Mutation of Position 52 in ERK2 Creates a Nonproductive Binding Mode for Adenosine 5′-Triphosphate[†,‡]

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ABSTRACT: Among the protein kinases, an absolutely conserved lysine in subdomain II is required for high catalytic activity. This lysine is known to interact with the substrate ATP, but otherwise its role is not well understood. We have used biochemical and structural methods to investigate the function of this lysine (K52) in phosphoryl transfer reactions catalyzed by the MAP kinase ERK2. The kinetic properties of activated wild-type ERK2 and K52 mutants were examined using the oncoprotein TAL2, myelin basic protein, and a designed synthetic peptide as substrates. The catalytic activities of K52R and K52A ERK2 were lower than that of wild-type ERK2, primarily as a consequence of reductions in k_{cat}. Further, there was little difference in K_m for ATP, but the $K_{m,app}$ for peptide substrate was higher for the K52 mutants. The three-dimensional structure of unphosphorylated K52R ERK2 in the absence and presence of bound ATP was determined and compared with the structure of unphosphorylated wild-type ERK2. ATP adopted a well-defined but distinct binding mode in K52R ERK2 compared to the binding mode in the wild-type enzyme. The structural and kinetic data show that mutation of K52 created a nonproductive binding mode for ATP and suggest that K52 is essential for orienting ATP for catalysis.

A lysine in subdomain II is absolutely conserved among the protein kinase family members (Hanks *et al.*, 1988). This lysine is required for efficient phosphoryl transfer reactions (Kamps & Sefton, 1986; Gibbs & Zoller, 1991). An examination of the structures of ATP-bound cyclic AMP-dependent protein kinase (cAPK),[1] cyclin-dependent kinase 2 (CDK2), and extracellular signal-regulated protein kinase 2 (ERK2) indicates that this lysine in subdomain II is positioned neither to act as the catalytic base nor to stabilize the γ-phosphoryl group in the transition state. Nevertheless, covalent modification and mutational analysis show that this lysine residue is essential for catalytic activity (Zoller & Taylor, 1979; Zoller *et al.*, 1981; Kamps & Sefton, 1986; Buechler & Taylor, 1989; Gibbs & Zoller, 1991).

Mutation of the conserved lysine in several protein kinases, notably cAPK and v-Src, resulted in residual protein kinase activity estimated to be less than 0.1% of the wild-type enzymes (Snyder *et al.*, 1985; Kamps & Sefton, 1986; Gibbs & Zoller, 1991). In the case of the MAP kinase ERK2, however, mutation of the subdomain II lysine, K52, to arginine creates a dominant interfering kinase in intact cells (Sontag *et al.*, 1993; Frost *et al.*, 1994), although the mutant retains approximately 5% of wild-type activity (Posada & Cooper, 1992; Robbins *et al.*, 1993). Surprisingly, introduction of the same mutation in the closely related kinase ERK1 yields a protein without detectable activity (Robbins *et al.*, 1993). The low but measurable kinase activity of K52R ERK2 makes ERK2 a good subject for examining the impact of this lysine on enzyme kinetics; in contrast to "kinase-dead" mutants of other protein kinases, quantitative information can be obtained with K52R ERK2. To examine the generality of data obtained with K52R ERK2, K52 was also mutated to alanine and the properties of this mutant were characterized. We report here biochemical data on K52R and K52A ERK2 and structural analysis of K52R ERK2 complexed with ATP.

MATERIALS AND METHODS

Purification of Wild-Type, K52R, and K52A ERK2. K52 was mutated to alanine using the Stratagene Chameleon double-stranded mutagenesis kit. The presence of the mutation was confirmed by DNA sequencing. K52R ERK2 was made previously (Robbins *et al.*, 1993). Histidine-tagged ERK2 proteins were expressed and purified from *Escherichia coli* as previously described (Robbins *et al.*, 1993). All proteins were stored at −80 °C.

Purification of Constitutively Active MEK1 (MEK1R4F). Constitutively active MEK1 in the pRSET expression vector was the generous gift of Dr. Natalie Ahn (Mansour *et al.*, 1993). It was expressed in the BL21-DE3 strain of *E. coli* from that vector or a modified pET16b vector encoding additional histidine residues to improve binding of the

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[1] Abbreviations: ERK2, extracellular signal-regulated protein kinase 2; cAPK, cyclic AMP-dependent protein kinase; CDK2, cyclin-dependent protein kinase 2; MEK, MAP/ERK kinase; MBP, myelin basic protein; GST, glutathione *S*-transferase; MEK1R4F, a constitutively active form of MEK1; ERKtide, peptide substrate for ERK1/2 with sequence ATGPLSPGPFGRR.

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histidine-tagged protein to the nickel resin. The bacteria were grown at room temperature in terrific broth in the presence of 0.02 mg/mL chloramphenicol and 0.1 mg/mL ampicillin until the OD_{600} was approximately 0.4−0.6. Cells were treated with isopropyl β-D-thiogalactopyranoside (0.05 mM) overnight at room temperature prior to harvest by centrifugation, washed in sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), and pelleted. The pellets were frozen in liquid nitrogen and thawed on ice. Thirty-five milliliters of sonication buffer with protease inhibitors was added per liter of original bacterial culture. Cells were lysed using a Stanstead cell disrupter, and the lysate was clarified at 30000 rpm for 30 min in a Beckman Ti35 rotor. The supernatant was adjusted to 1 M NaCl, 1% Tween 20, and 20% glycerol, then mixed with 0.5 mL of nickel NTA−agarose resin (Qiagen), and rocked at 4 °C for 2 h. The resin was washed with 10 mL of 50 mM sodium phosphate, pH 8.0, 10% glycerol, and 0.25% Tween 20 and then with 10 mL of 20 mM imidazole in sonication buffer. MEK1R4F was eluted with a 40 mL gradient of 20−250 mM imidazole, pH 7.0, in sonication buffer. Fractions with activity and immunoreactivity were pooled and dialyzed overnight against purification buffer (20 mM Tris, pH 7.5, 1 mM DTT, 1 mM EGTA, 10 mM benzamidine, and 10% glycerol). The protein was then applied to a MonoQ HR 5/5 column (Pharmacia) equilibrated in purification buffer and eluted with a 0−0.4 M gradient of NaCl in purification buffer. No impurities were detected in the constitutively active, MEK1R4F preparation by staining with Coomassie blue. Wild-type MEK1 phosphorylated with MEKK1 as described (Xu *et al.*, 1995) was generously provided by Andrei Khokhlatchev.

Activation of ERK2 Proteins with MEK1R4F or Phosphorylated MEK1. Phosphorylation reactions contained 20 mM Tris, pH 8.0, 1 mM DTT, 1 mM benzamidine, 10 mM $MgCl_2$, 100 μM ATP, 2−30 μg/mL ERK2 protein, and 0.2 unit of MEK1R4F or MEK1 and were incubated for 1.5 h at 30 °C (Mansour *et al.*, 1994; Xu *et al.*, 1995). After this time there was no further activation of ERK2. Reactions were then placed on ice until use in substrate assays. The stoichiometries of phosphorylation, ∼0.6−0.8 mol of phosphate/mol of ERK2, were equivalent for ERK2 and mutants.

Kinetic Analysis of ERK2 and Mutants. A synthetic peptide (ATGPLSPGPFGRR, ERKtide) was designed on the basis of the ERK1 substrate sequence preference from a peptide library screen (Z. Songyang and L. C. Cantley, submitted) and residues surrounding the ERK phosphorylation site in tyrosine hydroxylase (Haycock *et al.*, 1992). ERKtide was dissolved in water. Human GST−TAL2 (53−108) (Xia *et al.*, 1994) was purified using the method of Smith and Johnson (1988). Myelin basic protein (MBP) (0.6−30 μM) (Sigma), TAL2 (0.625−25 μM), or ERKtide (0.1−5 mM) was added to reactions containing 20 mM Tris, pH 8.0, 1 mM DTT, 1 mM benzamidine, 10 mM $MgCl_2$, 200 μM ATP ([γ-^{32}P]ATP, 25 μCi/reaction), and 0.2−3.0 μg/mL ERK2 protein that was freshly activated by phosphorylation with MEK1R4F or phosphorylated MEK1 as described above. No phosphorylation of substrate occurred in the presence of MEK1 or MEK1R4F alone (data not shown). To determine kinetic parameters for ATP, assays were performed as above except with 1 μM to 1 mM ATP ([γ-^{32}P]ATP, 20 μCi/reaction) andeither MBP or TAL2 at a concentration of 0.5 mg/mL or ERKtide at 2 mM. The concentration of ATP was determined by optical density. Reactions were incubated for 30 min (MBP), 1 h (TAL2), or 10 min (ERKtide) as 30 °C and stopped by the addition of concentrated sample buffer. Reactions with MBP or TAL2 were analyzed by electrophoresis, and bands corresponding to MBP or TAL2 were excised and counted by liquid scintillation.

Assays containing ERKtide were analyzed using P81 paper. ERK2 and mutants were activated without [γ-^{32}P]-ATP to eliminate counts on the P81 paper due to ERK2. Cpm bound to P81 paper in assays from which ERKtide was omitted were subtracted as background. Reactions were stopped by spotting on P81 paper. Papers were washed in dilute phosphoric acid, dried, and counted by liquid scintillation (Witt & Roskoski, 1980; Roskoski, 1983).

Apparent K_m and V_{max} values were derived by fitting the data into the Michaelis−Menten equation with the aid of Enzfitter (Elsevier-Biosoft) and k_{cat} (Biometallics, Inc.) kinetic analysis software.

Determination of the Crystal Structure of K52R ERK2. Crystals of K52R ERK2 were grown as previously described (Zhang *et al.*, 1993) by the vapor diffusion method, equilibrating a 6 mg/mL solution of the protein against 18% PEG 8000 and 0.2 M $(NH_4)_2SO_4$. The complex with ATP was prepared by soaking protein crystals in a stabilizing solution that contained 2 mM $MgCl_2$, 10 mM ATP, and 50 mM Tris, pH 7.0, for 4 h. The crystals of the mutant protein were isomorphous with those of wild-type ERK2.

Data were collected to 2 Å at 20 °C on an RAXIS (Rigaku) image plate detector using mirror monochromated Cu Kα X-rays generated by a Rigaku RU200 rotating anode (50 kV, 100 mA). The collected data were reduced using DENZ0 and SCALEPACK (Minor, 1993; Otwinoskwi, 1993). All crystallographic refinement was done in X-PLOR (Brünger, 1995), and model building was done using XtalView (McRee, 1994), with the exception of the final structure. As a test of the quality of the final structure, a simulated annealing omit map (SA-omit; Hodel *et al.*, 1992) was calculated in X-PLOR; the mutant side chain and Mg·ATP were removed from the calculation. The map and model were then visualized in "O" (Jones *et al.*, 1991) (see Figure 2A).

RESULTS

Kinetic Analysis of Wild-Type, K52R, and K52A ERK2. ERK2 phosphorylates a variety of substrates including MBP, tyrosine hydroxylase, c-Elk, c-Myc, c-Jun, and phospholipase A_2 (Erickson *et al.*, 1990; Alvarez *et al.*, 1991; Gille *et al.*, 1992; Haycock *et al.*, 1992; Lin *et al.*, 1993; Nemenoff *et al.*, 1993). It was necessary to identify substrates that contained a single phosphorylation site and were available in quantity. MBP was phosphorylated not only on T97, the published site (Erickson *et al.*, 1990), but also on T94 at 1 mM ATP (data not shown); thus it was useful only for qualitative comparisons. The TAL1 and TAL2 oncoproteins were tested because they each contain a single ERK phosphorylation site (Cheng *et al.*, 1993; Xia *et al.*, 1994). Of several TAL1 and TAL2 fragments tested, a GST−fusion protein, containing the 56 carboxy-terminal residues of human TAL2, was the best ERK2 substrate, although it was phosphorylated at only about 5% of the rate of MBP (Table

Table 1: Kinetic Constants of Wild-Type and Mutant ERK2s[a]

	wild-type ERK2	K52R ERK2	K52A ERK2
TAL2[b]			
$K_{m,app}$[ATP] (μM)	220 ± 60	550 ± 130	N/D[c]
$k_{cat,app}$ (min^{-1})	1.4 ± 0.15	>0.009 ± 0.001	N/D[c]
$K_{m,app}$[TAL2] (μM)	>40	N/D[c]	N/D[c]
$k_{cat,app}/K_{m,app}$ ($min^{-1}/\mu M$)[d]	0.31[e]	0.0016[e]	N/D[c]
ERKtide[f]			
$K_{m,app}$[ATP] (μM)	350 ± 60	400 ± 80	380 ± 160
$k_{cat,app}$ (min^{-1})	120 ± 8	>6.0	>1.0
K_m[peptide] (μM)	450 ± 230	>5000[g]	>5000[h]
$k_{cat,app}/K_{m,app}$ ($min^{-1}/\mu M$)[d]	0.27	0.0004[e]	N/D[c]
MBP[i]			
$K_{m,app}$[ATP] (μM)	190 ± 97	270 ± 30	300 ± 70
$k_{cat,app}$ (min^{-1})	80 ± 19	0.38 ± 0.02	0.88 ± 0.1
K_m[MBP] (μM)	55 ± 25	4 ± 1.5	1.8 ± 0.6
$k_{cat,app}/K_{m,app}$ ($min^{-1}/\mu M$)	1.47	0.09	0.49

[a] K_ms for ATP were measured at ~30 μM MBP, ~25 μM TAL2, and 2 mM ERKtide. K_ms for phosphoryl acceptors were measured at 200 μM ATP. [b] TAL2 data are the result of five to six independent experiments. Only at the higher TAL2 substrate concentrations tested could $K_{m,app}$ data be quantitated with the K52R mutant, and very little activity was detected even at the highest concentration of TAL2 tested with K52A ERK2 (25 μM) (Table 1). In addition, it was not possible to test higher TAL2 concentrations, as the protein solution could not be concentrated any further. [c] N/D = not enough detectable activity or data points available for quantitation. [d] k_{cat}/K_m values represent phosphoryl acceptor, not ATP. [e] These values were determined by calculating the slopes of the lines plotted from the V_0 vs [s] over the entire concentration range tested. [f] ERKtide data are the result of two to four independent experiments. [g] Estimates were based on the fact that, at the highest concentration tested, 5 mM, only slight curve saturation was evident. Actual K_m is likely to be at least 2-fold higher. [h] The K_m must be higher than 5 mM as K52A is only beginning to show activity at this concentration. The actual value may be at least 5-fold higher. [i] MBP data are the result of two to five independent experiments.

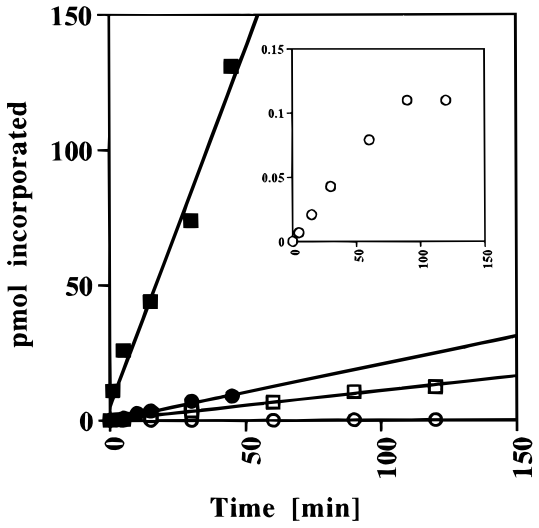

FIGURE 1: Phosphorylation of MBP (squares) and TAL (circles) by activated wild-type ERK2 (closed) or by activated K52R ERK2 (open). The plot shows picomoles of ATP incorporated into the substrate over time. Due to the low amount of phosphorylation of TAL2 by the activated wild type and of both proteins by activated K52R ERK2 shown in this figure, only values for the activated enzymes could be measured for kinetic analyses. Furthermore, in contrast to data with MBP, phosphorylation of TAL2 reached but did not exceed a stoichiometry of 0.4 mol of phosphate/mol of TAL2 (data not shown). Phosphorylation of TAL2 by activated K52R ERK2 is reproduced in the inset to better show these data.

1, Figure 1). A synthetic peptide (ERKtide) was designed on the basis of the work of Songyang *et al.* (1995), who determined an optimum substrate sequence for ERK1 using a peptide library screen (Z. Songyang and L. C. Cantley, submitted) and with certain residue choices based on the substrate tyrosine hydroxylase (Haycock *et al.*, 1992).

Previous experiments with MBP as substrate indicated that the specific activity for wild-type ERK2 was ~20-fold greater than that for the K52R mutant (Robbins *et al.*, 1993). To examine this difference more thoroughly, we determined apparent V_{max} and K_m values for ATP and a peptide substrate for the phosphorylated forms of wild-type ERK2, K52R ERK2, and K52A ERK2 (Table 1). With both GST−TAL2 and ERKtide, the $K_{m,app}$[ATP] of K52R ERK2 was not significantly higher than that of wild-type ERK2. With ERKtide, the $K_{m,app}$[ATP] of K52A ERK2 was also indistinguishable from wild-type ERK2. On the other hand, the k_{cat} with each substrate was much lower for K52R ERK2 and K52A ERK2 than for wild-type ERK2. The k_{cat} for K52R ERK2 was ~0.5−5% and for K52A ERK2 ~1% that of the wild-type enzyme for each peptide/protein substrate. However, the ratio of $k_{cat}/K_{m,app}$ for wild-type, K52R, and K52A ERK2 varied depending on the substrate (see Table 1).

On further analysis, the variability in peptide $k_{cat}/K_{m,app}$ was accounted for at least in part by the finding that the apparent K_m values for the peptide substrate are higher for K52R ERK2 and K52A ERK2 than for wild-type ERK2. The $K_{m,app}$[ERKtide] of wild-type ERK2 was ~0.5 mM. In comparison, the $K_{m,app}$[ERKtide] values of K52R and K52A ERK2 are greater than 5 mM, based on the observation that the rates were not approaching saturation at 5 mM, the highest peptide concentration that could be tested. As MBP is not phosphorylated on a single site, the K_m values for MBP of the K52 mutants and wild-type ERK2 cannot be directly compared. Thus, the kinetic findings suggest that mutagenesis of K52 affects both k_{cat} and K_m for peptide substrates.

Structural Analysis of K52R ERK2. A $F_{K52R} - F_{K52R\text{-}ATP}$ difference map using phases from wild-type ERK2 (Zhang *et al.*, 1994) clearly shows density for an ATP molecule, as well as density suggesting that R52 interacts with bound ATP. A model for the ATP plus a Mg^{2+} was fit into the density and subjected to positional refinement in X-PLOR (see the legend for Figure 2). Figure 2A shows a difference map calculated with phases derived from the final model, omitting Mg·ATP and the R52 side chain from refinement and phase calculations.

As in wild-type ERK2, ATP binds between the two domains of the enzyme (Figure 2B). The ribose takes up a 3′-endo, 2′-exo conformation. Many of the ATP binding interactions are identical to those observed in the wild-type enzyme; the adenine is in van der Walls contact with L154, I82, I29, V37, and M106. The adenine N1 is hydrogen-bonded to the M106, and N6 is hydrogen-bonded to D104. The ribose is oriented by hydrogen bonds of the O2′ and O3′ hydroxyl groups to D109 and K112, respectively, forming a hydrogen-bonding network.

The position of the triphosphate moiety, however, is different in the mutant enzyme as a result of direct interactions with the guanidinium group of R52. R52 is positioned differently from K52 in the wild-type kinase and is extended into the active site cleft (Figure 2B,C). The arginine side chain is hydrogen-bonded via a water molecule to N7 of the adenine ring in its major conformation (see Figure 2B) and is ion paired with the α-phosphate oxygens; large differences

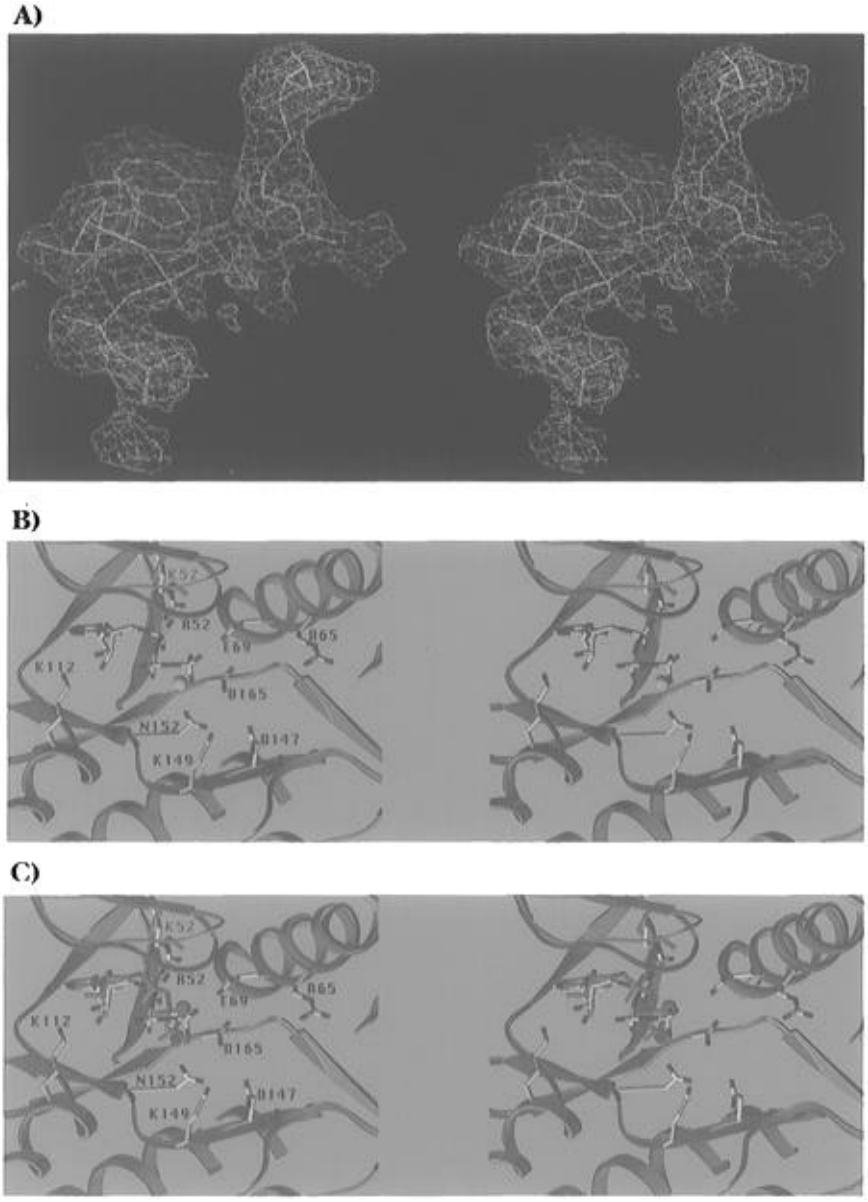

FIGURE 2: A model for Mg·ATP was fit into the density and subjected to positional refinement in X-PLOR. After refinement, the model was verified and rebuilt into a simulated annealing omit map calculated with the ATP, the Mg^{2+}, and the R52 side chain removed (panel A). The blue contours are 1.5σ; the magenta contours are 4σ. Density in the loop region of residues 29−36 suggested a movement of the loop, and the model was rebuilt in this region to fit the density. Subsequent to model verification and rebuilding, a round of simulated annealing (T_{max} = 500 °C), as well as positional and *B*-factor refinements, was carried out, and the final model was verified by $2F_o - F_c$ and difference maps. Panel B: Stereoview of the differences between the wild type ERK2 and the K52R ERK2 phosphate binding loop are shown. K52R is in magenta while wild-type ERK2 is drawn in green. The ATP shown is from the K52R refinement. The magnesium ion is shown in yellow. Panel C: Stereoview of the difference in binding mode for the ATP between K52R ERK2 and wild-type ERK2 is illustrated. [Panels B and C rendered using SETOR (Evans, 1993).]

are observed in the C5′−O5′ bond angle of ATP bound to wild-type (160°) and K52R ERK2 (−90°). The β- and γ-phosphate moieties take up different positions as compared to the wild-type enzyme, such that the γ-phosphate is in the position to be hydrogen-bonded to the ϵ-amino group of K149 and the hydroxyl group of S151. Arginine at position 52 does not form the ion pair with E69, which is observed for K52 in wild-type ERK2 (Figure 2).

Concomitant with the rearrangement of the triphosphate in the K52R mutant, the Mg^{2+} is coordinated differently. In the mutant, Mg^{2+} is coordinated by oxygens from all three of the phosphate groups, the carbonyl oxygen of N152, and the carboxylate group of D165. In the K52R mutant the phosphate binding ribbon (residues 30−35) is better ordered than in the wild type, and Y34 at the apex of the turn makes a hydrogen bond with the guanidinium group of R65.

From these differences in structure, it is clear that the introduction of the mutation K52R severely alters the position assumed by the triphosphate of the substrate ATP, destroys conserved side chain−side chain interactions, and disrupts the coordination sphere of Mg^{2+}. These structural results are consistent with the conclusion that this mutation has created a nonproductive binding mode for ATP. Interestingly, no structural differences between wild-type and K52R ERK2 other than those discussed above were found. It is unlikely, however, that the K52A mutation will introduce the same structural changes in ATP binding because most of the observed structural changes in K52R ERK2 arise from an overlap of the arginine side chain with the ATP binding site.

DISCUSSION

Role of the Conserved Lysine in Subdomain II. ERK2 and the protein kinases share a surprising number of conserved residues, including K52 in subdomain II, D147, K149, and N152 in subdomain Vlb, and D165 in subdomain VII. The functions of D147, which serves as the catalytic base, and D165, which coordinates Mg^{2+}, are relatively certain (Knighton *et al.*, 1991; Cole *et al.*, 1995). The functions of other residues, such as the lysine in subdomain II, have remained obscure. Mutation of the conserved lysine in subdomain II has become a standard method of inactivating protein kinases. For example, Kamps and Sefton found that neither histidine nor arginine could replace this lysine to support the protein kinase activity or transforming potential of Src (Kamps & Sefton, 1986). Comparable mutants of cyclin-dependent kinases, protein kinase C, MAP/ERK kinase, and other protein kinases have also been shown to be nonfunctional *in vivo* (Booher & Beach, 1986; Jove *et al.*, 1987; Ohno *et al.*, 1990; Lange-Carter *et al.*, 1993; Mansour *et al.*, 1994).

Alanine scanning mutagenesis of yeast cAPK demonstrated that mutation of the lysine in subdomain II was one of the most effective of the approximately 65 mutations tested in reducing kinase activity (Gibbs & Zoller, 1991). Examination of the kinetic parameters of crude yeast cell extracts containing the mutated yeast cAPK enzymes showed that mutation of the conserved lysine to alanine resulted in k_{cat}/K_m [ATP] that was ~0.02% of wild type, the lowest activity observed with any of the cAPK alanine-scanning mutants. The decrease in activity was due mostly to changes in k_{cat}, however, suggesting effects on catalysis and not just ATP recognition and binding (Taylor *et al.*, 1993). Our data also lead to the conclusion that mutation of this residue has a negligible effect on K_m for ATP but has a marked effect on k_{cat}, suggesting that interactions between ATP and K52 are important to binding of the transition state structure or facilitating conformational changes needed to promote the phosphoryl transfer reaction. Our results further demonstrate unexpected but significant changes in $K_{m,app}$ for peptide. The mechanism by which mutation of K52 influences peptide substrate binding could be a direct one in which the peptide is blocked by the displaced ATP. Alternatively, K52 may have an indirect role in peptide substrate binding, perhaps by promoting domain rotation that cannot occur in the mutant. The similarity of the effects on peptide substrate K_m caused by mutation of K52 to arginine or alanine is evidence against K52 having a direct role in peptide substrate binding.

It is highly improbable that the lysine side chain either stabilizes the γ-phosphoryl transition state intermediate or acts as the base catalyst. In the crystal structure of the ternary complex of cAPK (cAPK + Mg·ATP + protein kinase inhibitor), it was noted that the residue equivalent to K52 is within 3.5 Å of both the α- and β-phosphates, not the γ-phosphoryl. It is possible that the role of the lysine is to neutralize charges on ATP and to orient the triphosphate for maximum catalytic efficiency. Contacts made by the arginine side chain account at least in part for the differences in the ATP binding mode of K52R ERK2. These contacts would not be expected with alanine in the K52A mutant because of the smaller size of the alanine side chain. The structural data suggest that there might be differences in affinity for ATP among ERK2, K52R ERK2, and K52A ERK2. Interestingly, however, the K_m values for ATP of ERK2 and both K52 mutants are indistinguishable. Because of the differences in interactions of ATP with these different ERK2 molecules discerned and predicted from the structures, perhaps $K_m \neq K_s$. This is consistent with results of Adams and Taylor (1992), who reported that the rate-limiting step in cAPK was release of product ADP.

Structurally in K52R ERK2, the ATP is well ordered in the binding cleft. Nevertheless, the combination of structural changes induced by the arginine side chain, most noticeably the altered triphosphate conformation and concomitant change in Mg^{2+} coordination, leads to a nonproductive, bound complex in which ATP cannot assume the conformation required for phosphoryl transfer. However, while the observed structural changes may have been caused by steric problems from accommodation of the larger arginine side chain in K52R ERK2, the similar kinetic profile of K52A ERK2 suggests that substitution with this small uncharged side chain also may result in a nonproductive ATP binding mode. Although the ATP position and conformation in the low activity (unphosphorylated) structure is most likely different from that in the high activity (doubly phosphorylated form), a similar deviation in the ATP binding mode of the mutants as compared to wild-type ERK2 is expected to occur in both the low activity and high activity enzyme structures.

We have shown that the mutation of the conserved lysine in subdomain II creates a nonproductive binding mode for ATP, thus drastically lowering the activity of the enzyme. Interestingly, disruption of the ATP binding mode is a mechanism of regulation of protein kinases. In CDK2, it was found that the low activity conformation of that molecule has a nonproductive binding mode for ATP (DeBondt *et al.*, 1993); the position of the residue equivalent to K52 is also affected. Similarly, in the insulin receptor and twitchin kinase the low activity forms have a blocked ATP binding site (Hu *et al.*, 1994; Hubbard *et al.*, 1994). Apparently, it is easy to induce structural changes that interfere with the ATP binding site and thereby impair protein kinase function. The exquisite sensitivity to the position and conformation of ATP may serve a regulatory role in preventing protein kinases from hydrolyzing ATP in the absence of protein substrates.

We hope to be able to better understand the function of the several conserved catalytic residues in ERK2 and in all protein kinases when the structure of phosphorylated ERK2 is solved.

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