Multiple Activation Loop Conformations and Their Regulatory Properties in the Insulin Receptor’s Kinase Domain*

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Low catalytic efficiency of protein kinases often results from intrasteric inhibition caused by the activation loop blocking the active site. In the insulin receptor’s kinase domain, Asp-1161 and Tyr-1162 in the peptide substrate-like sequence of the unphosphorylated activation loop can interact with four invariant residues in the active site: Lys-1085, Asp-1132, Arg-1136, and Gln-1208. Contributions of these six residues to intrasteric inhibition were tested by mutagenesis, and the unphosphorylated kinase domains were characterized. The mutations Q1208S, K1085N, and Y1162F each relieved intrasteric inhibition, increasing catalytic efficiency but without changing the rate-limiting step of the reaction. The mutants R1136Q and D1132N were virtually inactive. Steric accessibility of the active site was ranked by relative changes in iodide quenching of intrinsic fluorescence, and A-loop conformation was ranked by limited tryptic cleavage. Together these ranked the openness of the active site cleft as R1136Q > D1132N > D1161A > Y1162F ~ K1085N > Q1208S ~ wild-type. These findings demonstrate the importance of specific invariant residues for intrasteric inhibition and show that diverse activation loop conformations can produce similar steady-state kinetic properties. This suggests a broader range of regulatory properties for the activation loop than expected from a simple off-versus-on switch for kinase activation.

Intrasteric inhibition is a regulatory feature used by many protein kinases to suppress catalytic activity (1–4). This form of inhibition is achieved when a polypeptide segment of the kinase blocks or distorts the active site, thereby preventing binding or proper orientation of one or both substrates. One frequently observed structural feature of intrasteric inhibition is a “pseudosubstrate” amino acid sequence that has the consensus recognition sequence of a true peptide substrate but does not always have the phosphoryl acceptor hydroxy-amino acid. A pseudosubstrate sequence is not a universal feature of intrasteric inhibition, as in the case of calcium/calmodulin-depen dent protein kinase I in which distortion, more than occlusion, of the active site occurs (5).

Catalytic activity of the insulin receptor’s kinase domain (IRKD)1,2 is suppressed through intrasteric inhibition by an activation loop (A-loop), which has a pseudosubstrate sequence between Asp-1161 and Lys-1165. The intrasterically inhibitory “gate-closed” conformation identified in a crystal structure of the unphosphorylated kinase domain (7) blocks both ATP and peptide substrate binding sites, as shown schematically in Fig. 1 (see below). The side chains of Phe-1151 to Met-1153 that block the adenine binding pocket probably do not contribute to stability of the gate-closed conformation, as suggested by high B values for the Phe-1151 ring and the undefined side chain of Met-1153. In contrast, binding of the pseudosubstrate sequence appears to be stable, as inferred from the lower B values of the pseudosubstrate side chains and the potential to form numerous hydrogen bonds (7). Taken together these observations suggest that intrasteric inhibition at both ATP and peptide binding sites would be determined by interactions at the peptide binding site.

Among residues shown in Fig. 1 that are potentially involved in stabilizing pseudosubstrate binding, six are invariant within the insulin receptor family.3 Three of the six hydrogen bonding residues are invariant in the tyrosine kinase superfamily (Asp-1132 and Arg-1136 in the catalytic loop, and the presence of one tyrosyl residue, either Tyr-1162 or Tyr-1163, in the A-loop), and three are variable in the tyrosine kinase superfamily (Lys-1085, Asp-1161, and Gln-1208; see Table I below). The latter residues are engaged in a triad of interactions in the basal state of the IR (see Fig. 1), which may be absent in other tyrosine kinases due to amino acid differences in at least one of these residues (see Table I, boldface letters). Importantly, the homologous residues of closely related tyrosine kinases such as the fibroblast growth factor receptor and Src family kinases do not appear to interact with each other in crystal structures in which the A-loops are unphosphorylated, and these A-loops do not show pseudosubstrate binding nor do they block the ATP binding site (8–12). Therefore, the residues participating in these interactions in the insulin receptor’s kinase domain may be necessary for maintenance of the gate-closed conformation and intrasteric inhibition.

To evaluate contributions of each residue to the gate-closed conformation, we made a set of single amino acid replacements

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1 The abbreviations used are: IRKD, insulin receptor’s kinase domain; Ac, acetate; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; IR, insulin receptor; IRS939, a synthetic peptide substrate for the IRKD; OE-PCR, overlap extension-polymerase chain reaction; A-loop, activation loop, WT, wild-type; ERK2, extracellular signal-regulated kinase 2.

2 The numbering of the IR and IRKD is from Ebina et al. (6).

3 R. A. Kohanski, unpublished.
Fig. 1. Schematic drawing of intrasteric inhibition at the insulin receptor kinase domain’s active site. The activation loop is shown as a solid line, the catalytic core is indicated by the dashed line, the ATP and peptide binding sites are shaded, amino acid side chains are shown as short lines ending in filled circles, and hydrogen bonds are indicated by heavy dotted lines. The A-loop backbone from Phe-1151 to Met-1153 blocks the ATP binding site, and the phenyl ring of Phe-1151 sits in the cavity that would bind the adenine ring of ATP. Residues Asp-1161 through Lys-1165 are bound at the peptide binding site, and the side chains fill subsites of the peptide substrate:\(^4\). Asp-1161 at the \(P_0\) site, Tyr-1162 at \(P_1\) Tyr-1162 at \(P_\text{number 5}\), and Lys-1165 at \(P_\text{number 6}\) (not shown). Hydrogen bonds between two of these A-loop residues and four residues from the catalytic core are indicated by the dashed lines, numbered 1 to 5. There are hydrogen bonds between the side chains of Gln-1208 and Lys-1085 (number 6) and between Asp-1132 and Arg-1136 (number 7). These features of intrasteric inhibition were described originally by Hubbard (7) in a crystal structure of the unphosphorylated wild-type kinase domain of the human insulin receptor.

Fig. 1. Schematic drawing of intrasteric inhibition at the insulin receptor kinase domain’s active site. The activation loop is shown as a solid line, the catalytic core is indicated by the dashed line, the ATP and peptide binding sites are shaded, amino acid side chains are shown as short lines ending in filled circles, and hydrogen bonds are indicated by heavy dotted lines. The A-loop backbone from Phe-1151 to Met-1153 blocks the ATP binding site, and the phenyl ring of Phe-1151 sits in the cavity that would bind the adenine ring of ATP. Residues Asp-1161 through Lys-1165 are bound at the peptide binding site, and the side chains fill subsites of the peptide substrate:\(^4\). Asp-1161 at the \(P_0\) site, Tyr-1162 at \(P_1\) Tyr-1162 at \(P_\text{number 5}\), and Lys-1165 at \(P_\text{number 6}\) (not shown). Hydrogen bonds between two of these A-loop residues and four residues from the catalytic core are indicated by the dashed lines, numbered 1 to 5. There are hydrogen bonds between the side chains of Gln-1208 and Lys-1085 (number 6) and between Asp-1132 and Arg-1136 (number 7). These features of intrasteric inhibition were described originally by Hubbard (7) in a crystal structure of the unphosphorylated wild-type kinase domain of the human insulin receptor.

to disrupt the hydrogen bonds shown in Fig. 1. A D1161A mutation has been reported, showing that loss of three interactions (1, 2, and 3, Fig. 1) produced an unphosphorylated kinase with increased catalytic efficiency (13, 14). The structure of this mutant IRKD displayed a “gate-open” conformation in which the ATP binding site was freed and pseudosubstrate binding of the A-loop was not observed; most residues in the A-loop were disordered. With this background information and the other mutants in hand, we tested the following three hypotheses: First, if each residue making up the bonding network in Fig. 1 is required to maintain intrasteric inhibition, then mutation of any one residue should yield a gate-open conformation and loss of intrasteric inhibition. Second, if intrasteric inhibition at the ATP binding site depends on interactions at the peptide binding site, then mutating residues at the peptide binding site will lower \(K_m,\text{ATP}\). Third, if a specific function of A-loop autophosphorylation is to lower the \(K_m,\text{peptide}\), then a gate-open conformation in these unphosphorylated kinase mutants will not improve \(K_m,\text{peptide}\). The steady-state kinetic results presented here suggest intrasteric inhibition of the insulin receptor requires each of these invariant residues. However, physical and biochemical evidence indicate that a range of A-loop conformations accompanies the changes in kinetic parameters.

### EXPERIMENTAL PROCEDURES

**Materials**—Dithiothreitol (Sigma Ultra grade), the disodium salt of ATP (from equine muscle, catalog number A-5394), ADP, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.; hydrogenated Triton X-100 (protein grade) was from Calbiochem; EDTA was from Fluka; Tris acetate, Tris base, Tris-Cl, the ATP analogs adenosine 5’-β,γ-imido triphosphate (AMP-PNP, tetrathylthium salt), and electrophoresis reagents were from Roche Molecular Biochemicals; magnesium acetate (MgAc\(_2\), Enzyme Grade) was from Fisher. Insect cell culture media and fetal bovine serum were from Life Technologies, Inc.

**Synthetic Peptides and Nucleotides**—The synthetic peptide IRS939 with a single phosphorylation site was prepared as the carboxyl-termin

### Table I

<table>
<thead>
<tr>
<th>RTK</th>
<th>1161</th>
<th>1085</th>
<th>1208</th>
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<tr>
<td>IR/IGF1R/IRK(^a)</td>
<td>D</td>
<td>K</td>
<td>Q</td>
</tr>
<tr>
<td>FGF-R</td>
<td>D</td>
<td>R</td>
<td>S</td>
</tr>
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<td>D</td>
<td>N</td>
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</tr>
<tr>
<td>LTK</td>
<td>S</td>
<td>K</td>
<td>M</td>
</tr>
<tr>
<td>7LESS</td>
<td>D</td>
<td>L</td>
<td>Q</td>
</tr>
<tr>
<td>AX1</td>
<td>D</td>
<td>H</td>
<td>P</td>
</tr>
<tr>
<td>MET</td>
<td>E</td>
<td>R</td>
<td>P</td>
</tr>
</tbody>
</table>

\(^a\) HIR, human insulin receptor.

\(^b\) IR, insulin receptor; IGF-1R, insulin-like growth factor-1 receptor; IRK, insulin receptor-related factor; FGF-R, fibroblast growth factor receptor; TRK, neurotrophin receptor; LTK, leukaocyte tyrosine kinase; 7LESS, sevenless receptor of *Drosophila melanogaster*; AX1, growth arrest-specific gene 6 receptor; MET, hepatocyte growth factor receptor.
Biotech) rather than a Waters DEAE 5R matrix. Other steps in the purification scheme were essentially the same as for the 48-kDa kinases.

**Iodide Quenching of Fluorescence—**Steady-state and time-resolved fluorescence measurements were made as described by Bishop et al. (23). Briefly, the emission spectra were obtained with an SLM 4800 spectrophotometer operating in the single-photon counting mode. For intrinsic tryptophan fluorescence spectra, an excitation wavelength of 300 nm was used and emission spectra were collected over the range of 310–420 nm in 1-nm increments. The final spectra were determined from the average of three spectral scans. Iodide quenching was done in the absence or presence of ADP as described by Ablooglu and Kohanski (15). The IRKD was 1 μM, and quenching was done with 0.0–0.6 mM KI in 50 mM TrisAc, 20 mM MgAc2, and 0.1 mM sodium thioulate, at pH 7.0; the potassium salt concentration was kept constant at 0.6 M by the addition of KCl, as needed. For each IRKD, the bimolecular collisional rate constant kcat was determined as described previously (15, 23).

**Limited Tryptic Cleavage—**Partial proteolysis of the IRKD was done according to Frankel et al. (21). Briefly, the IRKD was digested at 5 μl in cleavage buffer (50 mM TrisAc, 20 mM MgAc2, 1 mM DTT, 2 mM CaCl2, pH 7.0) with or without 10 mM ADP. Tryptsin was added to a ratio of 1:20 by mass, trypsin to IRKD. The reaction proceeded for selected times and was quenched by addition of sample buffer. The cleavage products were resolved by SDS-polyacrylamide gel electrophoresis. Gels were stained with AgNO3 and dried, scanned as grayscale images using an Arcus II scanner (Agfa) with Potolook and Photoshop software (Adobe), and quantified using ImageQuaNT software (Molecular Dynamics, Sunnyvale CA).

**Steady-state Kinetics and Viscosity Dependence of Peptide Substrate Phosphorylation—**Steady-state kinetic parameters for the unphosphorylated IRKD domains were determined as described previously (15). Reactions were performed, in triplicate, at room temperature with 50 μM TrisAc, 5 mM ATP, 10 mM MgAc2, 0.01% hydrogenated Triton X-100, 5 mM DTT, 50 mM TrisAc, pH 7.0, which produced maximum activation of each kinase (not shown). The activated kinase was diluted into peptide phosphorylation reaction mixtures to give a final concentration of 2–4 nM; dephosphorylation does not occur after dilution (i.e. during the assay). Peptide phosphorylation was assayed at 0.025–2 μM IRS939, at fixed 10 mM MgATP.

### RESULTS

**Relief of Intrinsic Inhibition Revealed through Steady-state Kinetics—**Low catalytic efficiency is the functional hallmark of intrasteric inhibition of the unphosphorylated IRKD. It is produced by low kcat and high Km for both substrates (15). The steady-state kinetic parameters for three mutants are summarized in Table II, together with previously published control data for the WT-IRKD and D1161A-IRKD. These results show relief of intrasteric inhibition for Q1208S-, K1085N-, and Y1162F-IRKD, based on increased catalytic efficiencies (kcat/Km). There were 4- to 6-fold increases in kcat and 12- to 22-fold decreases in Km for the Q1208S and, in particular, the K1085N and Y1162F-IRKD mutant compared with the WT-IRKD, but Km,peptide essentially unchanged in each of these mutant IRKDs. These features were observed previously in the D1161A-IRKD mutant (Table II), suggesting that a mutation breaking any subset of hydrogen bonds for pseudosubstrate recognition relieves intrasteric inhibition.

The Michaelis constant is often equated with a dissociation constant, although this does not apply in all kinetic mechanisms. Therefore, to determine if a lower Km,peptide represents an increased affinity for adenine nucleotide, we measured the inhibition constant for AMP-PNP, which is an analog of ATP that does not support phosphoryl transfer. Surprisingly, the Km,AMPPNP indicated slightly stronger nucleotide binding to the Q1208S-IRKD but 5- to 7-fold tighter nucleotide binding for the K1085N- and Y1162F-IRKD mutants. This result subdivides these kinase mutants in two groups, in which intrasteric inhibition is accompanied by (a) tighter nucleotide binding (K1085N-, Y1162F-, and D1161A-IRKD) or (b) occurs with only a modest change in affinity (Q1208S-IRKD).

The residues Lys-1085 and Gln-1208 interact with the P- residue of the peptide substrate, shown in structures of the activated IRKD (24, 25). Mutation of these residues could affect directly the value of Km,peptide. This was tested in the activated (autophosphorylated) forms of the mutant kinases (Table III). The value for Km,peptide for the activated Q1208S-IRKD was equivalent to activated WT-IRKD, but the Km,peptide for activated K1085N-IRKD was 5-fold higher. The data show that Lys-1085 is more important than Gln-1208 for Km,peptide. This result suggests the substitution of Ser for Gln-1208 does not disrupt pseudosubstrate binding of the A-loop as much as substitution of Asn for Lys-1085. The surprising result was the relatively high Km,peptide for autophosphorylated Y1162F-IRKD compared with the other autophosphorylated kinases. This probably resulted from a limiting stoichiometry of 1 mol of phosphate per mol of IRKD after autophosphorylation (not shown), and at least two phosphotyrosines are required for significant activation (26). The autophosphorylation of Q1208S- and K1085N-IRKD was equivalent to WT-IRKD (∼3 mol of phosphate per mol of 36 kDa IRKD; not shown).

The R1136Q- and D1132N-IRKD mutants were effectively relieved of intrasteric inhibition for Q1208S-, K1085N-, and Y1162F-IRKD, based on increased catalytic efficiencies (kcat/Km). There were 4- to 6-fold increases in kcat and 12- to 22-fold decreases in Km,AMPPNP compared with the WT-IRKD, but Km,peptide essentially unchanged in each of these mutant IRKDs. These features were observed previously in the D1161A-IRKD mutant (Table II), suggesting that a mutation breaking any subset of hydrogen bonds for pseudosubstrate recognition relieves intrasteric inhibition.

<table>
<thead>
<tr>
<th>Parametera</th>
<th>Units</th>
<th>WTb</th>
<th>Q1208S</th>
<th>K1085N</th>
<th>Y1162F</th>
<th>D1161Ad</th>
</tr>
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<tbody>
<tr>
<td>kcat</td>
<td>s⁻¹</td>
<td>(none)</td>
<td>(1, 6)</td>
<td>(2, 6)</td>
<td>(4, 5)</td>
<td>(1, 2, 3)</td>
</tr>
<tr>
<td>Km,MgATP</td>
<td>mM</td>
<td>1.0 ± 0.3</td>
<td>6.8 ± 3.8</td>
<td>4.8 ± 0.7</td>
<td>6.4 ± 1.6</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Km,peptide</td>
<td>mM</td>
<td>2.0 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>2.4 ± 0.9</td>
<td>3.7 ± 1.3</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

a Apparent parameters as defined under “Experimental Procedures”elm; b kcat from the determination of Km,peptide, at 10 mM MgATP. The peptide is IRS939.

b H bonds (shown in parentheses) are numbered in Fig. 1.

c From Ablooglu and Kohanski (15).

d From Frankel et al. (13).
inactive. Attempts to measure peptide phosphorylation indicated \(k_{\text{cat}}\) was decreased by a factor of at least 10\(^2\) for each mutant compared with WT-IRKD. The reduced \(k_{\text{cat}}\) for these mutants is similar to values determined for other protein kinases with mutations in the homologous residues (27–30). Because of these low turnover numbers, we made no attempt to determine \(K_m\) or \(k_{\text{cat}}/K_m\) for these two mutants.

**Rate-limiting Steps in the Mutant IRKD**—Previous studies showed that relief of intrasteric inhibition was accompanied by changes in the rate-limiting steps of the catalytic cycle, without (14) or with (15) activation loop phosphorylation. We probed these unphosphorylated mutant IRKDs for similar changes by measuring \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) at increasing solution viscosity. As shown in Fig. 2, neither parameter differed from the unphosphorylated WT-IRKD for any of the three mutants examined here, unlike the D1161A-IRKD. Therefore, the phosphoryl transfer reaction remains limited by a viscosity-insensitive step despite the relief of intrasteric inhibition from steady-state kinetics for the K1085N-, Y1162F-, and Q1208S-IRKD. In the functional patterns emerging from solution properties, these results distinguish D1161A-IRKD from the other IRKD mutants, K1085N-IRKD is grouped with Y1162F-IRKD, and Q1208S-IRKD remains intermediate between WT-IRKD and the other mutants.

**Identification of Gate-Open IRKD Mutants by Iodide Quenching of Intrinsic Fluorescence**—In previous work we had shown that collisional quenching of intrinsic tryptophan fluorescence by iodide is a sensitive indicator of solute exposure at the active site cleft, resulting from A-loop phosphorylation (23), from adenine nucleotide binding (13), or from mutagenesis (14). The observed increase in collisional quenching indicates greater openness of the catalytic cleft, in agreement with the crystal structures (14, 24). Here we test five IRKD mutants for gate-open conformations in the absence or presence of bound adenine nucleotide. The control experiments with unphosphorylated WT-IRKD are presented on each Stern-Volmer plot for comparison.

The A-loop tyrosine mutant Y1162F-IRKD showed iodide quenching of fluorescence in the absence of ADP that was similar to the WT-IRKD-ADP binary complex (Fig. 3A). There was virtually no change in iodide quenching measured in the presence of ADP. These results suggest this Tyr-to-Phe mutant adopts a gate-open conformation. Similarly, iodide quenching of fluorescence in D1132N-IRKD, with or without nucleotide, was similar to the WT-IRKD-ADP binary complex (Fig. 3B). A slightly different result was observed for the R1136Q-IRKD mutant; iodide quenching was significantly greater than WT-IRKD in the absence of ADP but did not appear to be as extensive as the two previously analyzed mutants. Nevertheless, the quenching was not affected significantly when ADP was present, which suggests a gate-open conformation has arisen in this mutant. Iodide quenching of fluorescence in the K1085N-IRKD was greater than the WT-IRKD, and it was increased slightly when ADP was bound (Fig. 3C). This mutant also can be considered gate-open. Within this cohort of mutants, iodide quenching of fluorescence in Q1208S-IRKD in the absence of ADP was most like quenching of the WT-IRKD in the absence of ADP (Fig. 3D). In addition, quenching in the presence of ADP was essentially the same for the Q1208S mutant and the WT-IRKD. These results suggest a mostly gate-closed conformation for Q1208S-IRKD, consistent with the weak affinity for adenine nucleotides described above by \(K_m\) (AMP-PNP) (Table II). Given the difference in iodide quenching due to bound ADP, we were able to titrate the equilibrium binding of adenine nucleotides to Q1208S-IRKD by the method described in Ablouglu and Kohanski (15) (titrations not shown). The dissociation constants were 2.0 ± 0.3 mM AMP-PNP and

### Table III

<table>
<thead>
<tr>
<th>IRKD</th>
<th>(K_m) peptide (mM)</th>
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<tr>
<td>WT</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>Q1208S</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>K1085N</td>
<td>0.27 ± 0.03</td>
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<tr>
<td>Y1162F</td>
<td>0.52 ± 0.09</td>
</tr>
<tr>
<td>D1161A</td>
<td>0.11 ± 0.01</td>
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</table>

\(a\) From Ref. 15.
\(b\) From Ref. 13.

**FIG. 2.** Relative \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) at increased solution viscosity. A, plot of \([k_{\text{cat}}]_{\text{rel}}\) versus \(\eta^{\text{rel}}\). B, plot of \([k_{\text{cat}}/K_m\]_{\text{rel}}\) versus \(\eta^{\text{rel}}\). The superscript “rel” indicates the ratio of the parameter measured with added sucrose (to increase viscosity) to the same parameter measured without added sucrose. Three mutants with catalytic activity were analyzed: K1085N-IRKD (circles), Y1162F-IRKD (triangles), and Q1208S-IRKD (squares). Control values for WT-IRKD (dashed lines) and for D1161A-IRKD (solid lines) are shown (taken from Refs. 23 and 14). Each condition was assayed in triplicate.

**FIG. 3.** Iodide quenching of IRKD mutants in the absence or presence of MgADP. The Stern-Volmer plots for five IRKD mutants, labeled on each panel, show quenching of intrinsic tryptophan fluorescence by increasing concentrations of KI at constant 0.6 mM potassium salts and 20 mM MgAc. Quenching was done at 1 mM IRKD alone (open symbols) or in the presence of 10 mM ADP (closed symbols). Data are averaged from duplicate experiments. A, Y1162F-IRKD (circles); B, D1132N-IRKD (triangles), R1136Q-IRKD (squares); C, K1085N-IRKD (diamonds); D, Q1208S-IRKD (hexagons). The quenching of wild-type IRKD, taken from a previous study (23), is shown for comparison on each panel: dashed line, WT-IRKD alone; solid line, WT-IRKD in the presence of 10 mM ADP.
Activation Loop Conformations

Quantitative changes in IRKD fluorescence parameters from mutations and MgADP binding. Fluorescence parameters were determined in the absence (open bars) or presence (closed bars) of 10 mM ADP; 20 mM MgAc2 was present in all samples. A, the intensity-weighted mean fluorescence lifetime (τ) was determined by time-resolved fluorescence. The average (τ) in the absence of ADP was 6.66 ± 0.55 ns, and in the presence of 10 mM MgADP it was 5.63 ± 0.39 ns (excluding R1136Q-IRKD, for reasons stated under ‘Discussion’). B, the bimolecular collisional rate constants were determined for iodide quenching, based on the relationship \( k_q = K_q k(\tau) \), using values for (τ) from A and the data shown in Fig. 2. Error bars show one standard deviation.

0.35 ± 0.03 mM ADP, corroborating the \( K_q \) measured from kinetics and showing almost 3-fold tighter binding for ADP than in the unphosphorylated WT-IRKD (15).

To make full use of the quenching data from steady-state fluorescence, we used fluorescence lifetimes to calculate bimolecular collisional rate constants for iodide quenching. This provides a good index of changes in solute exposure due to the mutations and/or nucleotide binding, and relative solute exposure is an indication of a gate-closed versus gate-open conformation (23). The intensity-weighted mean fluorescence lifetimes were determined from time-resolved fluorescence decay analysis of each variant in the absence and presence of saturating ADP (Fig. 4A). One pattern is immediately apparent: the lifetime decreases in WT-IRKD and in every mutant IRKD upon ADP binding, except for the R1136Q-IRKD. This could indicate a change in conformation or local electronic environment near the fluorescent tryptophan residues of the IRKD with bound nucleotide. A more subtle pattern can be discerned in which the WT-IRKD and Q1208S have similar decay lifetimes in their unliganded states, with an average value for (τ) of 7.43 ± 0.25 ns, compared with the four gate-open mutants with an average value for (τ) of 6.36 ± 0.18 ns.

The bimolecular collisional rate constants are arranged to suggest a gradient of increasing solute access between the gate-closed WT-IRKD and the gate-open D1132N-IRKD (Fig. 4B; open bars). The Q1208S-IRKD is more similar to the WT-IRKD than to other mutant IRKDs, with the Q1208S-IRKD appearing partially gate-open by this comparison. Quenching increased 9.3-fold upon nucleotide binding to the WT-IRKD and 3.4-fold for the Q1208S-IRKD, with the difference due mostly to the increased \( k_q \) for Q1208S-IRKD in the absence of bound nucleotide. In contrast, the -fold increase in \( k_q \) is only 1.3 ± 0.1 for the gate-open mutants upon ADP binding (except for R1136Q-IRKD, for which the change in \( k_q \) upon nucleotide binding is opposite to all the other IRKDs examined). These data demonstrate a range of IRKD conformations based on solute accessibility and reveal that bound nucleotide does have a further impact on the conformation of the kinase domain, in the gate-closed WT-IRKD and the gate-open mutant proteins.

Limited Proteolysis and Activation Loop Conformation—Trypsin cleaves the A-loop of the WT-IRKD, and the relative rate of cleavage is increased by bound adenine nucleotide (Ref. 21 and Fig. 5, lanes 1–3). Limited proteolysis of the mutant IRKDs, for a fixed time, provides a survey of A-loop conformations in the absence versus the presence of ADP (Fig. 5, lanes 3–18). Compared with WT-IRKD, in which A-loop cleavage was barely detectable, there was greater A-loop cleavage in the absence of ADP in each of the mutants, including the Q1208S mutant, although the difference was small in that case. The extent of cleavage appeared greatly enhanced when ADP was bound to the WT- and Q1208S-IRKDs, modestly increased for the K1085N- and Y1162F-IRKDs, and barely changed for the R1136Q- and D1132N-IRKDs, where cleavage was already extensive in the absence of nucleotide (Fig. 5, summarized in the last panel).

Quantitative analysis of A-loop cleavage over time is shown in the semi-logarithmic plots to better illustrate differences in cleavage rates among IRKDs and the effects of ADP (Fig. 6). The WT-IRKD and Q1208S-IRKD cleave very slowly in the absence of ADP, reaching barely 20% net cleavage by 20 min (Fig. 6A). In the presence of ADP, the WT-IRKD shows two approximately linear phases of cleavage, which suggests two conformational populations exist in the binary complex (each conformation is cleaved at a different rate). Similar results are obtained with the Q1208S-IRKD (Fig. 6A). Extrapolation of the slow phases to zero time indicates about 50–60% of the cleavage occurs via this slow phase; i.e. there is an ~50–50 split between two A-loop conformations in each of these binary complexes.

Cleavage of the A-loop in the K1085N- and Y1162F-IRKDs in the absence of ADP is faster than for the WT-IRKD (Fig. 6B). There is a slight increase in the rate of cleavage in each IRKD when ADP is bound. This finding confirms a conformational change in these binary complexes compared with IRKDs without bound nucleotide, as suggested by the fluorescence data. There appears to be one predominant A-loop conformation under each condition for both mutants, although slight curvature in these plots suggests a lower abundance second conformation could be present. The A-loop conformations in the K1085N- and Y1162F-IRKD are quite similar to each other, with or without nucleotide, and both differ from conformations apparent in the WT- and Q1208S-IRKDs.

Limited proteolysis of the A-loop in the R1136Q- and D1132N-IRKD mutants is very fast and affected only slightly by the presence of ADP (Fig. 6C). The single phase of cleavage appears to report a single A-loop conformation for each mutant in the absence or presence of ADP (Fig. 6C). Therefore, taking the iodide quenching and limited proteolysis data together, it appears that both of the catalytically inactive IRKD mutants have gate-open conformations in the absence of bound nucleotide, and the A-loop conformation is different in the presence versus the absence of nucleotide.

DISCUSSION

Intrastere inhibition of protein kinases has two components. The structural component is a polypeptide segment of the kinase that blocks or distorts the active site. The functional component is a low catalytic efficiency that will increase when the inhibitory segment moves away from the active site. A pseudosubstrate sequence of the insulin receptor’s unphospho-
The phosphorylatable activation loop is the intrasteric inhibitor of its kinase activity (7), shown schematically in Fig. 1. This gate-closed conformation produces low catalytic efficiency against both substrates, as defined by $k_{cat}/K_m$, MgATP and $k_{cat}/K_m$, peptide (15).

In this report, we studied the relationship between the structural and functional components of intrasteric inhibition using point mutations in the IRKD. Conclusions were reached for the three hypotheses stated in the introduction: First, each residue making up the hydrogen bond network in Fig. 1 is required to maintain intrasteric inhibition, based on the increased catalytic efficiency from each mutant. However, the structural component can be retained while the kinetic component of intrasteric inhibition using point mutations in the IRKD that has increased catalytic efficiency. Second, intrasteric inhibition at the ATP binding site depends on interactions at the peptide binding site. This is supported by the change in $K_m$, MgATP produced by mutations affecting substrate/pseudosubstrate recognition and the correlation between $K_m$, AMP-PNP and gate-closed versus gate-open conformations. Third, a specific function of A-loop autophosphorylation is to lower the $K_m$, peptide. This appears to be true because none of the gate-open mutants had a lower $K_m$, peptide than the WT-IRKD prior to autophosphorylation. In addition to addressing these hypotheses, the results demonstrate that a high $K_m$, peptide does not require filling of the peptide binding site by the pseudosubstrate sequence, because $K_m$, peptide remained high after the gate-closed conformation was lost. Furthermore, we show that multiple activation loop conformations can have similar steady-state kinetic features. These findings are summarized in Table IV.

The kinetic component of intrasteric inhibition requires each of the interactions shown in Fig. 1. This conclusion is based on the finding that catalytic efficiency was increased when any subset of interactions was broken, provided that residues in the catalytic loop, Arg-1136 and Asp-1132, were left intact. We measured increases in $k_{cat}/K_m$ of 50- to 130-fold for MgATP and 3- to 7-fold for peptide substrate (Table II). These shared kinetic changes resulted from the $8 \times$ 3-fold-increased $k_{cat}$ and $12 \times 3$-fold-lower $K_m$, MgATP, although the $K_m$, peptide showed an average 1.3-fold increase (essentially no change). However, $K_m$ and $K_a$ for a substrate are frequently not equivalent, and a change in $K_m$ may not be accompanied by a change in $K_a$. The best example of this important distinction, as applied to protein kinases, comes from work with cAMP-dependent protein kinase (compare Ref. 31 with Ref. 32), but it has also been made for the WT-IRKD (15). Therefore, we characterized binding affinity for adenine nucleotide based on the inhibition constant $K_i$, AMP-PNP. Thus, although the four mutants IRKDs had similar values of $K_i$, MgATP, the values of $K_i$, AMP-PNP indicated that affinity for AMP-PNP increased 5- to 7-fold for the K1085N-

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**Fig. 5.** The effects of mutations and MgADP on limited tryptic cleavage of the A-loop. The WT- and mutant IRKDs were cleaved with trypsin, in the absence or presence of MgADP, and the products were separated by SDS-polyacrylamide gel electrophoresis and silver staining. The IRKDs are indicated above the panels, grouped in sets of three lanes; the first lane showing the uncleaved kinases, the second lane showing cleavage in the absence of ADP, and the third lane showing cleavage in the presence of ADP. These conditions are indicated below the panels. The histogram shows the percent cleavage of the 37-kDa protein, in the absence (open bars) or presence (filled bars) of MgADP, in each panel (averaged from duplicate experiments).

**Fig. 6.** Rates of limited tryptic cleavage of the A-loop. The time courses of limited tryptic cleavage were analyzed from silver-stained gels similar to those shown in Fig. 5, using 36-kDa forms of the IRKD in all cases. Quantitation of the uncleaved kinase was done as described previously (21); all time courses were done in duplicate, and the averages are shown. Cleavages of the mutants were done at different times, as the kinases became available, and the WT-IRKD was used as an internal control. Data are presented as a “decay” process showing the fraction of kinase uncleaved, up to 90% complete cleavage. Open symbols show cleavage without MgADP. Closed symbols show cleavage in the presence of 10 mM MgADP. A, WT-IRKD (circles) and Q1208S-IRKD (squares); B, K1085N-IRKD (triangles, downward) and Y1162F-IRKD (triangles, upward); C, R1136Q-IRKD (hexagons) and D1132N-IRKD (diamonds).
Y1162F-, and D1161A-IRKD, but only a 1.5-fold increase was observed for Q1208S-IRKD. These observations suggest a gate-closed conformation of the A-loop in Q1208S-IRKD (same \( K_{\text{m,ATP}} \)) that is less tightly closed than in the WT-IRKD (lower \( K_{\text{a,AMP-PNP}} \)), and the other mutants are gate-open (lower \( K_{\text{a,AMP-PNP}} \)).

The K1085N-, Y1152F-, and D1161A-IRKD mutants share an increased affinity for adenine nucleotide, but they can be subdivided further using the patterns of viscosity dependence for steady-state kinetic parameters (Fig. 2). The parameter \( k_{\text{cat}} \) is given by the expression,

\[
k_{\text{cat}} = k_{3} \times k_{4} \frac{k_{5}}{k_{5} + k_{6}}
\]

where \( k_{3} \) is the rate constant for the chemical step of phosphoryl transfer and \( k_{4} \) is the net rate constant for product release, including conformational changes required to allow product dissociation (33). The viscosity dependence will reveal if \( k_{\text{cat}} \) is limited by the chemical step of the reaction \( (k_{3} < k_{4}) \) or net product release steps \( (k_{4} < k_{3}) \), if the slope in a plot such as shown in Fig. 2A is 0 or 1, respectively (see Ref. 34 for details, and Ref. 15 for specific details related to the steady-state kinetic mechanism of WT-IRKD). For the mutants K1085N- and Y1162F-IRKD, the slope was \(-1 \), and therefore, \( k_{\text{cat}} \approx k_{3} \), indicating that \( k_{3} \) is rate-limiting. In contrast, for D1161A-IRKD the slope was \(-0.8 \) and thus \( k_{4} < k_{3} \), and \( k_{3} \) is partially rate-limiting (14). In this respect, D1161A-IRKD is different from the other mutants and the WT-IRKD. The average value of \( k_{3} \) is \( 5.8 \text{ s}^{-1} \) for K1085N- and Y1162F-IRKD, but \( k_{3} \) is \( 59 \text{ s}^{-1} \) for D1161A-IRKD (14). Compared with \( k_{3} \) of \( 1.2 \text{ s}^{-1} \) for the WT-IRKD (15), these results show that relief of intrasteric inhibition produced similar increases in \( k_{\text{cat}} \) for each mutant (Table II), and that increase reflects an increased value of \( k_{3} \). However, the increase in \( k_{3} \) is another order-of-magnitude greater for D1161A-IRKD than the other mutants. Furthermore, if one relied exclusively on the change in \( k_{\text{cat}} \) from steady-state kinetics, this would not report the change in rate-limiting step that distinguishes the D1161A mutant; that change is revealed by the viscosity-dependence for \( k_{3} \).

The catalytic efficiency reported by \( k_{\text{cat}}/K_{\text{m,peptide}} \) is increased 5-fold above the WT-IRKD for all four active mutants \( (3.1 \pm 1.4 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \text{ versus } 0.6 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1}, \text{ calculated from data in Table I}) \). Again, there is an underlying mechanistic difference for D1161A-IRKD that is revealed by the viscosity dependence studies (Fig. 2B). The slope of zero indicates that peptide substrate equilibrates before the chemical transfer step in the reaction catalyzed by the K1085N-, Q1208S-, and Y1162F-IRKD mutants, as it does for the WT-IRKD; thus \( K_{\text{m,peptide}} \approx K_{\text{d,peptide}} \). Therefore, the K1085N, Q1208S, and Y1162F mutants have weak peptide binding, similar to the WT-IRKD basal state (Table IV). In contrast, the slope was \(-1 \) for D1161A-IRKD, which indicates that peptide substrate does not equilibrate with D1161A-IRKD prior to the chemical step (14), and \( K_{\text{d,peptide}} \) is not determined for the D1161A mutant from these experiments. However, the data in Table III indicated that Lys-1085 makes a greater contribution to \( K_{\text{m,peptide}} \) than does Gin-1208. This difference may explain the apparent gate-closed conformation displayed by the Q1208S mutant and the apparent gate-open conformation resulting from the K1085N mutation, as discussed below.

Participation of the A-loop in sterically blocked of the active site was examined by changes in iodide quenching. The trend in bimolecular collisional rate constants shown in Fig. 4B best illustrates the “gradient” of conformations that occur among the unphosphorylated wild-type and mutant IRKDs (\( k_{3} \), in the absence of adenine nucleotide; open bars). The larger values of \( k_{3} \) can be interpreted as indicating greater accessibility of the active site, suggesting the order D1132N > D1161A > Y1162F > K1085N > Q1208S > WT for relative openness of the cleft. In contrast to these mutant IRKDs, interpreting changes in fluorescence properties of the R1136Q-IRKD mutant is difficult, because the mutation affects directly the fluorescence of Trp-1175, the major fluorophore in IRKD (23). This effect is shown clearly by the lower mean fluorescence lifetime of R1136Q-IRKD (Fig. 4A), which is probably caused by the loss of an interaction between the guanidinium group of Arg-1136 and the indole ring of Trp-1175, which has been seen in all IRKD crystal structures (7, 14, 24, 25). In every other IRKD, bound adenine nucleotide appears to have an impact of conformation, because \( k_{3} \) always increases in each binary complex compared with the apo-protein. Cleft openness is based on two factors: relative orientation of the small and large lobes and position of the activation loop.

We addressed changes in A-loop conformation through limited proteolysis of the A-loop. We also measured the impact of bound adenine nucleotide, because this promotes a gate-open conformation in the WT-IRKD (Ref. 21 and Fig. 5), and because the binary complex differs from the unliganded IRKD according to the changes in fluorescence just described. These results yield the final grouping of IRKDs: the WT- and Q1208S-IRKD are mostly gate-closed in the absence of nucleotide, but at saturating nucleotide there are two nearly equal populations of A-loop conformations (Table IV). This is suggested by the biphasic progress curves of A-loop cleavage shown in the semi-
logarithmic plots of Fig. 6A, where each phase represents one cleavage rate and one underlying conformation. The K1085N- and Y1162F-IRKD are gate-open mutants, each showing one major conformational population, with a second population accounting for no more than 20–30% of A-loop conformations (Table IV). This interpretation applies in the absence or presence of ADP (Fig. 6B). The D1132N- and R1136Q-IRKDs are gate-open, and single A-loop conformations are sufficient to account for each cleavage rate in the absence or presence of bound nucleotide; D1161A-IRKD falls into this category as well (Table IV). The A-loop was disordered in the D1161A-IRKD structure (14), which could indicate disorder in solution. However, in other protein kinase structural studies, it has been suggested that the appearance of disorder comes from the limited extent of data refinement (cf. Refs. 10 versus 9) or perhaps from crystallization conditions (e.g. Refs. 12 versus 11). Our results indicate that multiple A-loop conformations occur among these IRKDs in solution. Importantly, different conformations occur without modification of the A-loop amino acid sequence (e.g. K1085N and D1132N versus WT).

To summarize the changes produced by these point mutants in the functional components of intrasteric inhibition, all the active mutants share increased catalytic efficiency and weak peptide binding. In terms of how the catalytic cycle proceeds, the D1161A-IRKD is unique: K1085N-, Q1208S-, and Y1162F-IRKDs are generally similar to WT-IRKD despite the increased catalytic efficiency of the mutants. The structural component of intrasteric inhibition is based on a gate-closed versus gate-open conformation. All the gate-open mutants have increased affinities for the adenine nucleotide AMP-PNP. The WT- and Q1208S-IRKD are gate-closed, but closure is weaker in the Q1208S mutant. Therefore, the hydrogen bond interactions 1 and 6 in Fig. 1 contribute least to the gate-closed conformation of the A-loop. It may be a general feature of the structural component of intrasteric inhibition that the A-loops become disordered when the gate-closed conformation is lost. In ERK2, Thr-183 and Tyr-185 have functions analogous to Tyr-1162 and Tyr-1163 of the IR, because their phosphorylation is essential for regulation, and the inhibitory properties of mobile and rigid activation loops are both significant in the regulation of protein kinase activity.

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Multiple Activation Loop Conformations and Their Regulatory Properties in the Insulin Receptor’s Kinase Domain
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