Measurement of a Brønsted Nucleophile Coefficient and Insights into the Transition State for a Protein Tyrosine Kinase

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Protein kinases are critical catalysts for cell signal transduction.[1] The nature of the transition state in ATP-dependent phosphoryl transfer reactions including protein kinases has been the subject of long-standing controversy.[2] Despite clear evidence that the corresponding nonenzymatic phosphoryl transfer reactions are dissociative, occuring with minimal nucleophile participation, it has been argued that enzyme reactions could follow an associative transition state using metals and extensive active site residue participation (Figure 1).[2] The catalytic cores of protein serine/threonine and tyrosine kinases are remarkably conserved.[1] The mechanistic details revealed below for protein tyrosine kinase Csk are thus likely to be general for protein kinases. The Brønsted nucleophile coefficient (β_{nuc}), a measure of the role of the nucleophile in the transition state, has never before been determined on an ATP-dependent kinase. Here we measure this value on the protein tyrosine kinase Csk catalyzed phosphorylation of a peptide substrate family and show strong evidence for a dissociative transition state.

Our current efforts in this area were sparked by the recent observation that a peptide with trifluorotyrosine in place of tyrosine was a catalytically efficient alternative substrate compared with the tyrosine-containing peptide.[3] This behavioral similarity was interpreted[3] as consistent with an associative model with the lower nucleophilicity of the trifluorotyrosine phenoxide anion possibly offset by its greater equilibrium bias toward the anion compared with the tyrosine phenoxide. Using the enzyme tyrosine phenol-lyase,[4] all nine possible aryl fluorinated tyrosine amino acids (**2**−**10**) (Figure 2) were prepared from the corresponding commercially available phenols.[5] The atomic radius of fluorine is only 0.2 Å larger than hydrogen but fluorine is much more electronegative and can cause marked pK_a effects.[6]

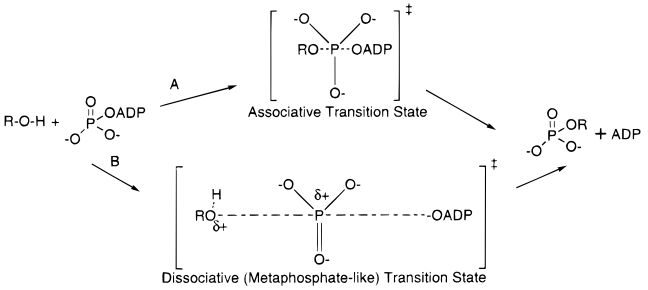

Figure 1. Associative vs dissociative transition states for phosphoryl transfer. ROH is the nucleophile (tyrosine phenol in this work) attacking the γ-phosphoryl group of ATP and ADP is the leaving group. Associative transition state (path A) is defined as more than 50% bond formation between the nucleophilic oxygen and the phosphorus occuring with at least 50% leaving group residual bond formation present.[2b] Dissociative transition state (path B) is defined as less than 50% bond formation between the nucleophile and the phosphorus occuring before the leaving group−phosphorus bond is at least 50% broken.[2b]

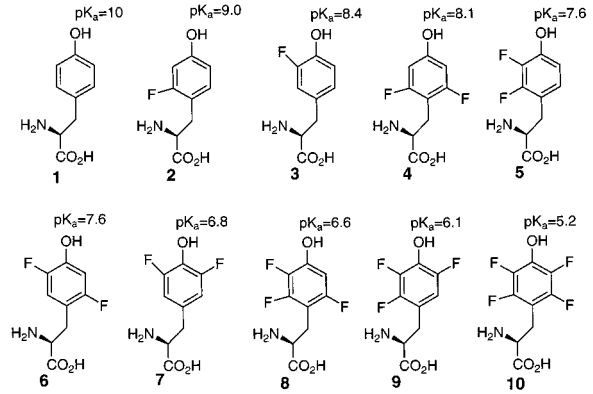

Figure 2. Fluorotyrosines (**1**−**10**) used in this work.

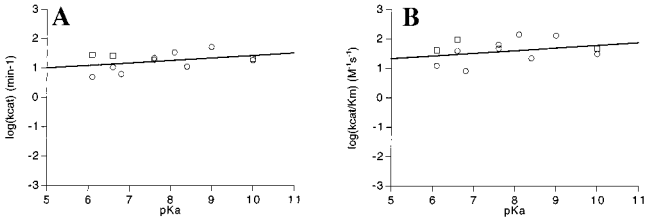

Figure 3. Brønsted plots of the rates of Csk phosphorylation of peptides containing tyrosine derivatives (**1**−**9**) vs the pK_a values of the tyrosine derivatives: (A) log(k_{cat}) vs tyrosine phenol pK_a and (B) log(k_{cat}/K_m) vs tyrosine phenol pK_a. Circles correspond to assays using Tris buffer, squares correspond to assays using MOPS buffer both at pH 7.4. Data with tetrafluorotyrosine (**10**) is omitted from the plots and calculations.

In the event, nine of these peptides (containing analogs **1**−**9**) proved to be well-behaved substrates for Csk with Mg as the divalent ion at pH 7.4, and the k_{cat} and K_m values were measured.[5] The overall correlation is reflected in the linear-free-energy plots of log(k_{cat}) vs pK_a and log(k_{cat}/K_m) vs pK_a in Figure 3. The slopes of these plots, the Brønsted coefficients $\beta_{nuc} = 0.08 \pm 0.06$ and 0.07 ± 0.08 for the k_{cat} and k_{cat}/K_m plots, respectively, quantitatively describe the weak relationship between rate and nucleophile pK_a and are very similar to a recently reported value for nonenzymatic nucleotide phosphoryl transfer reactions.[2l] Furthermore, these small values of β_{nuc}

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(5) Fluorotyrosine derivatives were prepared enzymatically and purified using the procedures of ref 4 and characterized by ^{1}H NMR, ^{19}F NMR, HRMS, and UV data. Tetrafluorotyrosine (**10**) required 10 times as much tyrosine phenol-lyase and a 14 day reaction time to achieve a comparable yield. Phenol pK_a values were determined spectrophotometrically (30 °C, see ref 3) on the free amino acids (except for tyrosine **1**, which was based on ref 11). It has previously been shown[3] that the pK_a of the phenol of **9** within the peptide is approximately 0.2−0.4 units higher than in the free amino acid. The peptides EDNEXTA (X = fluorotyrosine derivative) (see ref 3) were prepared as previously described. Kinase assays were performed as previously described[3] except using 15 mM $MgCl_2$ instead of $MnCl_2$. A minimum of five different concentrations varied 16-fold around K_m were used for k_{cat} and K_m determinations (in all cases except those peptides with derivatives **1** and **7**, concentrations greater than 2 times K_m were employed) and each assay was performed at least 2 times on separate occasions. Fits to the Michaelis−Menten equation showed $r \geq 0.99$ and standard errors for the kinetic constants were ±20% or less.

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Table 1. pK_a and Kinetic Data for Csk Phosphorylation of Peptides EDNEXTA (X = Tyrosine Derivatives (1, 8, 9, 10)) Comparing pH 6.6 and 7.4 with MOPS Buffer[a]

tyrosine analog	pH	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,min^{-1}$)	tyrosine analog	pH	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,min^{-1}$)
1	7.4	20	2900	**9**	7.4	27	2500
1	6.6	11	1300	**9**	6.6	30	3100
8	7.4	27	5800	**10**	7.4		210
8	6.6	46	5200	**10**	6.6	6.1	1300

[a] The k_{cat} for peptide with **10** could not be measured precisely.

strongly favor a dissociative mechanism, since it is very likely that in all cases (i) the chemical step is rate-determining[7] and (ii) the attacking species is the neutral phenol (see below). β_{nuc} values for dissociative, nonenzymatic phosphoryl transfer reactions of phosphate monoesters are 0−0.3.[8] β_{nuc} values for associative nonenzymatic phosphoryl transfer reactions (such as phosphate triesters) are 0.5 and larger.[8]

The similar reactivity of the differentially fluorinated tyrosines might simply represent the product of the proportion of phenoxide anion times its nucleophilicity as discussed above. As will be made clear, it is very likely that the attacking tyrosine is the neutral tyrosine phenol. So far, left out of the discussion are experiments with tetrafluorotyrosine (**10**) containing peptide. At pH 7.4, the rate of Csk phosphorylation of the peptide containing tetrafluorotyrosine (**10**) was very slow, not much above background and was difficult to quantitate (Table 1). Previous work with the insulin receptor tyrosine kinase also demonstrated that a peptide with tetrafluorotyrosine (**10**) was not an insulin receptor tyrosine kinase substrate.[9]

The intriguing possibility for this decreased activity was a difference in the protonation state of the tyrosine hydroxyl at pH 7.4 of tetrafluorotyrosine (**10**) compared to that of trifluorotyrosines **8** and **9**. That is, the more chemically reactive phenoxide anion species might actually be less reactive in the enzyme-catalyzed reaction. To test this proposal, an investigation of the rate of the Csk kinase reaction at more acidic pH was performed. Indeed, by decreasing the reaction buffer pH to 6.6, Csk reaction with tetrafluorotyrosine **10** was "rescued" and peptide phosphorylation rate (k_{cat}/K_m) increased dramatically (about 6-fold, Table 1). At this lower pH, about 6-fold more of the tetrafluorophenol should be in the neutral form relative to the phenoxide form. In contrast, the tyrosine peptide **1** exhibited a modest fall in rate at the lower pH (k_{cat} and k_{cat}/K_m showed a 50−60% decrease at pH 6.6 compared to at pH 7.4) and peptides with derivatives **8** and **9** showed intermediate effects. The simplest interpretation of these results is that the neutral phenol is the preferred enzymatic nucleophile compared to the phenoxide anion. That the preferred enzymatic nucleophile is the neutral phenol (a poor nucleophile) rather than the phenoxide anion (a good nucleophile) is most consistent with a dissociative mechanism.[10]

Thus, the dramatic rate acceleration (ca. 10^8-fold compared to the uncatalyzed rate)[12] effected by protein tyrosine kinase Csk and other protein kinases is probably not achieved by making the γ-phosphate more receptive to nucleophilic attack, or the tyrosine a better nucleophile, as has been widely speculated. How an enzyme can facilitate a dissociative transition state is still not known with certainty but presumably centers on activating the departure of the leaving group (in this case ADP).[2b] The function of a catalytic base if any is therefore likely to be deprotonating the tyrosine hydroxyl after tyrosine O−P bond formation is well advanced on the reaction coordinate. The roles of the residues and metal interactions previously considered to activate the γ-phosphate for attack by a nucleophile[13,14] must be reconsidered in light of this work. It is likely that they play an important role in active site orientation and organization. The negatively charged phenoxide anion is probably unable to orient for attack because of active site repulsion by other negative charges.

Protein kinase selectivity for tyrosine vs serine/threonine substrates is unlikely to be related to the nucleophilic differences between these substrate classes. Rather, it is probably based on geometric constraints. Intramolecular autophosphorylation might be more readily accommodated in a dissociative mechanism because of the greater predicted transition state distance between nucleophile and phosphate. Investigation into the role of active site residues in catalysis and regulation, and the design of transition state analog inhibitors,[15] should be guided by these transition state requirements.

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(7) The dissociative interpretation in this work presumes that the chemical step involving tyrosine attack is rate-determining for this enzyme reaction with this substrate family. Evidence for a rate-determining chemical step includes: (i) the reactions are all slower (k_{cat} 2−20-fold decreased) than Csk kinase reactions with other peptide substrates (see ref 2g, also unpublished data from P.A.C.) where viscosity effect measurements indicate product diffusional release is fast with Mg as the divalent ion, (ii) the peptides have high K_m values (millimolar), the K_m values likely equal K_d values,[2c,g,3] and the corresponding product phosphotyrosine peptides should have even lower affinity for the enzyme (Boerner, R. J.; Kassel, D. B.; Edison, A. M.; Knight, W. B. *Biochemistry* **1995**, *34*, 14852), (iii) subtle changes in the aromatic ring substitution lead to 2−3-fold *increases and decreases* in k_{cat} compared to tyrosine, arguing against an enzyme conformational step being rate-determining, (iv) thio effects [k_{cat}/K_m-ATP/k_{cat}/K_m-ATPγS] are nearly identical (ca. 20; using Mn as the divalent cation) for both the peptide containing **1** and the trifluorotyrosine analog **8**, (v) direct phosphoryl transfer occurs with no covalent enzyme intermediate (only 1 chemical step).[2c,f]

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(10) Since the attacking species is the neutral phenol, the linear free energy relationship depicted in Figure 3 should formally plot the pK_a values of the corresponding protonated phenols (ROH−H^+). These pK_a values (<−1) are very low and not precisely measurable; however, the contributions of the fluorines to the related anilines can be used as a surrogate. *m*-Fluorines lower the pK_a values of the anilines about 1 unit and *o*-fluorines about 1.4 units relative to hydrogen.[11] These fluorine substitution effects are likely additive, similar to their effects on phenols.[11] Thus, the slope of the Brønsted plot (β_{nuc}) where the horizontal axis profiles the pK_a values of the protonated phenols should be essentially identical to that of Figure 3. In principle, a general base could lower the value of β_{nuc} in an associative mechanism. If a general base were operative at the peak of the transition state, the transition state would be expected to be phenoxide-ion-like, which there is no evidence for with Csk. Evidence against this possibility is that the neutral phenol is the required attacking nucleophile. In a typical nonenzymatic associative mechanism (β_{nuc} = 0.6), the phenoxide anion would be expected to be 10^8-fold more reactive (11 kcal/mol) than the neutral phenol, which would be expected to overcome even significant (3−4 kcal/mol) orientational problems for the anion in the enzyme active site.

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