Peptides Corresponding to the N and C Termini of IκB-α, -β, and -ε as Probes of the Two Catalytic Subunits of IκB Kinase, IKK-1 and IKK-2*

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The signal-inducible phosphorylation of serines 32 and 36 of IκB-α is the key step in regulating the subsequent ubiquitination and proteolysis of IκB-α, which then releases NF-κB to promote gene transcription. The multisubunit IκB kinase (msIKK) responsible for this phosphorylation contains two catalytic subunits, termed IKK-1 and IKK-2. Using recombinant IKK-2, a kinetic pattern consistent with a random, sequential binding mechanism was observed with the use of a peptide corresponding to amino acids 26–42 of IκB-α. Values of 313 μM, 15.5 μM, and 1.7 min⁻¹ were obtained for Kₚ, K_ATP, and k_cat, respectively. The value of α, a factor by which binding of one substrate changes the dissociation constant for the other substrate, was determined to be 0.2. Interestingly, the recombinant IKK-1 subunit gave similar values for α and K_ATP, but values of 1950 μM and 0.016 min⁻¹ were calculated for K_peptide and k_cat, respectively. This suggests that the IKK-2 catalytic subunit provides nearly all of the catalytic activity of the msIKK complex with the IKK-1 subunit providing little contribution to catalysis. Using peptides corresponding to different regions of IκB-α within amino acids 21–47, it was shown that amino acids 31–37 provide most binding interactions (−4.7 kcal/mol of binding free energy) of the full-length IκB-α (−7.9 kcal/mol) with the IKK-2. This is consistent with the observation that IKK-2 is able to phosphorylate the IκB-β and IκB-ε proteins, which have consensus phosphorylation sites nearly identical to that of amino acids 31–37 of IκB-α. A peptide corresponding to amino acids 279–303 in the C-terminal domain of IκB-α was unable to activate IKK-2 to phosphorylate an N-terminal peptide, which is in contrast to the results observed with the msIKK. Moreover, the IKK-2 catalyzes the phosphorylation of the full-length IκB-α and the amino acid 26–42 peptide with nearly equal efficiency, while the msIKK catalyzes the phosphorylation of the full-length IκB-α 25,000 times more efficiently than the 26–42 peptide. Therefore, the C terminus of IκB-α is important in activating the msIKK through interactions with subunits other than the IKK-2.

The transcriptional activator NF-κB normally resides in the cytoplasm in unstimulated cells as an inactive complex with a member of the IκB inhibitory protein family. This class of protein includes IκB-α, IκB-β, and IκB-ε, which all contain ankyrin repeats necessary for complexation with NF-κB (for a review, see Ref. 1). In the case of IκB-α, the most carefully studied member of this class, stimulation of cells with agents which activate NF-κB-dependent gene transcription results in the phosphorylation of IκB-α at Ser-32 and Ser-36 (2). This is critical for subsequent ubiquitination and proteolysis of IκB-α, which then leaves NF-κB free to translocate to the nucleus and promote gene transcription (3–5). Indeed, a mutant in which both Ser-32 and Ser-36 have been changed to alanine prevents signal-induced activation of NF-κB and results in an IκB-α which is neither phosphorylated, ubiquitinated, nor proteolytically digested (5). Analogous serines have been identified in both IκB-β and IκB-ε, and phosphorylation at these residues appears to regulate the proteolytic degradation of these proteins by a mechanism similar to that of IκB-α (6, 7). Because the expression of many pro-inflammatory genes such as the cytokines tumor necrosis factor-α, interleukin-6, interleukin-8, and interleukin-1β; the adhesion molecules E-selectin and VCAM-1; and the enzyme nitric-oxide synthase is regulated by NF-κB (for reviews, see Refs. 8 and 9), the inhibition of this signal-inducible phosphorylation of IκB would be an important target for novel anti-inflammatory agents.

A high molecular mass (500–900 kDa) multisubunit IκB kinase (termed IKK) which phosphorylates at Ser-32 and Ser-36 of IκB-α has been isolated from HeLa cells (10–12). Two catalytic subunits (termed IKK-1 and IKK-2) of IKK have recently been identified, cloned, and shown to be widely expressed in human tissues (12–17). Demonstration that IKK is the kinase involved in the signal-inducible degradation of IκB-α was accomplished by both antisense inhibition of IKK-1 and the use of dominant-negative, catalytically inactive mutants of IKK-1 and IKK-2 (12, 14, 15). Both approaches abrogated cytokine- and lipopolysaccharide-induced activation of NF-κB. The signal-induced activation of IKK appears to proceed through phosphorylation of the IKK-1 and/or IKK-2 subunits by a mitogen-activated protein kinase kinase (such as MEKK1 or NIK), which greatly enhances the enzymatic activity (18).

Surprisingly, the multisubunit IKK recognizes and is stimulated by elements of the C terminus of IκBα (19). In this paper we provide a detailed characterization of the IKK-1 and IKK-2 catalytic subunits using peptides corresponding to the N- and C-terminal sequences of IκB proteins as probes. Interestingly, amino acids 31–37 of IκB-α provide for most of the binding free energy to the catalytic subunits, and regions outside of amino acids 21–47 provide for little interactions. Moreover, consider-

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1 The abbreviations are: IKK, IκB kinase; GST, glutathione S-transferase; GST–IκB-α, IκB-α fusion protein with glutathione S-transferase tag; msIKK, multisubunit IκB kinase from HeLa S3 cells; [32P]ATP, adenosine 5’-[γ-32P]triphosphate; HPLC, high performance liquid chromatography; Fmoc, N-9-fluorenylmethoxycarbonyl.
ably greater binding interactions between the C terminus of IkB-α and the regulatory subunits of the mIKK appear to be responsible for the activation of the multisubunit complex to accelerate catalysis by many orders of magnitude.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione S-transferase (GST)-IκBα was purchased from Santa Cruz Biotechnology and cleaved with thrombin to remove the GST tag. [33P]ATP (1000 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

**Peptide Synthesis**—Peptides corresponding to regions of IκB-α, IκB-β, and IκB-ε were synthesized on Fmoc-Knorr amide resin (N-9-fluorenyl)methoxycarbonyl-Knorr amide-resin; Midwest Biotech, Fisher, CA) and the Fmoc chemistry protocol (0.25 mmol scale) supplied with the instrument. Amino acids were double-coupled as their Nα-Fmoc derivatives and reactive side chains were protected as follows: Asp and Glu, t-butyloxycarbonyl; Arg, pentamethylenehromosulfonyl; Cys, triphenylmethyl; and Tyr, t-butyloxyl. After the final double-coupling cycle, the N-terminal Fmoc group was removed by the two-step treatment with piperidine in N-methylpyrrolidone described by the manufacturer. The N-terminal free amines were then treated with 10% acetic acid in N-methylpyrrolidone 5% diisopropylamine in N-methylpyrrolidone to yield the N-acetyl-derivative. The protected peptide-residues were simultaneously deprotected and removed from the resin by standard methods, except for peptides IκB-α 26–42 and 279–303, which were extracted as crude products from the resin by a modified procedure described previously (19). The lyophilized peptides were purified on C18 to apparent homogeneity, as judged by reverse phase-HPLC analysis. Predicted peptide molecular weights were verified by electrospray mass spectrometry.

**Expression of Human, Recombinant IκK-1 and IκK-2**—The full-length coding region of IκK-1 and IκK-2 were cloned using reverse transcription-polymerase chain reaction with mRNA from HeLa cells as a template. Identity was confirmed by sequencing. For the protein expression the coding region was cloned into pBM-S1, a derivative of pVL1393 that has the GST coding sequence inserted into the BarnHI site of the polynliner followed by a thrombin protease site and by the XbaI site of the polylinker. For this purpose, the initiation ATG of the kinases was replaced by GTG preventing potential internal translation initiation. Polymerase chain reaction primers were as follows for IKK: TCTCATGAACTCCTCTGGGACGACCCGGC and TCTATAGCGGCCGCTCATTCTGTTAACCAACTCCAATCAAG, cloned into EcoRI/Xhol, and for IKKB: TCTTATCTGATGTCGAGTCGTGTCACCTTCCTTACAAAGC and TCTTATAGCGGCCGCTCATTCTGTTAACCAACTCCAATCAAG, cloned into XbaI/Xhol site of the pBM-S1 vector.

**Spodoptera frugiperda Sf9**; ATCC CRL 1711) and Trichoplusia ni (BTI-Tn5B1-4, High Five Inc., Invitrogen Corp.) cells were grown in suspension cultures at 27 °C in S9 medium (Life Technologies, Inc.). Isolation of recombinant viruses was done as described by Summers and Smith. For the expression of recombinant proteins, High Five cells were grown in 1-liter suspensions and infected in the log phase of growth at a cell density of 1.0 × 10^9 to 1.5 × 10^9 cells per ml, using 10 plaque-forming units of virus per cell.

From pellets of High Five cells expressing the recombinant GST-fusion proteins, enzyme activity was accomplished by first lysing a cell pellet in insect cell lysis buffer (PharMingen) for 45 min at 4 °C and then centrifuging the lysate at 40,000 × g for 30 min at 4 °C. Glutathione-agarose beads (PharMingen) were added and allowed to incubate with gentle agitation for 5 min at 4 °C. The beads were then collected by centrifugation, washed twice with phosphate-buffered saline, and the GST proteins eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8, containing 1 mM diethiothreitol. SDS-polyacrylamide gel electrophoresis showed the presence of the desired GST-IκK fusion protein with the only significant contaminating being GST itself. Anti-GST and anti-IκK (Santa Cruz Biotechnology) immunoblots were used to verify the identity and relative concentrations of IκK-1 and IκK-2 in these isolated samples.

**Peptides as Substrates**—When using peptides as substrates for the IκK-1 or IκK-2, enzymatic assays were performed by adding the enzyme at 30 °C to solutions containing peptide and [33P]ATP (1000 Ci/mmol) in 50 mM Tris-HCl, 5 mM MgCl2, pH 8. After 60 min, the kinase reactions were quenched by addition of EDTA to a concentration of 10 mM. HPLC analysis was performed as described previously (19), and the amount of IκK-catalyzed incorporation of 33P into each peptide was quantitated by liquid scintillation counting. Under these conditions, the degree of phosphorylation of GST-IκBα was linear with time and concentration of enzyme.

**RESULTS**

**IKK-2 and IκK-1 Show Random, Sequential Binding Kinetics**—There are several kinetic mechanisms that have been described for two substrate enzyme systems. Using a peptide corresponding to amino acids 26–42 of IκB-α (GST-tagged) as substrate, the assay was allowed to proceed for 10 min before quenching with 2× Laemml sample buffer and heat treatment at 90 °C for 3 min. The samples were then loaded on to 10% Tris-glycine gels (NoveX, San Diego, CA). After completion of SDS-polyacrylamide gel electrophoresis, gels were dried on a slab gel dryer. The bands were then detected using a 445Si PhosphorImager (Molecular Dynamics), and the radioactivity quantified using the ImageQuant software while employing a mean background correction factor for each 33P-labeled IκB-α band. Radioactive standards were run to calculate the absolute (μCi) amount of radioactivity associated with the bands.

**Table I**

| Substrate with IκK | IKK-2 | IκK-1 | mIκK |
|-------------------|-------|-------|------|
| K<sub>ATP</sub><sup>a</sup> | 15.5 ± 4.5 μM | 9.7 ± 1.3 μM | 7.3 ± 3.4 μM<sup>b</sup> |
| K<sub>P,i</sub><sup>a</sup> | 313 ± 137 μM | 1950 ± 278 μM | 140 ± 28 μM<sup>b</sup> |
| K<sub>cat</sub> | 0.195 | 0.18 | 0.11<sup>b</sup> |
| k<sub>cat</sub> | 1.66 ± 0.25 min<sup>-1</sup> | 0.016 ± 0.009 min<sup>-1</sup> |  |

<sup>a</sup> Multisubunit IκB kinase isolated from HeLa S3 cells (19).
<sup>b</sup> See Scheme 1 for definition.
<sup>c</sup> Dissociation constant for N-terminal peptide substrate, see Scheme 1 for definition.
<sup>d</sup> Previously reported (19).

**Fig. 1. Initial velocity patterns for IκK-2 with varying levels of ATP and peptide substrate.** Hanes plot of the rate of IκK-2-catalyzed phosphorylation of a peptide corresponding to amino acids 26–42 of IκB-α at different ATP concentrations. Closed triangles, 7.74 μM peptide; open circles, 24.2 μM peptide; closed circles, 77.4 μM peptide. The solid lines represent nonlinear regression fits to sequential kinetics (21).
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FIG. 2. Initial velocity patterns for IKK-1 with varying levels of ATP and peptide substrate. Hanes plot of the rate of IKK-1-catalyzed phosphorylation of a peptide corresponding to amino acids 26–42 of IκB-α at different ATP concentrations. Closed triangles, 48.4 μM peptide; open circles, 193.6 μM peptide; closed circles, 484 μM peptide. The solid lines represent nonlinear regression fits to sequential kinetics (21).

and presence, respectively, of IκB-α bound to the active site. Using a non-linear regression analysis of the data from Fig. 1 (21), values for $K_{\text{cat}}$, $K_{\text{ATP}}$, and $\alpha$ were obtained and are shown in Table I. A value of $\alpha < 1$ demonstrates that the binding of one substrate increases the affinity for the second substrate (20).

Using recombinant IKK-1 as the enzyme, kinetics consistent with random, sequential binding were also obtained (see Fig. 2). However, the N-terminal peptide substrate bound 6 times less tightly to IKK-1 as compared with IKK-2. Moreover, the $k_{\text{cat}}$ with IKK-1 was 2 orders of magnitude smaller than that measured with IKK-2. Interestingly, the dissociation constants measured with IKK-2 agree well with the values measured previously with the multisubunit complex (nsIKK) isolated from HeLa S3 cells (see Table 1).

**N-terminal Peptides as Active Site Probes**—In order to determine the effect on active site binding and catalysis of amino acids around the region of Ser-32 and Ser-36 of IκB-α, peptides of various length were prepared and tested as substrates for IKK-2. As shown in Table II, the peptide corresponding to amino acids 26–42 of IκB-α bound with a dissociation constant of 23 μM, which corresponds to an apparent binding free energy of $-6.4$ kcal/mol. This apparent binding free energy may underestimate the intrinsic binding free energy since some of the binding energy may be utilized to accelerate catalysis (22). However, the apparent binding free energies calculated in Table II do provide for a reasonable comparison between peptides since the turnover number ($k_{\text{cat}}$) is relatively constant between peptides. Accordingly, because a peptide corresponding to amino acids 21–42 also bound with an apparent binding free energy of $-6.4$ kcal/mol, it would suggest that amino acids 21–25 contributes a negligible amount of binding interactions. Amino acids 43–47 provide for a small amount of additional binding interactions ($-0.9$ kcal/mol) as determined by peptide 26–47.

The use of a peptide corresponding to amino acids 26–39 demonstrated that nearly all of the binding free energy ($-6.2$ kcal/mol) with the longer peptides is provided by amino acids 26–39, with little contribution from amino acids 40–42. Indeed, the use of a peptide corresponding to amino acids 31–37 demonstrated that this region provided over two-thirds of the binding free energy of the longer peptides.

Peptides corresponding to amino acids 26–42 were synthesized with either Ser-32 or Ser-36 mutated to aspartate in an effort to probe the relative contributions of serines 32 and 36 to substrate binding interactions. As shown in Table II, these peptides bound to the active site with an avidity roughly equivalent to that of the “wild-type” peptide, demonstrating that having at least one of the serines present allowed for comparable binding interactions. Alternatively, the aspartate at either position may mimick phosphorylation at this site and provide for additional binding interactions lost from eliminating the serine. The turnover number ($k_{\text{cat}}$) for either of the mutant peptides was approximately half that of the wild-type peptide, probably owing to there being only one phosphorylation site available.

In order to further probe the contribution of the two serines to active site binding interactions, peptides corresponding to amino acids 26–42 were prepared with both serines replaced with either aspartates (S32D/S36D) or alanines (S32A/S36A). Since these “double mutants” could no longer be used as substrates, their active site affinities were determined by using them as inhibitors of the phosphorylation of the wild-type peptide (amino acids 26–42). As shown in Fig. 3, the S32D/S36D mutant showed dose-dependent inhibition of IKK-2, which gave a linear Dixon plot. By comparing the slope of this correlation to that shown by a C-terminal peptide inhibitor, which has a dissociation constant ($K_{\text{D}}$) of 1.5 μM (see below), the dissociation constant for this double mutant was calculated to be $102 \pm 2$ μM. The S32A/S36A mutant gave similar results (not shown). This corresponds to an apparent binding free energy of $-5.5$ kcal/mol, which indicates that the serines themselves only provide $-0.9$ kcal/mol of binding free energy for the 26–42 peptide ($-6.4$ versus $-5.5$ kcal/mol). This, of course, explains why the S32D or S32A “single mutant” peptides bind well to the enzyme.

Consistent with amino acids 31–37 of IκB-α providing most of the binding interactions of amino acids 21–47 with the enzyme, peptides corresponding to regions around the analogous serines of IκB-β and IκB-ε were also effective substrates as shown in Table III. Indeed, all three of these peptides have a highly conserved phosphorylation site (DSGX.X.S, where X1 is either leucine or isoleucine) corresponding to amino acids 31–36 of IκB-α and bind to the enzyme with roughly equivalent affinity. The three peptides also had dissociation constants roughly equal to each other when using IKK-1 as the enzyme source (results not shown).

**Full-length IκB-α Versus N-terminal Peptide as Substrate for IKK-2**—When comparing the N-terminal peptide to the full-length IκB-α as a substrate for IKK-2, the results shown in Table IV demonstrate that the dissociation constants and $k_{\text{cat}}$ values are similar to those of the 26–42 peptide. The dissociation constant of $2 \pm 0.2$ μM for IκB-α corresponds to an apparent free energy of binding of $-7.9$ kcal/mol. Therefore, the $-4.7$ kcal/mol of binding free energy from amino acids 31–37 (see above) represents most of the binding interactions of the full-length IκB-α with IKK-2. Interestingly, the GST-IκB-α fusion protein showed considerably different kinetic constants. As shown in Table IV, the presence of the GST tag greatly diminished the...
Dixon plot of the initial rate of rylation of a peptide substrate.

624.4

while keeping the concentration of peptide substrate fixed and

26–42) peptide substrate. From a nonlinear regression anal-

bacterially inhibition with respect to an N-terminal (amino acids

C-terminal peptide failed to potentiate the IKK-2- or IKK-1-

catalyzed phosphorylation of the N-terminal peptide. As shown

IKK-2, a random sequential mechanism was unequivocally

demonstrated by the use of these peptides. This is in agreement

with the mechanism determined for the msIKK isolated from

HeLa S3 cells (19). A sequential binding mechanism was also

recently determined by Li and co-workers (16) with recombi-

nant IKK subunits using full-length IκB-α. However, Li et al.

turnover number with a mild effect on the dissociation constant.

C-terminal Peptide as a Substrate and Inhibitor of IKK-

2—We have previously reported that a C-terminal peptide cor-

responding to amino acids 279–303 of IκB-α was able to acti-

vate the msIKK isolated from HeLa S3 cells to phosphorylate an

N-terminal peptide (19). In fact, the C-terminal peptide itself was a reasonable substrate for the msIKK.

When using the human, recombinant IKK-2 or IKK-1 as the

enzyme source, however, phosphorylation of the C-terminal peptide was not observed (results not shown). Moreover, the

C-terminal peptide failed to potentiate the IKK-2- or IKK-1-
catalyzed phosphorylation of the N-terminal peptide. As shown

in Fig. 4, this C-terminal peptide instead showed pure competi-
tive inhibition with respect to an N-terminal (amino acids

26–42) peptide substrate. From a nonlinear regression analysis

of the data represented in Fig. 4, a $K_{\text{app}}^\text{pp}$ value of 1.5 ± 0.3

$\mu$m was obtained. Thus, the C-terminal peptide binds well to

the active site, but does not act as either a substrate or activa-
tor of the IKK-2.

That the C-terminal peptide showed competitive inhibition

with respect to the N-terminal peptide substrate is expected

from a mechanism detailed in Scheme 2, where the binding of

the inhibitor (i.e., the C-terminal peptide) competes with the

binding of IκB-α, but not ATP. Also consistent with this mech-

anism, the inhibition observed with the C-terminal peptide while keeping the concentration of peptide substrate fixed and

varying the concentration of ATP showed mixed-type (noncom-

petitive) inhibition as shown in Fig. 5. As expected from

Scheme 2, infinitely large concentrations of ATP are unable to
completely overcome the inhibition produced by the C-terminal peptide. However, this analysis does not rule out an ordered

binding mechanism, which would have shown a similar inhibi-
tion pattern if the peptide substrate binds before ATP (23).

Verification of a random sequential binding mechanism comes

from the use of staurosporine as an inhibitor. Staurosporine,

which is known to bind to the ATP binding site of kinases (24,

25), shows competitive inhibition with respect to ATP (Fig. 6)

and mixed-type noncompetitive inhibition with respect to pept-

ide substrate (see Fig. 7). This unequivocally demonstrates a

random sequential binding mechanism since an ordered bind-

ing mechanism would have shown uncompetitive inhibition

with respect to peptide substrate (23).

\[
E \cdot I + \text{ATP} \rightarrow E \cdot I \cdot \text{ATP} \\
E \cdot I \cdot \text{ATP} + K_{\text{ATP}} \rightarrow E \cdot I \cdot \text{ATP} + aK_{\text{ATP}} \\
E \cdot I \cdot \text{ATP} + aK_{\text{ATP}} \rightarrow E + \text{products}
\]

Scheme 2 shows equilibria in a random sequential mechanism

showing an inhibitor (I), which competes with IκB-α (or peptide

substrate) but allows ATP to bind (20). Here, $K_I$ and $\beta K_T$

represent the dissociation constants of the inhibitor in the absence

and presence, respectively, of ATP; and $K_{\text{ATP}}$ and $\beta K_{\text{ATP}}$

represent the dissociation constants of ATP in the absence

and presence, respectively, of inhibitor. The other constants are
defined elsewhere.

**DISCUSSION**

**Random Sequential Kinetics**—The use of peptide substrates

and inhibitors in the present work has provided valuable insights into the interactions between IκB and IKK. In the case of

IKK-2, a random sequential mechanism was unequivocally

demonstrated by the use of these peptides. This is in agreement

with the mechanism determined for the msIKK isolated from

HeLa S3 cells (19). A sequential binding mechanism was also

recently determined by Li and co-workers (16) with recombi-

nant IKK subunits using full-length IκB-α. However, Li et al.


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*Ser-32 and Ser-36 are underlined.

* Apparent dissociation constant for peptide at 10 μM ATP.

* Apparent $V_{\text{max}}$ at 10 μM ATP, 370 ng/ml IKK-2.

* Observed binding free energy obtained from the $K_{\text{app}}$ ($\Delta G_{\text{app}} = RT\ln(K_{\text{app}})$).

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**TABLE II**

 Kinetic and binding constants for peptide substrates of IKK-2

| Peptide sequence | Corresponding amino acids of IκB-α | $K_{\text{app}}$ | $V_{\text{max}}$ | $\Delta G_{\text{app}}$ |
|-----------------|-----------------------------------|---------------|---------------|-----------------|
| LDDRHDGLDSMKDEEY | 26–42 | 23 ± 4 | 4.1 ± 0.3 | −6.4 |
| LDDRHDGLDSMKDEEY | 26–42 (S32D) | 15 ± 7 | 1.2 ± 0.2 | −6.7 |
| LDDRHDGLDSMKDEEY | 26–42 (S36D) | 10 ± 5 | 1.6 ± 0.2 | −6.9 |
| KKKLDDRHDGLDSMKDEEY | 21–42 | 26 ± 6 | 3.0 ± 0.3 | −6.4 |
| LDDRHDGLDSMKDEEYPEMV | 26–47 | 6 ± 3 | 3.4 ± 0.5 | −7.3 |
| LDDRHDGLDSMKD | 26–39 | 33 ± 1 | 2.14 ± 0.02 | −6.2 |
| DGLDSMK | 31–37 | 390 ± 20 | 1.6 ± 0.5 | −4.7 |

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2 Nonlinear fit to noncompetitive inhibition (21) gave a value for $K_o$ of 24.4 ± 25.2 μM, which is not significantly different from zero.
measured an apparent dissociation constant of 0.13 μM for ATP and failed to show a cooperative binding between ATP and IκB-α (i.e., α = 1). This is in contrast to the present work, where values of 0.2 and 2 μM were determined for α and K_{ATP}, respectively. While the basis of this difference is not clear, we have found that the use of NaCl in the kinase assay (Li and co-workers used 100 mM NaCl) quite often failed to give linear reaction rates for more than a few minutes, especially when using the full-length IκB-α as substrate. In contrast, the use of peptide substrates in the present research gave reaction rates that were linear for ≥2 h. It is interesting to note that the K_{Ap, max} values determined here agree well with the value determined with the msIKK (19) and are more in line with the dissociation constants determined with other protein kinases such as mitogen-activated protein kinase (26) and cAMP-dependent protein kinase (27), which have ATP dissociation constants in the 5–10 μM range.

**Differences between IKK-1 and IKK-2 Catalytic Subunits**—

While the kinetics of the IKK-1 subunit are also consistent with a random sequential binding mechanism, this catalytic subunit proved to be a much less effective catalyst for the phosphorylation of IκB-α peptides than the IKK-2 catalytic subunit as others have also noted (16, 41). This resulted from a 6-fold increase in the peptide substrate dissociation constant as well as a 100-fold decrease in the turnover number when compared with IKK-2. This translates into an apparent second order rate constant (k_{cat}/K_{m}) for IKK-1 that is 650 times smaller than with IKK-2.

\[ \text{FIG. 5. Mixed inhibition by the C-terminal peptide with respect to ATP.} \]

Varying concentrations of ATP were used in the assay at a fixed peptide substrate (corresponding to amino acids 26–42 IκB-α) concentration of 90 μM with C-terminal peptide (corresponding to amino acids 279–303 IκB-α) concentrations of 0 (closed circles), 1 (open circles), 3 (closed triangles), and 10 (open triangles) μM. The solid lines represent nonlinear regression fits to competitive inhibition (21).

**FIG. 6. Competitive inhibition by staurosporine with respect to ATP.**

Varying concentrations of ATP were used in the assay at a fixed peptide substrate (corresponding to amino acids 26–42 IκB-α) concentration of 90 μM with staurosporine concentrations of 0 (closed circles), 60 (open circles), 130 (closed triangles), and 280 (open triangles) μM. The solid lines represent nonlinear regression fits to competitive inhibition (21).
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For instance, the translation of a catalytically inactive mutant of IKK-1 resulted in a multisubunit complex that was only slightly less active than with wild-type IKK-1, while the translation of a catalytically inactive mutant of IKK-2 obliterated the activity of the multisubunit complex (13). Moreover, the use of these catalytically inactive, dominant-negative mutants of the two catalytic subunits expressed in cells showed that the IKK-2 played a larger role in the tumor necrosis factor-α-stimulated NF-κB translocation in HeLa cells and lipopolysaccharide-induced NF-κB-dependent transcription in monocyte cells (14, 28). However, the IKK-1 and IKK-2 subunits appear to contribute equally in CD28-dependent and HTLV-1 tax-mediated activation of NF-κB in Jurkat cells (29, 30).

While it is possible that a heterodimer of IKK-1 and IKK-2 may show different characteristics than the homodimers of each catalytic subunit used in the present study, this is unlikely since it has been shown that the two catalytic subunits are not affected by each other when present as heterodimers (16).

Binding Free Energies—Using peptides corresponding to amino acids 21–47 of IκB-α, the present research indicates that most of the binding interactions come from amino acids 31–37, the region encompassing the serines (32 and 36) that are phosphorylated by the IKK (see Table II). This explains the ability of IKK to phosphorylate IκB-β and IκB-ε as well, since these proteins have highly conserved phosphorylation sites as shown in Table III (31).

It has previously been shown that a peptide corresponding to amino acids 279–303 (the “C-terminal peptide”) activates the multisubunit IKK to catalyze the phosphorylation of an N-terminal peptide containing serines 32 and 36 (19). The present research shows that the isolated IKK-1 and IKK-2 was not activated by this C-terminal peptide. This indicates that only the msIKK, and not the catalytic subunits, is able to bind to and be activated by the C terminus of IκB-α. Consistent with this conclusion is the observation that the dissociation constants and $V_{\text{max}}$ for the 26–42 peptide substrate and the full-length IκB-α are similar when using the recombinant IKK-2 as enzyme. This is in contrast to the msIKK, which showed a 10-fold increase in $V_{\text{max}}$ and a 2500-fold decrease in the $K_m$ when comparing the peptide to the full-length length IκB-α (19). This corresponds to a 25,000-fold greater apparent second order rate constant ($k_{\text{cat}}/V_{\text{max}}$) for the full-length IκB-α as compared with the peptide substrate.

Therefore, the effects observed with the msIKK must be due to either C-terminal peptide interactions with other subunits within the msIKK, or that the presence of other regulatory subunits within the complex affects the conformation of the catalytic subunits so that activation by the C-terminal peptide can occur. Indeed, two other subunits of the msIKK, termed IKAP and IKK-γ, have recently been identified, and the IKK-γ in particular has been shown to interact with and regulate the activity of the catalytic subunits (32, 33). Interestingly, the IKK-γ appears to interact preferentially with IKK-2. We are currently investigating how IKK-γ affects the kinetics of IKK-2-catalyzed phosphorylation of IκB.

The conclusion that the C terminus of IκB-α activates the msIKK is also consistent with reports that the presence and phosphorylation state of the C terminus of IκB-α play a role in the signal-induced degradation of IκB-α (34–36). An analogous C-terminal region rich in proline, glutamate, aspartate, serine, and threonine residues (termed the PEST domain) has also been identified in IκB-β (37, 38). Interestingly, a recent report has indicated that recombinantly transferring the C terminus (along with the N terminus) of IκB-α to an unrelated protein such as GST enables GST to be phosphorylated and degraded in a signal-responsive way (39), although this may result from a recognition of the C terminus by the proteasome (40). Transferring only the N terminus of IκB-α to GST did not result in a recombinant protein that underwent signal-induced proteolysis.

Also related to the conclusion that regions of IκB-α outside of amino acids 21–47 interact with the msIKK to affect catalysis, it has been recently reported that IκB-α complexed with NF-κB is a more efficient substrate (lower $K_m$ and greater $V_{\text{max}}$) than IκB-α alone (41). This may result from a conformational change in IκB-α, which may further maximize binding interactions that are utilized for rate acceleration. It should be noted, however, that the GST fusion protein of IκB-α was used in those studies. The present research indicates that the GST tag reduced the effectiveness of the IκB-α as a substrate. Since untagged IκB-α is a better substrate (lower $K_m$ and greater $V_{\text{max}}$), the effects with the GST-tagged protein as substrate when complexed with NF-κB may simply reflect a change in the conformation of GST-IκB-α that removes the detrimental effects of the the GST tag. We are currently investigating these possibilities.

In summary, the present work demonstrates that both the IKK-2 and IKK-1 catalytic subunits follow a random, sequential binding mechanism as was observed with the msIKK. The much greater catalytic efficiency observed with IKK-2 as compared with IKK-1 suggests that the IKK-2 subunit contributes nearly all of the catalytic activity of the msIKK complex with the IKK-1 subunit providing little contribution to catalysis. In addition, amino acids 31–37 of IκB-α, which contains the consenssus phosphorylation site, appear to contribute more of the binding interactions of the full-length IκB-α with the IKK-2. However, areas in the C terminus of IκB-α contribute greatly to binding interactions with the msIKK, which provides for extremely large rate accelerations.

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