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Published on Web 05/02/2002

Proton Demand Inversion in a Mutant Protein Tyrosine Kinase Reaction

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Received February 21, 2002

Phosphoryl-transfer reactions are involved in many important biological processes and have been the subject of investigation in enzymatic and nonenzymatic systems for over 40 years.[1] Elucidating the mechanism of phosphoryl transfer is consequently not only of fundamental chemical importance, it may also be valuable for the design of specific enzyme inhibitors with therapeutic potential.[2] Although there has been mounting experimental support for a dissociative transition state for nonenzymatic[3] and, to a lesser extent, enzymatic phosphoryl-transfer reactions[4] (Figure 1A), there is not universal agreement on these mechanisms.[5] In fact for phosphorylation reactions involving hydroxy nucleophiles, recent ab initio calculations and other theoretical arguments have been put forward in favor of an alternative model involving initial proton transfer from hydroxy to phosphate oxygen (diester-like intermediate) preceding nucleophile−phosphorus bond formation via an associative pathway (Figure 1B).[6,7] This model is difficult to rigorously exclude because of the presumed instability of the proposed intermediate. While a small β_{nuc} (Brønsted nucleophile coefficient) and requirement of the substrate neutral phenol form rather than the chemically more reactive phenoxide anion in protein tyrosine kinase-catalyzed reactions have been interpreted to support a dissociative pathway,[8] these results could also be explained by a hydroxy nucleophile to phosphate proton-transfer model (Figure 1B).[6] In fact, the molecular basis for the paradoxical neutral phenol preference in the tyrosine kinase reactions is not well understood. In this report, we use the complementarity of site-directed mutagenesis and synthetic substrate analogues to shed light on this unusual neutral phenol requirement and further strengthen the dissociative mechanistic model for protein tyrosine kinases.

All protein tyrosine (and serine/threonine) kinases have a highly conserved catalytic loop aspartate which is sometimes called the catalytic base.[9] Earlier studies have shown that mutating this residue leads to a significant reduction in the rate of catalysis.[9,10] Previously, it was proposed that the carboxylate group of the aspartate side chain could contribute to the requirement for the neutral phenol form of the substrate because of its electrostatic repulsion of the phenoxide ion.[8] We hypothesized that mutation of this aspartate residue in the protein tyrosine kinase Csk to a neutral asparagine might change the substrate preference.

To enhance the possibility of observing residual activity, we employed the optimized[11] substrate peptide KKKKEEIYFFF and developed a sensitive radioactive assay.[12] The rate of D314N Csk kinase activity decreased to below the level of detection (>10^5-fold slower than the WT-promoted reaction) with the tyrosine-containing undecapeptide. In contrast, the identical sequence containing tetrafluorotyrosine (**6**) (Figure 2A) in place of tyrosine (**1**) was phosphorylated by D314N Csk (k_{cat}/K_m = 0.94 M^{-1} min^{-1}), albeit ~5000-fold more slowly than with wild-type Csk (k_{cat}/K_m = 4900 M^{-1} min^{-1}) at pH 7.4.[12] On the basis of the limit of detection, the catalytic efficiency (k_{cat}/K_m) of the D314N Csk-promoted reaction with (**6**)-containing peptide substrate was at least 15-fold greater than the tyrosine-containing peptide at pH 7.4. In contrast, there is a significant preference for (**1**) (k_{cat}/K_m = 95 000 M^{-1} min^{-1}) versus (**6**) (k_{cat}/K_m = 4900 M^{-1} min^{-1}) with the wild-type enzyme at pH 7.4.

One explanation for the switch in substrate preference is that D314N Csk employs the substrate phenoxide anion form rather than

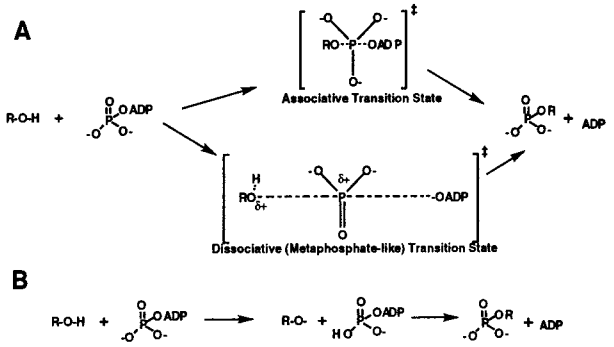

Figure 1. (A) Schematic of an associative vs dissociative transition state for phosphoryl transfer. The associative transition state involves substantial bond formation between nucleophile and phosphorus, while the dissociative transition state occurs with minimal nucleophile participation. For further details, see refs 3−5. (B) Proposed mechanism of nucleophile hydroxy-to-phosphate proton transfer occurring prior to nucleophile O−P bond formation.[6]

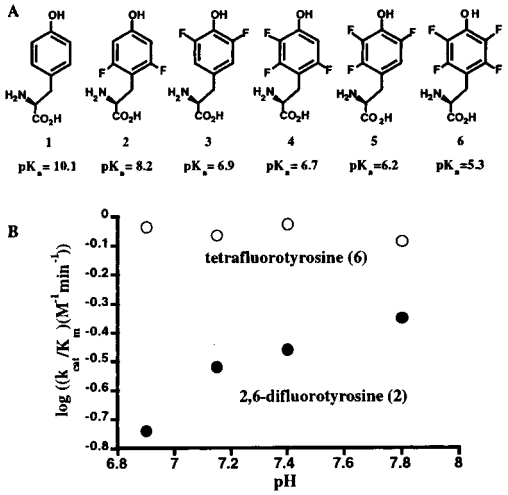

Figure 2. (A) Fluorotyrosine analogues incorporated into KKKKEEI**X**FFF (**X** = tyrosine analog). The pK_a shown is that of the tyrosine phenol in the undecapeptide. (B) The pH rate profile of D314N Csk-promoted phosphorylation of **6**-containing (○) and **2**-containing (●) undecapeptide.

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10.1021/ja025993a CCC: $22.00

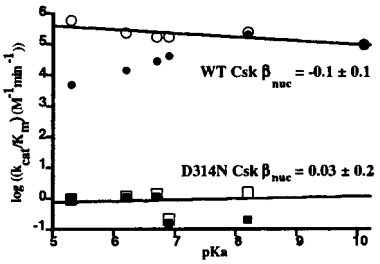

Figure 3. Brønsted plot of log (k_{cat}/K_m) versus undecapeptide phenol pK_a for WT (circles) and D314N (squares) Csk-catalyzed phosphorylation of tyrosine by ATP. The darkened circles and squares show uncorrected data, while the open circles and squares are data-corrected by the Henderson−Hasselbalch equation to show the rate of the neutral phenol-requiring reaction for WT Csk and the rate of the phenolate ion-requiring reaction for D314N Csk.

the neutral phenol form (required by the wild-type enzyme) for catalytic processing. To explore this possibility further, pH-rate studies were carried out between pH 6.9 and 7.8. Whereas wild-type Csk-catalyzed reactions showed a pronounced k_{cat}/K_m decrease with increasing pH with the substrate peptide containing **6**[12] in accord with previous studies,[2a,8b] the k_{cat}/K_m with D314N Csk was pH-independent in this region (Figure 2), consistent with the requirement for a substrate phenoxide anion nucleophile with the mutant enzyme. In comparison, kinase reactions with the peptide containing **1** and wild-type Csk showed a k_{cat}/K_m that was pH-independent in this pH range[12] (analogous to earlier studies),[2a,8b] while the D314N Csk-promoted reaction with peptide containing **2** displayed a k_{cat}/K_m that dropped steeply with decreasing pH (Figure 2). Taken together, these results strongly suggest that with D314N Csk, in contrast to wild-type Csk, the phenoxide form of the substrate is required.

One possibility for the requirement of the phenoxide anion nucleophile in catalysis by D314N Csk is that the reaction mechanism involves an associative transition state, since the negatively charged species is much more reactive as a nucleophile than the neutral phenol. We investigated the effect of the basicity of the nucleophile on the reaction rate by measuring the β_{nuc} with the KKKKEEIXFFF undecapeptide containing five different (di-, tri-, and tetra-) fluorotyrosine derivatives (Figures 2, 3). As discussed previously,[13] the β_{nuc} is simpler to interpret as a test of the nature of the enzymatic transition state when unencumbered by differences in the nucleophile protonation state of the various substrates. Since the KKKKEEIYFFF substrates show high K_m,[11] these measurements were made under k_{cat}/K_m conditions.[12] After correcting for the concentration of the phenoxide ion form, the β_{nuc} was close to zero[14] (0.03 ± 0.20) which places it in a range (<0.3) that is characteristic of dissociative transition states (Figure 3). An analogous plot with wild-type Csk shows a similar near zero β_{nuc}, assuming the neutral form of the nucleophile is active (Figure 3), similar to previous results with a different peptide substrate.[8] Given the slowness of the D314N Csk-catalyzed reaction, it was very unlikely that a diffusional step or enzyme conformational change would be rate-determining,[12] which could be an alternative explanation of a small β_{nuc}.

On the basis of the measurement of a small β_{nuc}, D314N Csk-catalyzed phosphoryl transfer is likely to follow a dissociative transition state. It is interesting to consider why D314N Csk is catalytically defective. The role of Asp-314 may be critical in orientation of the substrate phenol. Similar orientation effects have been observed with Csk Arg-318.[15,16] In addition, the catalytic deficiency of D314N Csk could result from Asp-314 playing a role in deprotonation late in the transition state.[8,17]

Since the phenoxide-containing peptide substrate in the D314N Csk-catalyzed reaction lacks a proton to be transferred to phosphate, it appears implausible for the reaction mechanism to involve substrate-to-phosphate proton transfer (Figure 1B). Even if proton transfer from another proton donor to phosphate were occurring, the low β_{nuc} is still supportive of a dissociative transition state. Although the D314N mutant enzyme is significantly impaired when compared to the wild-type Csk, the rate by which it still catalyzes phosphoryl transfer is $\sim 10^4$-fold faster than a comparable spontaneous reaction rate.[18] Additionally, no major structural reorganization is expected on the basis of the change from aspartic acid to asparagine. Thus, the evidence here for a dissociative transition state for the mutant enzyme reaction strongly supports the involvement of a dissociative transition state for the wild-type enzyme as well.

Acknowledgment. We thank NIH for financial support and the Cole lab for helpful discussions.

Supporting Information Available: Experimental details, Csk WT pH rate profile (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA025993A