cells are stimulated by TNFα or interleukin-1, IκB proteins (IκBα and IκBβ) are rapidly phosphorylated at Ser residues in the N-terminal region (2, 3). Phosphorylated IκBα and IκBβ are subsequently ubiquitinated and undergo ubiquitin-dependent degradation by the 26 S proteasome (3, 4). Degradation of IκB results in the release of NF-κB which then translocates to the nucleus where it up-regulates the transcription of target genes (1).

IκBo and IκBβ are phosphorylated by a 500–900-kDa IκB kinase (IKK) (5, 6). Two kinases in the IKK complex, denoted IKKα and IKKβ (or IKK-1 and IKK-2), phosphorylate IκBα at the specific Ser residues that target the protein for ubiquitination and degradation (5–9). Both IKKα and IKKβ contribute to the activity of the IKK complex and are involved in NF-κB activation (5–9). The physiological function of these protein kinases was recently explored by analysis of IKKα-deficient or IKKβ-deficient mice (10–15). Mouse embryonic fibroblast cells that were isolated from IKKβ(−/−) embryos showed a marked reduction in TNFα- and interleukin-1-induced NF-κB activity and enhanced apoptosis in response to TNFα (11, 14, 15). In contrast, IKKα was not required for activation of IKK and degradation of IκB by pro-inflammatory stimuli (10, 12). These results show that IKKβ, not IKKα, is the target for pro-inflammatory stimuli. On the other hand, IKKα is essential for development of skin and skeleton during embryogenesis (10, 12, 13). NF-κB activation is impaired in the basal layer of epidermal cells in IKKα-deficient mice (12). Since IKKα and IKKβ have distinct functions, it is informative to compare the kinetic mechanisms of both kinases. Inhibitors with selectivity between these two kinases would help to elucidate further their different functions in cells and in animal models.

IKKα and IKKβ share ~50% overall homology, and both contain a conserved N-terminal Ser/Thr kinase domain, a leucine-zipper region, and a C-terminal helix-loop-helix (HLH) motif (6–9). Such folding is unique among the known kinases. It has been shown that the HLH domain of IKKβ is required for its kinase activity and the HLH domain can activate the truncated IKKβ (HLH deletion) mutant in trans (16). This suggests a functional interaction between the HLH domain and the kinase domain of IKKβ. IKKα and IKKβ also share a distinguishing feature in that they have a strong preference for Ser versus Thr on the substrates (5, 6). It is important to understand the kinetic mechanisms of these two unique members of the Ser/Thr kinase family.

Several naturally existing kinase inhibitors have been reported to inhibit the NF-κB pathway. Quercetin, a flavonoid that occurs in many fruits and vegetables (17), is a nonspecific inhibitor of protein kinases (18) and suppresses TNF-induced NF-κB activation (19). The inhibitor blocks the degradation of IκBα and the consequent translocation of the NF-κB p65 subunit (19). Staurosporine, a microbial alkaloid that was isolated from Streptomyces staurospores (20), has shown potent inhibition of both tyrosine and Ser/Thr kinases (18, 21). In THP-1 monocytic cells, staurosporine inhibits LPS-dependent NF-κB activation, suggesting that staurosporine-sensitive kinase(s) are involved in LPS-mediated NF-κB activation (22). The inhibitory effects of quercetin and staurosporine in the NF-κB pathway are consistent with their anti-inflammatory responses as observed in various animal models including experimental arthritis and experimental colitis (23–26). However, the molecular target(s) of staurosporine and quercetin in the NF-κB

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1 The abbreviations used are: HLH, helix-loop-helix; IKK, IκB kinase; TNF, tumor necrosis factor; TRX, thioredoxin; LPS, lipopolysaccharide.
signaling cascade have not been identified. Since IKK is essential for activation of NF-κB by both TNFα and LPS (6–9, 27), it is important to know whether quercetin and staurosporine inhibit IKKα and IKKβ. It was recently shown that high concentrations of the anti-inflammatory agent aspirin inhibits IKKβ (IC50 = ~50 μM) (28), consistent with its inhibitory effect on the NF-κB pathway (29).

Previously, we have demonstrated that purified recombinant IKKα and IKKβ are direct kinases of IκBα and function independently in vitro (30). We have also shown that both IKKα and IKKβ display a sequential bi bi mechanism (30). However, our previous report did not discriminate between the possibilities of a random sequential or an ordered sequential mechanism. In the current study, we perform product and substrate inhibition experiments that demonstrate that both IKKα and IKKβ proceed by a random sequential mechanism. We also demonstrate that the natural compounds quercetin and staurosporine inhibit both IKKα and IKKβ with compound-specific mechanisms. Thus, the inhibitory effects of quercetin and staurosporine on the NF-κB pathway are at least partially through their inhibitions of IKKα and IKKβ.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—IKKα and IKKβ were expressed as N-terminal FLASH-tagged fusion proteins in baculovirus. The recombinant FLASH-tagged IKKα and IKKβ were purified to apparent homogeneity by affinity chromatography using M2 anti-FLASH affinity gel (Sigma). The procedures for expression and purification have been described previously (30). IκBα was expressed as a His6-tagged thioredoxin fusion protein (Trx-IκBα-(1–54)) in Escherichia coli and purified by a Ni2+–nitrilotriacetic acid affinity column, as described (30).

In Vitro Phosphorylation Assays—The kinase assays were performed in a plate assay format as described previously (30). Briefly, reactions (55 μl) were performed at 23 °C in 20 mM HEPES, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 100 mM NaCl, 100 μM Na3VO4, 20 mM β-glycerophosphate, and 1 mM dithiothreitol. The amount of substrates ATP, [γ-33P]ATP (2000 Ci/mmol, NEN Life Science Products) and IκBα are specified for each individual experiment. Samples were analyzed by trichloroacetic acid precipitation on a microtiter plate (Millipore), followed by liquid scintillation counting (30). Assay conditions were controlled so that the degree of phosphorylation of IκBα was linear with time and concentration of enzyme. The counts represent initial velocity of IKK-catalyzed phosphorylation (<10% of total ATP conversion). All experiments were performed in duplicate.

Kinetic Analysis—Initial velocity studies were performed with varying concentrations of IκBα at a constant ATP concentration and several fixed inhibitor concentrations. Conversely, initial velocity studies were performed with varying ATP concentrations at a constant IκBα concentration and several fixed inhibitor concentrations. All enzyme activity data are reported as the average of duplicate determinations. The initial rate V was recorded as femtomoles of phosphate transferred to IκBα during the reaction period. Lineweaver-Burk double-reciprocal plots were generated by linear least square fits of the data. From inhibition experiments were fitted to either a linear competitive model (Equation 1) or a non-competitive (or mixed inhibition) model (Equation 2) (31–33).

\[
\frac{1}{V} = \frac{K_m}{V_{max}} \left[ \frac{1}{[I]} + \frac{1}{K_i} \right] + \frac{1}{V_{max}} \quad \text{(Eq. 1)}
\]

\[
1 + \frac{[S]}{K_m} \frac{1}{V_{max}} = \frac{1}{V} \quad \text{(Eq. 2)}
\]

Accordingly, secondary plots were generated by replotted the slopes, the x intercepts, and the y intercepts of the lines as a function of [inhibitor] (32). The values of Kι and Kι can be determined from the secondary plots. Kι is the apparent Ki value that accounts for the change of the slope, Kι is the apparent Ki value that accounts for the change of the y intercept.

Materials—The peptide IκBα-(21–41) was ordered from Ana Spec Inc. (San Jose, CA).

RESULTS

Various models of kinetic mechanisms have been described for enzymes that catalyze two substrates (34, 35). For IKKα and IKKβ, our previous study had eliminated a ping-pong mechanism and demonstrated that both enzymes followed a sequential bi bi mechanism (30). Scheme I describes the three possible sequential mechanisms: ordered sequential mechanism with ATP binding first (Model 1), ordered sequential mechanism with IκBα binding first (Model 2), and a random sequential mechanism (Model 3). Validations of these mechanisms are described below.

Inhibition of IKKα and IKKβ by the Product Inhibitor ADP—First, the kinase activities of IKKα and IKKβ were determined as a function of varying concentrations of ATP at various fixed concentrations of ADP. The Lineweaver-Burk plots of the data for both IKKα and IKKβ followed Michaelis-Menten kinetics (Fig. 1A and 2A). For both IKKα and IKKβ, a series of double-reciprocal straight line plots intersected on the ordinate, indicating a competitive inhibition mechanism (32). Furthermore, the data were plotted as the slope of the reciprocal plot versus the concentration of the inhibitor. The replots for both IKKα and IKKβ are linear (Figs. 1A and 2A, insets), and yielded Kι values of 156 and 147 μM for IKKα and IKKβ, respectively.

We subsequently investigated the inhibition mechanism of ADP toward the substrate IκBα. The kinase activities of IKKα and IKKβ were determined as a function of varying concentrations of IκBα at various fixed concentrations of ADP. The Lineweaver-Burk plots of the data for both IKKα and IKKβ yielded a series of straight lines that crossed on the abscissa, to the left side of the ordinate (Figs. 1B and 2B), indicating a non-competitive inhibition mechanism (32).

As can be seen, the product ADP is a competitive inhibitor of IKKα and IKKβ with respect to ATP and a non-competitive inhibitor with respect to IκBα. This behavior is incompatible with an ordered sequential mechanism with IκBα binding first (Scheme I, Model 2), since otherwise ADP would have been an un-competitive inhibitor with respect to IκBα. However, the results do not exclude a random sequential mechanism or an ordered sequential mechanism with ATP binding first (Scheme I, Model 1 or 3).

Inhibition of IKKα and IKKβ by a Peptide Analogue of IκBα—The peptide corresponding to amino acids 21–41 of IκBα would compete with IκBα for binding to the enzymes, since the peptide can be phosphorylated by both IKKα and IKKβ (6, 30). Thus, this peptide is an alternative substrate for IKKα and IKKβ with respect to IκBα. Since the 21-amino acid peptide is not retained during trichloroacetic acid precipitation and membrane filtration in the phosphorylation assay (data not shown), the assay only monitors the appearance of the radioactive 32P on recombinant protein Trx-IκBα. Therefore, we are able to use this peptide as an alternative substrate inhibitor to study the kinetic mechanisms of IKKα and IKKβ. In an effort to further elucidate the sequential mechanism (Scheme I, Model 1 or Model 3), we inhibited the phosphorylation of IκBα with this
peptide using approaches similar to that employed for the ADP inhibition studies as described above. As shown in Figs. 3A and 4A, double-reciprocal plots of $v$ versus $1/[ATP]$ at various fixed peptide concentrations yielded straight lines that crossed on the ordinate, confirming its being a competitive inhibitor toward the substrate IxEa for both IKK$\alpha$ and IKK$\beta$. The apparent $K_i$ values of 139 and 90 $\mu$m for IKK$\alpha$ and IKK$\beta$, respectively, were obtained from linear secondary plots (Figs. 3A and 4A, insets).

The kinase activities of IKK$\alpha$ and IKK$\beta$ were also measured as a function of varying concentrations of ATP at several different fixed concentrations of peptide IxEa-(21–41). The Lineweaver-Burk plots of the data for both IKK$\alpha$ and IKK$\beta$ yielded a series of straight lines that intersected on the abscissa, to the left side of the ordinate, indicating a non-competitive inhibition mechanism (Figs. 3B and 4B).

The different patterns of product inhibition and substrate inhibition for bi bi sequential reactions have been derived (34, 35). The inhibition patterns obtained for IKK$\alpha$ and IKK$\beta$ in this study are summarized in Table I. The fact that the product ADP was a competitive inhibitor $versus$ ATP but a non-competitive inhibitor $versus$ IxEa indicates either a random sequential mechanism (Scheme I, Model 3) or an ordered sequential mechanism with ATP binding first (Scheme I, Model 1). The peptide IxEa-(21–41) behaves as a competitive inhibitor $versus$ IxEa but as a non-competitive inhibitor $versus$ ATP. This eliminates the possibility of an ordered sequential mechanism with ATP binding first (Scheme I, Model 1), which would give an un-competitive inhibition pattern with respect to ATP. In conclusion, the kinetics of IKK$\alpha$ and IKK$\beta$ follow a random-ordered sequential bi bi mechanism (Scheme I, Model 3).

**Staurosporine Is an ATP-competitive Inhibitor of IKK$\alpha$ and IKK$\beta$**—The natural kinase inhibitor staurosporine has been implicated to inhibit the NF-$\kappa$B pathway since it blocks LPS-stimulated NF-$\kappa$B activation in THP-1 monocytic cells (22). Since LPS activates NF-$\kappa$B through IKK in THP-1 cells (27), we decided to test whether staurosporine inhibits IKK$\alpha$ or IKK$\beta$. Staurosporine inhibited both IKK$\alpha$ and IKK$\beta$ in a dose-dependent manner, with an apparent IC$_{50}$ of 0.85 and 1.6 $\mu$m for IKK$\alpha$ and IKK$\beta$, respectively (Fig. 5A). The effect of staurosporine on the initial velocity patterns for IKK$\alpha$ and IKK$\beta$ are shown in Fig. 5, B and C. Double-reciprocal plots of $v$ versus $1/[ATP]$ at different fixed concentrations of staurosporine intersect on the ordinate, indicating that the inhibitor is competitive with ATP for both IKK$\alpha$ and IKK$\beta$ (Fig. 5, B and C). As represented in Fig. 5D, increased concentrations of IxEa did not reduce the inhibition of IKK$\alpha$ and IKK$\beta$ by staurosporine, indicating that staurosporine is non-competitive with IxEa. This is consistent with staurosporine being a competitive inhibitor with ATP (Fig. 5, B and C). Global fitting of the data in Fig. 5, B and C, to a competitive inhibition model (EnzFitter program, Biosoft) yielded $K_i$ values of 86 $\pm$ 17 and 172 $\pm$ 39 nM for IKK$\alpha$ and IKK$\beta$, respectively. The potent inhibition of IKK$\alpha$ and IKK$\beta$ by staurosporine is consistent with its potent inhibition of NF-$\kappa$B activation (22).

**IKK$\alpha$ and IKK$\beta$ Are Inhibited by Quercetin**—Quercetin has been reported as an inhibitor of both tyrosine kinases and Ser/Thr kinases (18, 36). Since quercetin inhibits TNF-induced nuclear translocation of NF-$\kappa$B (19), we investigated whether it acts upon IKK$\alpha$ and IKK$\beta$. Quercetin inhibited both IKK$\alpha$ and IKK$\beta$ (Fig. 6, A and B), with an apparent IC$_{50}$ value of 11 and 4 $\mu$m, respectively. Daidzein, a structural analogue of quercetin...
Mechanisms of IKKα and IKKβ

Fig. 3. Inhibition of IKKα by peptide IxBa-(21–41). A, double-reciprocal plots of 1/v versus 1/[IxBa] were generated at 5 fixed peptide concentrations of 0 μM (open triangles), 35 μM (closed circles), 100 μM (closed diamonds), 200 μM (closed triangles), and 400 μM (closed squares). Reactions were performed at 23 °C for 15 min with 200 ng of IKKα, 533 nCi of [γ-32P]ATP, 200 nM ATP, and varying concentrations of IxBa as indicated. B, double-reciprocal plots of 1/v versus 1/[ATP] were generated at 5 fixed peptide concentrations of 0 μM (open triangles), 50 μM (closed circles), 100 μM (closed diamonds), 200 μM (open triangles), and 500 μM (closed squares). Reactions were performed at 23 °C for 15 min with 200 ng of IKKα, 7 μM IxBa, 563 nCi of [γ-32P]ATP, and varying concentrations of ATP as indicated. Insets, the slopes of the plots in A and B were replotted versus [peptide].

Fig. 4. Inhibition of IKKβ by peptide IxBa-(21–41). A, double-reciprocal plots of 1/v versus 1/[IxBa] were generated at 5 fixed peptide concentrations of 0 μM (closed circles), 62.5 μM (open circles), 125 μM (closed triangles), 250 μM (open triangles), and 500 μM (closed squares). Reactions were performed at 23 °C for 15 min with 25 ng of IKKβ, 546 nCi of [γ-32P]ATP, 200 nM ATP, and varying concentrations of IxBa as indicated. B, double-reciprocal plots of 1/v versus 1/[ATP] were generated at 5 fixed peptide concentrations of 0 μM (closed circles), 50 μM (open circles), 100 μM (closed triangles), 200 μM (open triangles), and 500 μM (closed squares). Reactions were performed at 23 °C for 15 min with 25 ng of IKKβ, 2 μM IxBa, 566 nCi of [γ-32P]ATP, and varying concentrations of ATP as indicated. Insets, the slopes of the plots in A and B were replotted versus [peptide].

Table I

| Enzyme | Fig. | Inhibitor | Varied substrate | Inhibition pattern | $K_i$ (μM) | $K_m$ | $K_{in}$ | $\beta$ |
|--------|------|-----------|------------------|-------------------|-------------|-------|---------|-------|
| IKKα   | 1A   | ADP       | ATP              | C                 | 0.156       | -     | -       | -     |
|        | 1B   | ADP       | IxBa             | NC                | 0.420       | 0.281 | 0.125   | 0.7   |
|        | 3A   | IxBa(21–41) | IxBa             | C                 | 139         | -     | -       | -     |
|        | 3B   | IxBa(21–41) | ATP              | NC                | 174         | 174   | 136     | 1.0   |
| IKKβ   | 2A   | ADP       | ATP              | C                 | 0.147       | -     | -       | -     |
|        | 2B   | ADP       | IxBa             | NC                | 0.346       | 0.351 | 0.136   | 1.0   |
|        | 4A   | IxBa(21–41) | IxBa             | C                 | 90          | -     | -       | -     |
|        | 4B   | IxBa(21–41) | ATP              | NC                | 172         | 180   | 29      | 1.0   |

The abbreviations used are: C, competitive; NC, noncompetitive. The values of $K_i$ and $\beta$ were derived from the equation $K_i^{1/\beta} = K_i(1 + |A|/K_a)$ (37). The values of $K_{in}$, $K_m$, and $K_{in}$ that were used in the calculations were taken from the previous report (30). The $\beta$ represents the ratio of $K_{in}$ in the presence and absence of inhibitor.

(Scheme II), showed no significant inhibitory effects on the activities of IKKα and IKKβ (Fig. 6, A and B). Since daidzein failed to block TNF-mediated NF-κB activation at 80 μg/ml (19), this result is consistent with IKKα and IKKβ being involved as molecular targets of quercetin in the TNF pathway.

We further investigated the inhibition mechanism of quercetin on IKKα and IKKβ. We first examined kinase inhibition by quercetin in the presence of various amounts of ATP. Fig. 7, A and B, shows double-reciprocal plots of 1/v versus 1/[ATP] at several fixed concentrations of quercetin. The Lineweaver-Burk plots of the data for both IKKα and IKKβ are linear, indicating Michaelis-Menten kinetics at each individual concentration of quercetin (Fig. 7, A and B). For both IKKα and IKKβ, quercetin significantly reduced the apparent $V_{max}$ (1/v intercept) and increased the apparent $K_m$ (1/v intercept), indicating a mixed type inhibition mechanism. However, both series of double-reciprocal plots did not intersect at a single point to the left of the ordinates (Fig. 7, A and B), suggesting a more complicated mechanism than the standard linear mixed type inhibition mechanism (33). In contrast to that observed for staurosporine (Fig. 5D), the inhibition of IKKα and IKKβ by quercetin was protected by increased amounts of substrate IxBa (Fig. 7C). This result is consistent with quercetin being a noninhibitory inhibitor with respect to ATP and IxBa as indicated by Fig. 7, A and B. These observations suggest that the binding site of quercetin may overlap with both the ATP- and IxBa-binding sites.

DISCUSSION

Previous kinetic studies of IKKα and IKKβ did not discriminate between a random sequential or an ordered sequential mechanism (30). The results of the present inhibition studies...
Data are presented as percentage of inhibition by staurosporine. Reactions were performed at 23 °C for 15 min with 200 nM ATP, and 2 μM IκBα. The IC_{50} curves were generated by SigmaPlot regression fitting using the equation: \( y = 100 - \left( \frac{L_{\text{max}}}{IC_{50} + x^\alpha} \right) \) (x = [compound], y = % activity, and L_{\text{max}} is the maximum percentage of inhibition). B and C, inhibition pattern of staurosporine with respect to ATP. Double-reciprocal plots of 1/\( v \) versus 1/[ATP] were generated at 4 fixed staurosporine concentrations of 0 nM (closed triangles), 125 nM (open circles), 250 nM (closed circles), and 500 nM (closed squares). Reactions were performed at 23 °C for 15 min with 2 μM IκBα, 554 nCi of [\( \gamma^{33}\text{P} \)]ATP, varying concentrations of ATP as indicated, and either 200 ng of IKKα (B) or 25 ng of IKKβ (C). D, effect of various concentrations of IκBα on staurosporine-mediated inhibition of IKKα and IKKβ. Both IKKα (200 ng, closed circles) and IKKβ (25 ng, closed triangles) were assayed in the presence or absence of 500 nM staurosporine. Reactions were performed at 23 °C for 15 min with 200 nM ATP, 550 nCi of [\( \gamma^{33}\text{P} \)]ATP, and varying concentrations of IκBα as indicated. Data are presented as percentage of inhibition by staurosporine.

![Mechanisms of IKKα and IKKβ](image)

**FIG. 5. Inhibition of IKKα and IKKβ by staurosporine.** A, IC_{50} plots of IKKα (200 ng, closed circles) and IKKβ (100 ng, closed triangles). IC_{50} assays employed a 5-min preincubation of enzyme plus inhibitor at 23 °C prior to initiation of reaction with substrates. Data are presented as a percentage of control activity (no inhibitor). For IKKα, reactions were performed at 23 °C for 60 min with 550 nCi of [\( \gamma^{33}\text{P} \)]ATP, 250 nM ATP, and 7 μM IκBα. For IKKβ, reactions were performed at 23 °C for 30 min with 550 nCi of [\( \gamma^{33}\text{P} \)]ATP, 250 nM ATP, and 2 μM IκBα. The IC_{50} curves were generated by SigmaPlot regression fitting using the equation: \( y = 100 - \left( \frac{L_{\text{max}}}{IC_{50} + x^\alpha} \right) \) (x = [compound], y = % activity, and L_{\text{max}} is the maximum percentage of inhibition). B and C, inhibition pattern of staurosporine with respect to ATP. Double-reciprocal plots of 1/\( v \) versus 1/[ATP] were generated at 4 fixed staurosporine concentrations of 0 nM (closed triangles), 125 nM (open circles), 250 nM (closed circles), and 500 nM (closed squares). Reactions were performed at 23 °C for 15 min with 2 μM IκBα, 554 nCi of [\( \gamma^{33}\text{P} \)]ATP, varying concentrations of ATP as indicated, and either 200 ng of IKKα (B) or 25 ng of IKKβ (C). D, effect of various concentrations of IκBα on staurosporine-mediated inhibition of IKKα and IKKβ. Both IKKα (200 ng, closed circles) and IKKβ (25 ng, closed triangles) were assayed in the presence or absence of 500 nM staurosporine. Reactions were performed at 23 °C for 15 min with 200 nM ATP, 550 nCi of [\( \gamma^{33}\text{P} \)]ATP, and varying concentrations of IκBα as indicated. Data are presented as percentage of inhibition by staurosporine.

![Scheme II](image)

**SCHEME II.**

clearly demonstrate that both IKKα and IKKβ proceed through a random sequential mechanism. The equilibria shown in Scheme III describe the kinetic parameters in a random sequential bi bi system. In our previous report (30), we had fitted the two-substrate profiling data of IKKα and IKKβ to a random sequential model as described in Scheme III. As a result, for IKKα, values of 85 nM, 25 μM, 0.09/min, and 1.0 were obtained for \( K_{\text{ATP}} \), \( K_{\text{IκBα}} \), \( k_{\text{cat}} \), and \( \alpha \), respectively. For IKKβ, values of 130 nM, 1.4 μM, 0.30/min, and 1.0 were obtained for \( K_{\text{ATP}} \), \( K_{\text{IκBα}} \), \( k_{\text{cat}} \), and \( \alpha \), respectively (30). Thus, as we have proven the random sequential model in this study, the kinetic mechanisms and parameters of IKKα and IKKβ are now complete.
Since the native 500–900-kDa IKK complex is composed of both IKKα and IKKβ (6, 7), the kinetics of the IKK complex is likely to proceed through a random sequential mechanism. Consistent with this assumption, it has been shown that a multisubunit IkB kinase complex isolated from HeLa cells displays a random sequential mechanism (38), although it has not been demonstrated whether it is the same IKK complex that contains IKKα and IKKβ.

The product ADP is a potent inhibitor for both IKKα and IKKβ, with a $K_i$ value of 125 and 136 nm, respectively (Table I). These values are slightly higher than the corresponding $K_{ATP}$ values (85 nm for IKKα and 130 nm for IKKβ) (30). This suggests that the binding of ATP to IKKα and IKKβ is predominantly mediated by the ADP portion of the molecule. It should be noted that, within the kinase family, a distinguishing feature for IKKα and IKKβ is their low $K_m$ (ATP) value for ATP (−100 nm) (30). In comparison, much higher $K_m$ (ATP) values have been reported for other Ser/Thr protein kinases, such as cAMP-dependent protein kinase ($K_m$ = 10 μM) (39) and p38 mitogen-activated protein kinase ($K_m$ = 23 μM) (40). Similarly, IKKα and IKKβ have unprecedented low $K_j$, with a $K_j$ value of 1.0 for the inhibitor IκB-(21–41), respectively (Table I). These values, with allowance for experimental error, are comparable to the 1.0 $a$ value for IKKα (30). Taken together, for both IKKα and IKKβ, the binding of one substrate has no effect on the affinity for the other substrate.

The native cytokine-inducible IKK complex contains both IKKα and IKKβ (5, 6). By using purified recombinant IKKα or IKKβ, we have previously demonstrated that IKKα and IKKβ are direct kinases of IκBα but that they have no synergistic kinase activity (30). Since these two kinases share ~50% homology, it is possible to inhibit both kinases with a small molecule compound. This possibility is supported by our observation that staurosporine and quercetin are potent inhibitors of both kinases. On the other hand, IKKα and IKKβ have distinct physiological functions (10–15). Specific inhibition of each individual kinase may be preferred. Inhibitors that show selectivity between these kinases would allow characterization of their physiological functions in vivo.

Staurosporine inhibits widely divergent members of the protein kinase family (21). This suggests that staurosporine functions by binding to a region that is conserved throughout the protein kinase family. The inhibition of the mammalian small heat-shock protein (HSP25) kinase by staurosporine and its analogue K252a is competitive with respect to ATP (44). In addition, an ATP-competitive mechanism has been observed in the inhibition of protein kinase C and cAMP-dependent protein kinase by the staurosporine analogue K252a (45). The same mechanism is now shown in the inhibition of IKKα and IKKβ by staurosporine. This is not surprising since both IKKα and IKKβ contain a conserved catalytic kinase domain at the N-terminal region which includes the conserved ATP-binding site (5–9). At this time, staurosporine is the most potent compound inhibitor of IKKα ($K_i$ = 86 nm) and IKKβ ($K_i$ = 172 nm) ever reported. Such potent inhibitions by staurosporine provide a starting point for building more selective inhibitors of IKKα and IKKβ. In fact, several staurosporine derivatives such as CGP 41251 (4′-N-benzoyl staurosporine) and Ro 318425 show significant selectivity for protein kinase C over cAMP-dependent protein kinase and epidermal growth factor receptor tyrosine kinase (26, 46). The inhibition mechanism of quercetin on various kinases appears to be diverse. Quercetin inhibits pp66$	ext{^{Glu}}$ tyrosine kinase as an ATP-competitive inhibitor (47). In contrast, the inhibition of phosphatidylinositol 3-kinase-1 kinase and phosphatidylinositol 3-kinase II by quercetin is non-competitive versus ATP (48). In our studies of IKKα and IKKβ, quercetin showed a mixed inhibition mechanism toward ATP (Fig. 7). The binding site of quercetin is likely to overlap with both the ATP and IκBα binding pockets.

Several tyrosine kinase inhibitors, such as quercetin, genistein, staurosporine, and herbimycin, are able to inhibit NF-κB activation (19, 22). Thus, it has been implicated that tyrosine kinase(s) are involved in NF-κB regulation. However, there is a lack of direct evidence that tyrosine kinases participate in the NF-κB pathway. We have now shown that quercetin and staurosporine inhibit IKKα and IKKβ, the two key regulated serine kinases in the NF-κB pathway, consistent with their inhibitory effects on NF-κB activation. In addition, IKKα and IKKβ were not inhibited by daidzein (Fig. 6), a quercetin analogue without inhibitory effects on TNF-induced NF-κB activation (19). The tyrosine kinase inhibitor genistein also inhibits IKKβ. Since kinase inhibitors usually have poor selectivity, their inhibitory effects on certain signaling pathways are likely to be a combination of inhibitions of several kinase targets within multiple signaling cascades. This study suggests that the inhibitory
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effects of staurosporine and quercetin on NF-κB activation are at least partially due to the inhibition of IKKα and IKKβ. As NF-κB is a key cellular regulator of the inflammatory response, the anti-inflammatory properties of quercetin and staurosporine (23–26) may be partially due to their inhibition of IKKα and IKKβ. A correlation between the anti-inflammatory effects and the inhibition of IKKβ has been observed for aspirin and salicylate (28).

The recent in vivo knock-out studies of IKKβ imply that IKKβ is a valid target for inflammatory diseases (11, 14, 15). Thus high throughput screening for inhibitors of IKKα and IKKβ could yield small molecules of therapeutic value. Here we have demonstrated the kinetic mechanism of both IKKα and IKKβ to be random sequential, with each substrate binding independently of the other. This characterized kinetic mechanism will help in the evaluation of potential drug leads. Based on the potent inhibition of IKKβ by ADP, staurosporine, and quercetin, these compounds may be considered starting points for designing specific inhibitors. The different inhibition mechanisms of staurosporine and quercetin also indicate that potent inhibition of the enzyme can be achieved by targeting different parts of the ATP-binding site. However, it is challenging to create tight-binding inhibitors that are selective between IKKα and IKKβ, the two homologous kinases that have similar kinetic mechanisms. Comparison of x-ray crystal structures of both kinases will help us to accomplish this goal.

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