Crystallographic and Solution Studies of an Activation Loop Mutant of the Insulin Receptor Tyrosine Kinase

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The tyrosine kinase domain of the insulin receptor is subject to autoinhibition in the unphosphorylated basal state via steric interactions involving the activation loop. A mutation in the activation loop designed to relieve autoinhibition, Asp-1161 → Ala, substantially increases the ability of the unphosphorylated kinase to bind ATP. The crystal structure of this mutant in complex with an ATP analog has been determined at 2.4 Å resolution. The structure shows that the active site is unobstructed, but the end of the activation loop is disordered and therefore the binding site for peptide substrates is not fully formed. In addition, Phe-1151 of the protein kinase-conserved DFG motif, at the beginning of the activation loop, hinders closure of the catalytic cleft and proper positioning of α-helix C for catalysis. These results, together with viscometric kinetic measurements, suggest that peptide substrate binding induces a reconfiguration of the unphosphorylated activation loop prior to the catalytic step. The crystallographic and solution studies provide new insights into the mechanism by which the activation loop controls phosphorylation transfer as catalyzed by the insulin receptor.

The insulin receptor is an αβ2 heterotetrameric glycoprotein possessing intrinsic protein-tyrosine kinase (PTK) activity (1, 2). Upon insulin binding to the α subunits, the insulin receptor undergoes a poorly characterized conformational change that results in autophosphorylation of specific tyrosine residues in the cytoplasmic portion of the β subunits. Three regions in the β subunits are sites of autophosphorylation: the juxtamembrane region, the activation loop (A-loop) within the tyrosine kinase domain, and the C-terminal tail (3–6). Autophosphorylation of tyrosine residues stimulates receptor catalytic activity (7, 8) and creates recruitment sites for downstream signaling molecules such as the insulin receptor substrate (IRS) proteins (9) and Shc (10, 11).

Previous crystallographic studies of the tyrosine kinase domain of the insulin receptor (IRKD) have demonstrated that upon autophosphorylation, the kinase A-loop undergoes a major change in conformation (12, 13). In the crystal structure of the unphosphorylated, low activity form of IRKD (IRKD(3P)) without ATP (apo), Tyr-1162 in the A-loop is situated in the active site, blocking access to peptide substrates (12). In this A-loop configuration, the beginning (proximal end) of the A-loop interferes with ATP binding. The crystal structure of the tris-phosphorylated, activated form of IRKD (IRKD(3W)) reveals how autophosphorylation of Tyr-1158, Tyr-1162, and Tyr-1163 stabilizes a specific A-loop configuration in which the substrate binding sites (MgATP and peptide) are accessible and the important catalytic residues are properly positioned (13, 14).

Solution studies of IRKD indicate, however, that in the presence of millimolar quantities of ATP (as are present in a cell), the A-loop of unphosphorylated IRKD is in equilibrium between inhibiting, “gate-closed” conformations, as represented by the apoIRKD(3W) crystal structure, and “gate-open” conformations in which Tyr-1162 is displaced from the active site (15). When the A-loop adopts a gate-open conformation, the kinase is competent to serve as either enzyme or substrate in a trans-autophosphorylation reaction. Prior to A-loop autophosphorylation, gate-open conformations of the A-loop would exist in which the majority of the A-loop has no particular conformation (because of lack of phosphotyrosine-mediated interactions), but is nevertheless disengaged from the active site. After autophosphorylation, the A-loop is stabilized in the gate-open conformation observed in the IRKD(3W) structure.

A detailed understanding of the mechanism by which insulin triggers the initial autophosphorylation event in the insulin receptor requires a structural description of the basal state (unphosphorylated) kinase with bound substrates (ATP and protein). Ideally, this would be provided by a crystal structure of IRKD(3W) with bound ATP analog and peptide substrate. To date, attempts to obtain crystals of such a ternary (or binary) complex have been unsuccessful. Steady-state kinetic studies of IRKD provide a plausible explanation for this failure: the $K_{m}$ values for ATP and peptide substrate...
Crystallographic and Solution Studies of IRKD\textsuperscript{D1161A}

are elevated prior to autophosphorylation, 0.9 and 2 mm, respectively, decreasing to 0.04 and 0.05 mm upon autophosphorylation.\textsuperscript{2}

These data are consistent with the autoinhibitory mechanism suggested by the apoIRKD\textsuperscript{DP} structure and underscore the inherent difficulty of loading the kinase with substrates prior to A-loop autophosphorylation.

Recently, a substitution in the A-loop of IRKD, Asp-1161 → Ala (IRKD\textsuperscript{DAA}), has been introduced that dramatically alters the A-loop equilibrium in the unphosphorylated kinase.\textsuperscript{3} This particular substitution was motivated by the apoIRKD\textsuperscript{DP} crystal structure in which the Asp-1161 side chain participates in several hydrogen bonds that stabilize the gate-closed configuration of the A-loop (Fig. 1). Steady-state kinetic experiments demonstrate that the $K_m$ (ATP) in the basal state is ~10-fold lower for this mutant than for wild-type IRKD, suggesting that the A-loop equilibrium is shifted toward gate-open conformations.\textsuperscript{3} Interestingly, this mutation does not affect $K_m$(peptide) in the unphosphorylated state, which remains high (several millimolar). The kinetic properties of IRKD\textsuperscript{DAA} after autophosphorylation are indistinguishable from those of the wild-type kinase.

The lower $K_m$(ATP) for this mutant affords the possibility of structurally characterizing IRKD with ATP bound prior to insulin-stimulated A-loop autophosphorylation. Indeed, crystals of IRKD\textsuperscript{DAA} with a bound ATP analog (AMP-PCP) were readily obtained. Here we present the structure of the binary complex of IRKD\textsuperscript{DAA} with MgAMP-PCP at 2.4-Å resolution. This structure and the accompanying viscometric and denaturation data provide insights into the structural rearrangements that occur within the basal state kinase to promote catalysis.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—The 46-kDa form (residues 953–1355) of the Asp-1161 → Ala mutant cytoplasmic domain of the insulin receptor, IRKD\textsuperscript{DAA}, was generated and purified as described.\textsuperscript{3} This form of the kinase was used for denaturation studies. To generate the 35-kDa form (residues 978–1283) of the mutant (IRKD\textsuperscript{DAA}) used in the crystallographic and viscometric studies, the 0.65-kb Xhol-NotI fragment from pX-D1161A-IRKD was inserted into the Xhol-Stul sites of pALTER-IRK vector, which includes the point mutations Cys-981 → Ser and Tyr-984 → Phe described previously (16). The fragment swap was verified with NheI digestion; this restriction enzyme site was introduced previously to replace a Stul site by a silent mutation at Ala-1048 and Ser-1049 (17). The resulting pALTER-IRKD\textsuperscript{DAA} was digested with HindIII, filled in with the large Klenow fragment of DNA polymerase, and a 0.9-kb fragment was released with BamHI. This was inserted into the BamHI-SmaI sites of the baculovirus expression vector pVL1393 (PharMingen), producing pVL1393-IRKD\textsuperscript{DAA}, and the recombinant virus was generated using a BaculGold kit (PharMingen); the mutation was reconfirmed in pVL1393-IRKD\textsuperscript{DAA} by DNA sequencing. Proteins were expressed and purified as described (18).\textsuperscript{3} The absence of A-loop phosphorylation in each form (46 kDa and 35 kDa) of the mutant was determined by endoproteinase Lys-C digestion and peptide mapping by reverse-phase high performance liquid chromatography.

**Crystallographic Studies**—Crystals of the binary complex of IRKD\textsuperscript{DAA} and MgAMP-PCP were grown at 20 °C by vapor diffusion in hanging drops containing 2.0 \mu l of protein solution (9 mg/ml IRKD\textsuperscript{DAA}, 1.5 mm AMP-PCP (Sigma), and 4.5 mm MgCl\textsubscript{2}) and 2.0 \mu l of reservoir buffer (18% polyethylene glycol 8000, 100 mm Tris-HCl, pH 7.5, 100 mm NaCl, and 5 mm dithiorethiol). Crystals belong to the orthorhombic space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, with unit cell dimensions $a = 57.9$ Å, $b = 69.6$ Å, and $c = 89.3$ Å when frozen. There is one molecule in the asymmetric unit, and the solvent content is 51% (assuming a protein partial specific volume of 0.74 cm\textsuperscript{3}/g). Crystals were transferred into a cryosolvent consisting of 30% polyethylene glycol 8000, 100 mm Tris-HCl, pH 7.5, 100 mm NaCl, and 15% ethylene glycol. A data set was collected from a flash-cooled crystal on a Rigaku RU200 rotating anode equipped with a Rigaku

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**RESULTS**

**Crystal Structure of IRKD\textsuperscript{DAA} in Complex with MgAMP-PCP**—In the original structure of unphosphorylated (low activity) IRKD, the A-loop traverses the ATP-binding cleft between...
the N- and C-terminal lobes of the kinase, and Tyr-1162 in the A-loop is bound in the active site, hydrogen-bonded to Asp-1132 and Arg-1136 in the catalytic loop (12). Asp-1161 contributes to the stabilization of this inhibitory conformation of the A-loop by participating in four hydrogen bonds (Fig. 1). In the crystal structure of the Asp-1161 → Ala mutant IRKD in complex with MgAMP-PCP (Fig. 2), the A-loop adopts a conformation in which the active site is unobstructed (gate-open), consistent with solution studies measuring the accessibility of the active site.

In the IRKDDA structure, the proximal end of the A-loop, containing the protein kinase-conserved 1150DFG sequence, is positioned more similarly to that in the activated IRKD3P structure than that in the apoIRKD0P structure (Fig. 3A). A-loop residues 1155–1171, which include the three autophosphorylation sites Tyr-1158, Tyr-1162, and Tyr-1163, have no supporting electron density in the IRKD DA structure and are presumed to be disordered. The A-loop becomes ordered again with solution studies measuring the accessibility of the active site (3).

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The A-loop is shown in blue, and selected side chains are shown in stick representation (12). From its position in the IRKD0P structure, the N-terminal lobe is rotated 11° toward the C-terminal lobe in the IRKD DA structure. An additional 8° is required for the N-terminal lobe to reach the position observed in the IRKD3P structure. Analysis of the changes in backbone φ, ψ torsion angles shows that the hinge points for the N-terminal lobe rotation are at Arg-1061, before β-strand 4 (b4), and Met-1079, in the segment linking the N- and C-terminal lobes.

When the N-terminal β sheet is superimposed for the three IRKD structures, α-helix C (αC) in the IRKD DA structure is observed to be in essentially the same position with respect to the β sheet as it is in the IRKD3P structure. Thus, in the transition from the autoinhibited, gate-closed conformation of the A-loop (IRKD0P) to a gate-open conformation with bound ATP (IRKD DA), the entire N-terminal lobe rotates as a rigid body. However in the transition to the activated state (IRKD3P), αC undergoes an independent (from the β sheet) motion that entails a 12° rotation toward the C-terminal lobe and a 28° rotation about the helical axis (Fig. 3D). These movements of αC, which mainly occur through φ, ψ changes at Phe-1054 and Thr-1055 at the base of the helix, are necessary to position protein kinase-conserved Glu-1047 (in αC) proximal to conserved Lys-1030 in (in β3). Lys-1030 coordinates the α- and β-phosphates of ATP in an active protein kinase configuration (30, 31).

The IRKD DA structure suggests that the rotation of αC required for a properly configured active site relies on the precise positioning of Phe-1151 in the DFG motif. Although the position of Phe-1151 in the IRKD DA structure is roughly similar to that in the IRKD3P structure, there are critical differences. In the IRKD3P structure, Phe-1151 is buried deep in a hydrophobic pocket underneath αC (Fig. 4A). This pocket is composed of residues from αC (Glu-1047, Val-1050, Met-1051), from the αC-β4 loop (Phe-1054, Val-1059), from αE (Leu-1123), and from β8 (Ile-1148). In contrast, the side chain of Phe-1151 in the IRKD DA structure points upward toward αC and is situated in a shallow hydrophobic pocket comprising the same residues as above (some with different side-chain rotamers) and additionally Phe-1128 in the segment preceding the catalytic loop (Fig. 4B). With Phe-1151 in this position, αC is sterically hindered from undergoing the movements that bring Glu-1047 into the

FIG. 1. Interactions of Asp-1161 in the apoIRKD0P structure (12). The A-loop is shown in blue, and selected side chains are shown in stick representation. The remainder of the structure is represented as a molecular surface (grey). Carbon atoms are colored orange, nitrogen atoms blue, and oxygen atoms red. Hydrogen bonds are shown as dashed black lines. Asp-1161 and Tyr-1162 are in the A-loop, Asp-1132 and Arg-1136 are in the catalytic loop, Lys-1085 is in αD, and Gln-1208 is in the loop between αF and αG (Fig. 2).

FIG. 2. Ribbon diagram of the IRKD DA structure. β strands (numbered) are shown in cyan and α helices (lettered) in red and yellow. The nucleotide analog AMP-PCP is shown in ball and stick representation (black). The dashed grey line indicates the portion of the A-loop that is disordered (residues 1155–1171).
active site. Moreover, conserved Asp-1150, which coordinates Mg\(^{2+}\), is pulled back from the active site \( \textit{vis à vis} \) its position in the IRKD\(^{DA}\) structure (Fig. 3B).

The ATP analog (AMP-PCP) that was co-crystallized with IRKD\(^{DA}\) is bound in the cleft between the two kinase lobes (Fig. 2) and is ordered throughout, including the \(\gamma\)-phosphate. The conformation of AMP-PCP in the IRKD\(^{DA}\) structure is different from the conformation of AMP-PNP observed in the ternary
IRKD<sup>3P</sup> structure and closely resembles the conformation of ATP and AMP-PNP in crystal structures of cyclic AMP-dependent protein kinase (30, 31). In the ternary IRKD<sup>3P</sup> structure, the γ-phosphate of AMP-PNP is swung away from the hydroxyl group of the tyrosine substrate in the active site, presumably due to the imperfect fit with nitrogen rather than oxygen as the bridging atom between the β- and γ-phosphates.

Due to the incomplete rotation of the N-terminal lobe toward the C-terminal lobe in IRKD<sup>DA</sup>, AMP-PCP binds to the “roof” of the cleft (N-terminal residues) but not to the “floor” (C-terminal residues). Lys-1030 in β3 is hydrogen-bonded to the α-phosphate, but the ribose hydroxyl groups are not within hydrogen-bonding distance to Asp-1083 in the C-terminal lobe, as in IRKD<sup>3P</sup>. Moreover, only one Mg<sup>2+</sup> ion is evident in the IRKD<sup>DA</sup> structure, coordinated by Asp-1150 and the β- and γ-phosphates of AMP-PCP. Because of the retracted position of Asp-1150, the coordination of this Mg<sup>2+</sup> is weak: Mg–O distances ≥ 2.4 Å. Due to the lack of lobe closure and the consequent positioning of AMP-PCP at the roof of the cleft, the second Mg<sup>2+</sup> ion present in the IRKD<sup>3P</sup> structure, coordinated by Asn-1137 of the catalytic loop (C-terminal lobe), is absent in the binary IRKD<sup>DA</sup> structure.

Although the A-loop in the IRKD<sup>DA</sup> structure does not occlude the peptide binding site as in the IRKD<sup>3P</sup> structure, <i>K<sub>cat(peptide)</sub></i>, unlike <i>K<sub>cat(ATP)</sub></i>, is not decreased in the Asp-1161 → Ala mutant. In the structure of ternary IRKD<sup>3P</sup>, residues 1169–1171 at the distal end of the A-loop are hydrogen-bonded via main-chain atoms to peptide substrate residues P<sub>-1</sub> through P<sub>3</sub> (P<sub>0</sub> is the acceptor tyrosine), forming two short, antiparallel β strands (13). In addition, the side chains of Leu-1170 and Leu-1171 are constituents of the binding pockets for the P<sub>0</sub> and P<sub>3</sub> side chains, respectively. Thus, the disorder in the IRKD<sup>DA</sup> A-loop at the distal end results in a peptide binding site that is not fully formed, which is reflected in the high <i>K<sub>cat(peptide)</sub></i>. In contrast, in the gate-open conformation stabilized by A-loop autophosphorylation, the distal end of the A-loop is ordered even in the absence of peptide substrate.

**Viscometric Analysis**—The binding and chemical steps associated with an IRKD-catalyzed phosphorylation reaction are summarized in Scheme 1 for the experimental conditions where enzyme (E) is saturated with ATP (T), tyrosyl peptide (Y) binds with on- and off-rate constants <i>k<sub>1</sub></i> and <i>k<sub>-2</sub></i>, the rate constant for the chemical step is given by <i>k<sub>2</sub></i>, and the net rate constant for release of products ADP (D) and phospho-tyrosyl peptide (pY) is given by <i>k<sub>4</sub></i>.

\[
E \cdot T + Y \overset{k_1}{\rightarrow} E \cdot T \cdot Y \overset{k_2}{\rightarrow} E \cdot D + pY \overset{k_3}{\rightarrow} E + D + pY
\]

**Scheme 1**

The stepwise rate constants present in the steady-state kinetic parameters were derived using Cleland’s method (32) and were established from the viscosity dependence of <i>k<sub>cat</sub></i> and <i>k<sub>cat/K<sub>cat</sub>(peptide)</sub></i> (Fig. 5).

\[
k_{cat} = k_3 \cdot k_4 / (k_2 + k_4)
\]

(Eq. 1)

\[
h_{cat/K<sub>cat</sub>(peptide)} = k_5 \cdot k_7 / (k_5 + k_7)
\]

(Eq. 2)

The principle behind viscometric analysis is that increased solution viscosity will affect the diffusion-dependent steps of substrate binding (<i>k<sub>1</sub></i> and <i>k<sub>-2</sub></i>) and product release (<i>k<sub>3</sub></i>) but not the chemical step (<i>k<sub>2</sub></i>), because the latter does not involve solute (substrate) exchange between the bulk phase and the enzyme’s active site (23).

![Figure 5. Viscosity dependence of the IRKD<sup>DA</sup>-catalyzed reaction.](http://www.jbc.org/)

To identify whether chemistry is the rate-limiting step in steady-state phosphorylation of the peptide substrate Y-IRS939 by IRKD<sup>DA</sup>, the viscosity dependence of <i>k<sub>cat</sub></i> and <i>k<sub>cat/K<sub>cat</sub>(peptide)</sub></i> was determined. The parameters (<i>k<sub>cat</sub></i><sup>rel</sup> and (<i>k<sub>cat</sub>/K<sub>cat</sub>(peptide)</i>)<sup>rel</sup>) are the ratio of <i>k<sub>cat</sub></i> and <i>K<sub>cat</sub>(peptide)</i> in the presence of viscogen using the control reaction in aqueous buffer without viscogen. If the rate constant for a diffusion-dependent step is much smaller than for a diffusion-independent step, then the plot of (<i>k<sub>cat</sub></i><sup>rel</sup> or (<i>k<sub>cat</sub>/K<sub>cat</sub>(peptide)</i>)<sup>rel</sup> versus <i>η</i> will have zero slope. For IRKD<sup>DA</sup>, both global parameters were sensitive to changes in viscosity, increasing linearly with increasing viscogen (Fig. 5). The slope for (<i>k<sub>cat</sub></i><sup>rel</sup> or (<i>k<sub>cat</sub>/K<sub>cat</sub>(peptide)</i>)<sup>rel</sup> versus <i>η</i>) was 0.8 ± 0.1, and the slope for (<i>k<sub>cat</sub>/K<sub>cat</sub>(peptide)</i>)<sup>rel</sup> versus <i>η</i>) was 1.2 ± 0.2. For IRKD<sup>DA</sup>-catalyzed reactions without viscogen, <i>k<sub>cat</sub></i> = 9.6 ± 0.4 s<sup>-1</sup> and <i>k<sub>cat</sub>/K<sub>cat</sub>(peptide) = 4.2 ± 0.7 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup>. From these values, we calculate <i>k<sub>3</sub></i> = 59 s<sup>-1</sup> and <i>k<sub>5</sub></i> = 14 s<sup>-1</sup>. Because these rate constants differ by only 4-fold, the steady-state rate constant of the reaction (<i>k<sub>cat</sub></i>) is partially limited by chemistry and partially by product release (Equation 1). These are approximately the same values of <i>k<sub>3</sub></i> and <i>k<sub>5</sub></i> determined for the activated kinase, IRKD<sup>3P</sup>, 46 s<sup>-1</sup> and 11 s<sup>-1</sup>, respectively.

**Denaturation Experiments**—Denaturation of IRKD<sup>DA</sup> (46-kDa form of the mutant) in guanidinium chloride was monitored using fluorescence and is presented (Fig. 6) as the change in centroid of the emission spectrum (defined in Ref. 18). The data are compared with denaturation profiles from unphosphorylated and phosphorylated wild-type IRCD taken from previous work (18). As before, there are three transitions in the denaturation profile. Denaturation over transition I follows the same pattern observed for phosphorylated wild-type IRCD, and denaturation over transition III follows the same pattern as observed for unphosphorylated wild-type IRCD. This indicates that the Asp-1161 → Ala substitution, while releasing the A-loop from the gate-closed conformation, does not otherwise alter the intrinsic conformational flexibilities within the A-loop or the two kinase lobes.

The conformational free energy in the unphosphorylated, gate-open mutant (IRCD<sup>DA</sup>) was calculated from these results: Δ<i>GH</i><sub>H2O</sub> = −2.8 kcal/mol over transition I and −8.5 kcal/mol over transition III, for a net free energy of unfolding Δ<i>GH</i><sub>H2O,net</sub> = −11.3 kcal/mol. Compared with values published previously for the basal and activated state wild-type kinase (Δ<i>GH</i><sub>H2O,net</sub> = −14.1 and −10.2 kcal/mol, respectively) (18), the overall differ-

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5 These parameters were calculated from <i>k<sub>4</sub></i> = <i>k<sub>cat</sub></i>/slope of (<i>k<sub>cat</sub></i><sup>rel</sup> versus <i>η</i>)<sup>rel</sup>; <i>k<sub>3</sub></i> = <i>k<sub>cat</sub></i><sup>rel</sup><i>k<sub>4</sub></i> / (<i>k<sub>4</sub></i> − <i>k<sub>cat</sub></i>).
ever, in these two structures, the ATP analog is not bound in the nucleotide binding site. However, which an A-loop tyrosine is not mimicking a tyrosine substrate, fibroblast growth factor receptor kinase (34) structures, in the Src (38, 39), and Abl (40), exhibit a wide range of conformations. In the IRKD and Abl structures, one of the tyrosines in the A-loop (Tyr-1162 in IRKD, Tyr-393 in Abl) is bound in the active site, mimicking an exogenous tyrosine substrate. In the IRKD3P structure, conserved Glu-1047 in Abl is proximal to conserved Lys-1030 in IRKD. Comparing reactions catalyzed by the unphosphorylated enzyme, the IRKD3P structure suggests that peptide substrate binding (13), is disordered in the IRKD3P structure, providing a rationale for the elevated $K_m$(peptide) for this mutant (see below).

The IRKD3P structure indicates that proper positioning of conserved Phe-1151 is critical for the downward and inward rotation of $\alpha$C and suggests that binding of ATP to the basal state kinase is not sufficient to induce the $\alpha$C transition. In the IRKD3P structure, conserved Glu-1047 in aC is proximal to conserved Lys-1030 in $\beta$, orienting the lysine side chain for ATP (phosphate) binding (Fig. 3B). The optimal position (for catalysis) of conserved Asp-1150, which coordinates an essential Mg$^{2+}$ ion, is also dependent on the proper positioning of neighboring Phe-1151.

Upon autophosphorylation, the conformation of the A-loop is stabilized by short $\beta$-strand interactions between the A-loop and other C-terminal lobe residues and by electrostatic interactions involving the phosphoryl groups of Tyr(P)-1162 and Tyr(P)-1163 (13). In this configuration, the proximal and distal ends of the A-loop are “pulled taut,” which favors the buried position of Phe-1151 beneath aC (Figs. 3A and 4A). Prior to autophosphorylation, the IRKD3P structure suggests that peptide substrate binding to the distal end of the A-loop is sufficient to reconfigure, at least transiently, the A-loop for catalysis.

**Integration of Solution and Structural Studies**—The IRKD3P crystal structure is the third structure of the kinase domain of the insulin receptor, each of which represents a different conformational state. For each structure, we also have available kinetic parameters (Fig. 5A) and conformational free energies (Fig. 6 and Ref. 18). Each type of analysis (structural, kinetic, and thermodynamic) shows that IRKD3P is intermediate between basal IRKD3P and fully activated IRKD3P and together provide further insights into the mechanism by which A-loop conformation regulates this kinase.

Every catalytic cycle comprises binding and chemical steps and conformational changes. These may be discrete or overlapping, depending on the relative rate constants for individual steps. For IRKD these steps were summarized in Scheme 1 (see “Results”). Comparing reactions catalyzed by the unphospho-
rylated forms, IRKDDA and IRKDP, the dissociation constants for peptide substrate are nearly the same, but the rate constants for the chemical step ($k_3$) differ by almost 50-fold. Peptide binding equilibrates prior to the chemical step in the IRKDP-catalyzed reaction but not in the IRKDDA reaction (see “Results”). The smaller rate constant for $k_3$ in the IRKDP versus IRKDDA reaction indicates a higher free energy barrier to phosphoryl transfer for IRKDP, making it 50 times less likely that a ternary complex will convert to a transition state complex. This barrier in IRKDDA may arise structurally if the A-loop reconfiguration were less advanced than observed in the IRKDDA-AMP-PCP binary complex. The conformational change in the A-loop needed to complete cleft closure and $\alpha C$ rotation in the IRKDP basal state would occur after peptide binding has equilibrated. If the conformational change is folded into the chemical step in this way, it could yield the 50-fold smaller rate constant in the basal state IRKDP catalytic cycle.

When IRKDDA and IRKDP are compared, $k_3$ is virtually the same for both. The same $k_3$ indicates that an equivalent free energy barrier exists between the respective ternary and transition state complexes. This suggests that the terminal IRKDP complex should resemble the ternary IRKDP complex (13), with repositioning of the N-terminal $\beta$ sheet and $\alpha C$ accomplished in the IRKDP catalytic complex prior to the actual phosphoryl transfer event. A similar ternary complex for IRK-

We envision that peptide binding begins at the $P_1$ and $P_0$ sites, which are fully open in the IRKDDA structure, and proceeds until the $P_1$ and $P_3$ residues become seated properly. The peptide substrate is captured by the faster chemical step of the catalytic cycle ($k_3 > k_0$), and thus binding does not equilibrate before the phosphoryl transfer occurs. Backbone hydrogen bonding and hydrophobic interactions involving the $P_1$ and $P_3$ side chains could impose order on Gly-1169–Leu-1171 before the chemical step for the latter but not the former (see “Results”). These kinetic features of unphosphorylated IRK-

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