Probing the Catalytic Mechanism of the Insulin Receptor Kinase with a Tetrafluorotyrosine-containing Peptide Substrate*

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The interaction of a synthetic tetrafluorotyrosyl peptide substrate with the activated tyrosine kinase domain of the insulin receptor was studied by steady-state kinetics and x-ray crystallography. The pH-rate profiles indicate that the neutral phenol, rather than the chemically more reactive phenoxide ion, is required for enzyme-catalyzed phosphorylation. The pKᵦ of the tetrafluorotyrosyl hydroxyl is elevated 2 pH units on the enzyme compared with solution, whereas the phenoxide anion species behaves as a weak competitive inhibitor of the tyrosine kinase. A structure of the binary enzyme-substrate complex shows the tetrafluorotyrosyl OH group at hydrogen bonding distances from the side chains of Asp^{1132} and Arg^{1136}, consistent with elevation of the pKᵦ. These findings strongly support a reaction mechanism favoring a dissociative transition state.

Protein kinases are enzymes that catalyze transfer of the γ-phosphoryl group from ATP to the alcoholic side chains of amino acid residues on their substrate proteins and peptides. They play important roles in signaling and have been subject to intensive cell biologic, enzymologic, structural, and kinetic scrutiny. Elucidation of the chemical reaction mechanisms of these enzymes is proving to be important in defining the details of their regulation and for designing inhibitors.

The reaction mechanism of phosphoryl transfer reactions can favor either an associative or dissociative pathway (Fig. 1). In the extremes, these are distinguished by dependence on versus independence from nucleophilicity of the phosphoryl acceptor, respectively, distances between the γ-phosphorus atom and the attacking and leaving group oxygens, and other factors (1–7). The associative pathway would benefit from proton abstraction prior to formation of the transition state so that a more reactive oxygen anion is formed, whereas that proton is retained in the dissociative transition state. Earlier kinetic and crystallographic studies of protein kinases, especially the cAMP-dependent protein kinase, demonstrated the importance of a conserved aspartyl residue that is essential for the enzyme-catalyzed reaction (8–10). The location of this Asp^{106} side chain in the cAMP-dependent protein kinase crystal structure was close enough to the serine hydroxyl that it could create a more reactive nucleophile by removal of the seryl or threonyl proton, in accordance with an associative mechanism (8, 9), but other kinetic evidence was interpreted as favoring a dissociative pathway (5, 11, 12, 14). Never the less, similar positioning of the conserved aspartyl side chain in other protein kinase structures has been offered as further supporting evidence for proton abstraction, thus generating a more reactive entering oxygen as a basis for catalysis (e.g. see Ref. 15). On the other hand, as discussed by Mildvan (2), distances as short as 2.7 Å between the entering oxygen and the γ-phosphorus could be estimated from modeling studies (16) and as long as 5.3 Å from NMR (17); the latter indicates a reaction pathway with a more dissociative character. Furthermore, the mechanism of phosphoryl transfer in chemical reactions of phosphate monoesters, as models for enzyme-catalyzed reactions, appears to be dissociative with relatively little contribution of nucleophilicity to the transition state (18, 19). On the premise that chemical and enzymatic reactions differ in stabilization but not character of the transition state (20–26), it would seem that protein kinases should follow a more dissociative pathway.

For protein tyrosine kinases, the ability to vary nucleophilicity by aromatic ring substitution on tyrosine facilitated a chemical analysis of the phosphoryl transfer reaction catalyzed by C-terminal Src kinase (Csk).1 Analyzing the pH-independent kinetic parameters for a series of substituted tyrosine derivatives with pKᵦ's ranging from 5.2 to 10, it was shown that kₐₑₐₚ and kₑₐₚ/Kᵦ were relatively independent of nucleophilicity of the attacking phenolic residue, yielding a very small Bronsted nucleophile coefficient (0 < βₑₐₚ < 0.1) (6). The pH-rate profiles also demonstrated that the chemically more reactive phenoxide anion species was enzymatically less reactive than the neutral phenol species. Further studies with Csk revealed that the reverse reaction, where the fluorotyrosyl peptide is the leaving group, gave a Bronsted leaving group coefficient (βₑₐₚ) of −0.3 (3). This supports protonation of the phenolic OH in both directions. Thus, evidence from the forward and reverse reactions together suggest that the character of the transition state for Csk is mostly dissociative (26).

Fluorotyrosyl analogs have also been used with the insulin receptor (IR) tyrosine kinase (27, 28), but the opposite conclusion had been reached in those studies; i.e. the transition state...
for phosphoryl transfer seemed to be associative. Therefore, the analysis reported here was undertaken to specifically address this discrepancy between Csk and IR, making use of the following three advances since the earlier work of Graves and co-workers (27, 28): 1) There is now a better understanding of the substrate-specificity determinants for the insulin receptor (29–32), which was a factor in design of the peptide substrates. 2) The recombinant cytoplasmic kinase domain of the insulin receptor (IRKD) can be expressed as a highly purified protein whose autophosphorylation state can be fixed experimentally (31–34). 3) The ternary complex of the catalytic core with a tyrosyl peptide substrate and MgATP analog has been determined, which establishes a distance between O\(\gamma\) of the reactive Tyr and the γ-phosphorus atom of the adenine nucleotide (32). Our results with the autophosphorylated and maximally activated IRKD support a dissociative mechanism for this protein kinase, show an increase in \(k_{\text{cat}, \text{obs}}\) of the enzyme-bound fluorotyrosyl group compared with aqueous solution, and demonstrate that this fluorotyrosyl side chain can form hydrogen bonds virtually identical to those between the unmodified tyrosyl residue of the substrate and the catalytic core of the kinase.

**EXPERIMENTAL PROCEDURES**

**General—**Dithiothreitol (Sigma Ultra), the disodium salt of ATP (from equine muscle, catalog number A-5394), and bovine serum albumin (radioimmunoassay grade) were purchased from Sigma; hydroxylated Trition X-100 (protein grade) was from Calbiochem; EDTA was from Fluka; Tris aceate, Tris base, HCl, and electrophoresis reagents were from Roche Molecular Biochemicals; 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-tris propane) was from Sigma; magnesium acetate (MgAc\(_2\), enzyme grade) was from Fisher. Insect cell culture media and fetal bovine serum were from Life Technologies, Inc.

**Peptide Synthesis—**Tri-Fluorotyrosine was enzymatically prepared with tyrosine phenol-lyase as described previously and converted to the corresponding Fmoc derivative (6). Fmoc-L-tetrafluorotyrosine was prepared with tyrosine phenol-lyase as described previously and converted spectrophotometrically at 280 nm (\(\epsilon = 40,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the unphosphorylated IRKD, and \(\epsilon = 35,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the autophosphorylated IRKD). The autophosphorylation stoichiometry for the IRKD was 5.4 mol phosphate/mol enzyme, and the enzyme was determined to be maximally activated (specific activity of 800 mol-phospho-IRS939/min/mol IRKD at 1 mM ATP, 20 mM MgAc\(_2\)). The absence of an unphosphorylated or monophosphorylated activation by peptide substrate analogs was determined spectrophotometrically at 280 nm, at a peptide concentration of 0.25 mM.

**IRKD Expression and Purification—**Baculovirus-encoding amino acid residues 953–1355 of the insulin receptor’s cytoplasmic kinase domain\(^{34\text{w}}\) was used to express the IRKD in High Five\textsuperscript{TM} cells (Invitrogen). Enzyme purification, autophosphorylation, and storage were essentially as described previously (38). The purified protein was quantified spectrophotometrically at 280 nm (\(\epsilon = 40,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the unphosphorylated IRKD, and \(\epsilon = 35,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the autophosphorylated IRKD). The autophosphorylation stoichiometry for the IRKD was 5.4 mol phosphate/mol enzyme, and the enzyme was determined to be maximally activated (specific activity of 800 mol-phospho-IRS939/min/mol IRKD at 1 mM ATP, 20 mM MgAc\(_2\)). The absence of an unphosphorylated or monophosphorylated activation by peptide substrate analogs was determined spectrophotometrically at 280 nm, at a peptide concentration of 0.25 mM.

**Peptide Phosphorylation Assays—**Steady-state kinetic parameters were determined with the autophosphorylated IRKD as the enzyme, using an HPLC-based assay (36). Quantification of the apo and phosphopeptide were done by peak area integration with Chrome Perfect Software (Justice Innovations, Palo Alto, CA). Dead-end inhibition studies were done over 0.0–0.63 mM fluoro-IRS727, constant 0.08 mM ATP, and variable IRS939 (0.04–0.2 mM). Reactions were performed at room temperature in 50 mM bis-tris propane, 5 mM dithiothreitol, 0.05% bovine serum albumin (w/v), 2–8 nM phospho-IRKD, and 20 mM MgAc\(_2\) and at the pH values indicated in the figure legends. The pH of stock solutions of peptide, ATP, dithiothreitol, and bovine serum albumin solutions were adjusted accordingly. The reactions were initiated with the addition of peptide substrate. Kinetic parameters were determined from the best global fits of the data. The pH dependence of the observed rate constant \(k_{\text{cat}, \text{obs}}\) was fit assuming a single proton dissociation. Plots of \(\log(\text{v}_{\text{max}})\) versus pH values for fluoro-IRS727 were fit to the equation,

\[
\log(k_{\text{cat}, \text{obs}}) = \log(k_{\text{cat}}(1 + K_H))
\]  
(Eq. 1)

where \(k_{\text{cat}}\) is the pH-independent rate constant, \(H\) is the proton concentration, and \(K_H\) is the dissociation constant for the ionizable group that is active in the protonated state (\(pK_e = \log(1/K_H)\)).

Competitive inhibition kinetic analysis was done by fitting all of the data points to the linear competitive inhibition equation of KinaseAssay II\textsuperscript{TM} based on the algorithms of Cleland (39),

\[
v = V_p[S/K_{\text{cat}}(1 + IK)] + S
\]  
(Eq. 2)

using a non-linear least squares approach. The fixed substrate was assumed to be saturating. Kinetic constants \(\pm\) S.E. are reported here.

**X-ray Crystallographic Studies—**Expression and purification of the tri-phosphorylated core kinase domain of the human insulin receptor (IRK3P, residues 978–1283) has been described previously (32). Crystals were grown at 4 °C by vapor diffusion in hanging drops containing 2.0 μl of protein solution (6.5 mg/ml IRK3P and 1 mM fluoro-IRS727) and 2.0 μl of reservoir buffer (18% polyethylene glycol 6000, 100 mM Tris-HCl, pH 8.5, and 15% ethylene glycol). Small crystals (<50 μm) were obtained initially and used for microseeding to grow larger crystals (150–200 μm). Crystals belong to the trigonal space group \(P_3_1\) with unit cell dimensions \(a = b = 71.8\, \text{Å}, c = 125.8\, \text{Å}\) when frozen. There is one molecule in the asymmetric unit, and the solvent content is 48% (assuming a partial specific volume of 0.74 cm\(^3\)/g).

The 3.0 Å atomic resolution data set was collected on a Rigaku RU-200 rotating anode equipped with a Rigaku R-AXIS IIC image plate detector. The other data set was collected on a Rigaku RU-200 rotating anode equipped with an ADSC Quantum-4 CCD detector. Crystals were flash-cooled in a dry

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\(^{34\text{w}}\) Numbering of the IR and IRKD is from Ebina et al. (37).

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**FIG. 1.** Associative and dissociative reaction mechanisms for phosphoryl transfer from ATP to the phenolic hydroxyl of tyrosine. The upper pathway depicts an associative transition state in which the entering and leaving groups are closer to the γ-phosphorus atom and where the axial bond number to the entering O\(\gamma\) could be as high as one, versus a dissociative transition state, shown in the lower pathway, in which these groups are farther apart, and the axial bond number could be as low as zero.
RESULTS

The insulin receptor is not a soluble kinase; it is a hetero-
meric transmembrane protein whose intracellular kinase ac-
tivity is regulated by extracellular insulin binding (44). To
perform these studies in a manner similar to those done with
the soluble enzyme Csk, we have utilized the highly purified
and activated (autophosphorylated) cytoplasmic portion of the
IR. The IRKD was previously shown to be a good model for the
holomeric receptor’s basal and activated states, and these fea-
tures are retained by the conserved catalytic core used in
crystallization (33, 45, 46). The tetrafluorotyrosyl peptide sub-
strate (fluoro-IRS727) was chosen for these studies, because
the unmodified form (IRS727) was cocrystallized with the cat-
alytic core of the IRKD (32). The peptide-inhibition studies
were done using IRS939 as the substrate because of signal to
noise considerations in the peptide phosphorylation assays.

Kinetic Analysis—Kinase assays of IRS727 using the IRKD
revealed somewhat different kinetic parameters than those
reported previously for the full-length IR (35). This was possi-
ibly because of differences in assay procedures and kinase or
reagent purity. Here, global fitting of the data from our steady-
state assays gave the following kinetic parameters at pH 7:

\[
k_{\text{cat}} = 14 \pm 2 \text{ s}^{-1}, \quad K_m = 0.18 \pm 0.02 \text{ mM IRS727.}
\]

Analysis of fluoro-IRS727 showed that it was apparently a poorer sub-
strate than IRS727 at pH 7, with \(k_{\text{cat}} = 0.5 \pm 0.1 \text{ s}^{-1}\), and \(K_m = 0.21 \pm 0.05 \text{ mM}\). Given the observed rate reduction, and
previous claims that no phosphorylation was detected with a
peptide would be minimally active as substrate (\(k_{\text{cat}} = 0.04 \text{ s}^{-1}\))
compared with the varied peptide substrate IRS939 (\(k_{\text{cat}} = 12 \pm 2 \text{ s}^{-1}\); \(K_m = 0.040 \pm 0.002 \text{ mM}\)). Fluoro-IRS727 is a linear
competitive inhibitor against IRS939, with \(K_i = 0.59 \pm 0.14 \text{ mM}\)
determined from a global fit of the data (Fig. 3). Therefore the
phenolate form of fluoro-IRS727 binds weakly to the kinase.

To determine whether the phenolate form of the peptide
would bind to the enzyme, we measured the inhibitory proper-
tries of fluoro-IRS727 at pH 9, where the tetrafluoro phenolic
substrate would be fully deprotonated in free solution, and
the peptide would be minimally active as substrate (\(k_{\text{cat}} = 0.04 \text{ s}^{-1}\))
compared with the varied peptide substrate IRS939 (\(k_{\text{cat}} = 12 \pm 2 \text{ s}^{-1}\); \(K_m = 0.040 \pm 0.002 \text{ mM}\)). Fluoro-IRS727 is a linear
competitive inhibitor against IRS939, with \(K_i = 0.59 \pm 0.14 \text{ mM}\)
determined from a global fit of the data (Fig. 3). Therefore the
phenolate form of fluoro-IRS727 binds weakly to the kinase.

DISCUSSION

The transition states in phosphoryl transfer reactions cata-
yzed by enzymes have been the subject of lively discourse over
the past several years (1–7, 18–25, 47). Mounting evidence
suggests that these enzymes affect catalysis by enhancing but
not fundamentally altering the non-enzymatic mechanisms.
proach has provided compelling evidence to support a dissociative transition state for the reaction catalyzed by Csk (6, 26) but an associative transition state for the reaction catalyzed by IR (27, 28). There were two important concerns not addressed specifically in the study on Csk that are addressed here and that allowed us to re-evaluate the basis for an apparent discrepancy between Csk and IRK catalytic mechanisms. First, because the fluorine atomic radius is only 0.2 Å greater than the hydrogen atomic radius, these substitutions should not impose steric constraints on orientation of the P0 residue in the active site. That has been confirmed by comparison of two structures, showing the virtual absence of any positional difference between the enzyme-bound P0 tetrafluorotyrosine, reported here, and the enzyme-bound P0 tyrosine reported previously by Hubbard (32). Indeed, this shows that positioning of the phosphorylextender oxygen occurs even in the absence of MgATP (or analog), at least in a ground state binary complex. This structural observation (Fig. 4) allows greater confidence in the significance of kinetic studies using fluorine-substituted tyrosyl peptides with respect to the phosphoryltransfer mechanism of the natural tyrosine-containing substrates.

Second, it was presumed that the $pK_a$ of the fluorotyrosyl derivatives may be altered in the enzyme-bound state (27). The $k_{cat}$ versus $pH$ profile likely represents the $pK_a$ of the phenolic group when the substrate is bound to the enzyme (in the ternary complex). It is apparently elevated 1.5–2 $pH$ units when compared with aqueous solution (Fig. 2). The increased $pK_a$ in the bound state is also supported by the X-ray structure, which shows a hydrogen bond present between Asp1132 and the tetrafluorotyrosine phenol. These crystals were grown at pH 8.5, and in order for the neutral phenol to be the major species in the complex at this pH we would expect a higher $pK_a$ in this complex (perhaps 2–3 units greater than for the free peptide). These crystals lack an ATP analog that could affect the $pK_a$ of the phenolic group. Additionally, the $pK_a$ in the solid state may be harder to assess given the presence of large quantities of polyethylene glycol and other factors that might govern crystallization. However, the physical basis for $pK_a$ elevation observed in these kinetic experiments could be the electrostatic repulsion that would result from simultaneous deprotonation of the substrate-hydroxyl and the conserved catalytic aspartyl side chain, whose proximity has been established for IRK (see Ref. 32 and Fig. 4).

The elevated $pK_a$ may appear surprising at first glance, because the enzymatic reaction ultimately catalyzes proton removal concomitant with phosphoryl transfer. However, we can estimate $\beta_{nuc} \approx 0.1$ by taking into account the fact that $k_{cat}/K_m$ should represent all steps prior to the first irreversible step and that $k_{cat}/K_m$ of fluoro-IRS727 (solution $pK_a = 5.7$) is only 2-fold less than IRS727 (the tyrosyl group in solution will have a $pK_a = 10$). This small value is in good agreement with the $\beta_{nuc}$ determined for protein tyrosine kinase Csk using a broader range of fluorotyrosyl derivatives (6, 26). Therefore, the reaction mechanism for IRK favors a dissociative pathway such that facilitation of proton removal early in the reaction coordinate is unnecessary, and apparently unfavorable, as discussed above. Our finding that the phenolate form of fluoro-IRS727 was a weak competitive inhibitor and not a substrate further supports this view. It seems clear, therefore, that a neutral phenolic group of the P0 residue is required for enzymatic processing by IRK, as with Csk, and the transition states for IRK and Csk are likely to be similar.

This and other published evidence (1–3) suggests the protein-tyrosine and serine-threonine kinases, as do other enzymes involved in phosphoryl transfer of phosphate moieties, likely favor dissociative transition states.

Among the experimental approaches most consistently applied to chemical and enzymatic reactions has been linear free energy relationships to probe the mechanistic role of nucleophilicity (1, 18). Measurements of $\beta_{nuc} \leq 0.3$ for non-enzymatic phosphoryl transfer reactions involving phosphate monoesters suggested only a small impact of nucleophilicity in formation of the transition state and therefore favored a dissociative mechanism (1, 13, 18, 48–50). Extending this conclusion to protein kinases would suggest that proton abstraction, to generate the associative transition state for the reaction catalyzed by Csk (6, 26) and allows Bronsted coefficients to be determined. This approach has provided compelling evidence to support a dissociative transition state for the reaction catalyzed by Csk (6, 26) but an associative transition state for the reaction catalyzed by IR (27, 28).
Establishing the nature of the protein kinase transition states provides geometric and functional constraints for understanding the roles of individual residues in regulation of kinase activity, the factors that govern protein substrate selectivity, and the design of transition state analogs as selective inhibitors.

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