The Role of the Catalytic Base in the Protein Tyrosine Kinase Csk*

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A potential distinguishing feature between protein tyrosine kinases and homologous serine/threonine kinases is the function of the catalytic base in these enzymes. In this study, we show that a peptide containing the unnatural amino acid trifluorotyrosine shows remarkably similar efficiency as a substrate of the tyrosine kinase Csk (C-terminal Src kinase) compared with the corresponding tyrosine-containing peptide despite a 4-unit change in the phenolic pKₐ. These results argue against the importance of early tyrosine deprotonation by a catalytic base in Csk. To further explore the role of the proposed catalytic base, the Csk mutant protein D314E was produced. This mutant displayed a significant reduction in kₐ (approximately 10⁴) but relatively little effect on substrate kₗ values compared with wild-type Csk. Examination of the thio effect (kₐ-ATP/kₐ-adenosine 5'-O-(thiotriphosphate)) for D314E Csk led to the suggestion that a role of aspartate 314 may be to enhance the reactivity of the γ-phosphate of ATP toward electrophilic attack. These results may have significant impact on protein tyrosine kinase inhibitor design.

Protein tyrosine kinases play central roles in cell proliferation, cell differentiation, and signaling processes (1). While their enzymatic activity, transfer of a phosphoryl group from ATP to tyrosines in proteins, was first described over 15 years ago, details of tyrosine kinase catalytic mechanism have largely remained elusive. In contrast, their homologous family member protein serine/threonine kinases have been more intensively studied and are better characterized enzymically (2–8). In order to rationally develop specific inhibitors for these enzymes (9) as well as to delineate the biochemical consequences of their in vivo regulation, a more complete understanding of tyrosine kinase catalysis is needed.

Recently, we carried out a preliminary mechanistic evaluation of the protein tyrosine kinase Csk (C-terminal Src kinase) from humans (10), overproduced in and purified from Escherichia coli (11). It was shown that, like the better studied serine/threonine kinase protein kinase A, the Csk-catalyzed kinase reaction obeys a ternary complex mechanism, most likely with rapid and random binding of the substrates ATP and 4:1 poly(Glu,Tyr). This argued against a covalent phosphoenzyme intermediate. Moreover, using ATP·γS and microviscosity effects, it was shown that in the Csk ATP reaction, product ADP diffusional release is partially rate-determining for the overall reaction (10). The lack of a large deuterium solvent kinetic isotope effect (actually a slightly inverse, kₗ HD/kₗ D = 0.74, was seen) in the ATP·γS reaction (where the chemical step appeared fully rate-determining) suggested that tyrosine hydroxyl deprotonation is asymmetric in the transition state. Because a tetrafluorotyrosine-containing peptide was previously reported to be a tight binding inhibitor (and not detectably a substrate) for insulin receptor tyrosine kinase (12), a phenoxide anion was reasoned to be transition state like (10). The phenoxide anion was proposed to be the species attacking the γ-phosphate of ATP (see Fig. 1). In principle, the role of a catalytic base may be a mechanistically distinguishing feature between serine/threonine and tyrosine kinases because the pKₐ values of tyrosine hydroxyls (approximately 10) are significantly lower than those of serine or threonine hydroxyls (approximately 13–15) (2–5, 7, 8).

MATERIALS AND METHODS

Synthesis of Trifluorotyrosine(F₃Y)—Reaction between trifluorophenol, pyruvate, and NH₄OH catalyzed by tyrosine phenol-lyase was carried out analogous to previously described methods (13, 14). Pure trifluorotyrosine (0.14 g) was obtained and gave satisfactory spectroscopic and analytical data.

pKₐ Measurements—The pKₐ measurements of the trifluorotyrosine and EDNE(F₃Y)TA phenolic hydroxyls were performed by monitoring the UV absorption at 274 nm. Buffers used included 150 mM NaCl and 30 mM sodium acetate (pH 4.1–4.8), Na-MES (pH 5.1–6.6), Na-MOPS (pH 6.6–7.3), Tris-HCl (pH 7.6–9.0). Hill coefficients for the pKₐ values obtained in this way were 0.99–1.03.

Peptide Synthesis—Peptides were made on a Biosearch 9600 automated peptide synthesizer. They were purified by high pressure liquid chromatography using C-18 reverse-phase chromatography (with a H₂O/acetonitrile/trifluoracetic acid solvent system) and were characterized by mass spectrometry.

Inhibition of Csk Phosphorylation of Poly(Glu,Tyr) by Peptides EDNEYTA and EDNE(F₃Y)TA—Enzyme reactions were carried out as previously described (10).

Csk-mediated Phosphorylation of EDNE(F₃Y)TA Monitored by 19F NMR—Reactions (0.4 ml) that included EDNE(F₃Y)TA (3 mM), ATP (0.5 mM), PEP (5 mM), MnCl₂ (5 mM), NaDH (5 mM), DTT (10 mM), pyruvate kinase-lactate dehydrogenase (20 μg, 3:1), Tris-HCl (60 mM, pH 7.4). The reactions were incubated without or with wild-type Csk (2.6 mg, 3:1), Tris-HCl (60 mM, pH 7.4) were incubated without or with wild-type Csk (2.6 mg) at 30°C for 45 h. 19F NMR (376 MHz) spectra were obtained on the mixtures after adding 25 μl of D₂O (0.5 ml) with 0.2 ml of D₃O.

Kinase Assays of Csk with EDNE(F₃Y)TA and EDNE(Y)TA as Substrates—Assays were carried out with 0.5 mM ATP, 3 mM MnCl₂, PEP (1 mM), DTT (10 mM), NaDH (0.19 mM), pyruvate kinase-lactate dehydrogenase (20 μg, 3:1), Csk (800 nm), Tris-HCl (60 mM, pH 7.4), and variable peptide concentrations (for EDNEYTA, 1–30 mM; for EDNE(F₃Y)TA, 1–16 mM) in 0.4 ml at 30°C and quantitatively monitored by UV absorbance change at 340 nm (15). Calibration experiments with poly(Glu,Tyr) showed that the radioactive assay gave results that were quantitatively similar (within 30%) compared with this coupled assay, with no evidence of uncoupling.

The abbreviations used are: ATP, adenosine 5'-O-(3'-thiotriphosphate); BSA, bovine serum albumin; DTT, dithiothreitol; Fmoc, (9-fluorenyl)methylxycarbonyl; F₃Y, trifluorotyrosine; MES, 2-(N-morpholinol)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate.
plating” (ADP formation in the absence of phosphorylated poly(Glu,Tyr)). Kinetic constants were calculated using the rectangular hyperbola curve fit in Kinetasyst and displayed in the text ± S.E.

D314E Csk Construction—The D314E Csk mutant was generated by PCR mutagenesis using standard molecular biological procedures and confirmed by DNA sequencing. Expression and purification to near homogeneity of the D314E Csk were carried out analogous to previously described methods (11). Enzyme concentrations were measured by Bradford assay, which for wild-type Csk has been standardized to the method of von Hippel (16).

D314E Csk Kinase Assays—These assays were performed as previously described (10) for wild-type Csk with minor modifications. Enzyme activity proved to be linear versus time up to 45 min and linear versus enzyme concentration up to 10.5 μM, and autophosphorylation was insignificant.

RESULTS AND DISCUSSION

To further explore the role of a proposed active site catalytic base, we decided to pursue studies on Csk using an unnatural fluoro-tyrosine-substituted peptide. The goal was to determine the inhibitory properties toward Csk of an isoster of tyrosine analog with a reduced pKₐ. Using tyrosine phenol-lyase (13, 14), trifluorotyrosine was generated (see Fig. 2A) and after conversion to the Fmoc derivative (17) incorporated into the Src autophosphorylation peptide EDNE(F₃Y)TA by solid phase peptide synthesis. EDNEYTA has previously been reported to be a Csk substrate (18), and we confirmed that here (kₓ = 14 ± 0.4 min⁻¹, Kₓ = 6.1 ± 0.4 mW, see Fig. 2D). The enzymatically synthesized trifluorotyrosine was presumed enantiomerically pure (with L-configuration) and was measured by spectrophotometric titration to have a phenolic pKₐ of 6.2 ± 0.3. The pKₐ of the trifluorotyrosine in EDNE(F₃Y)TA was found to be 6.5 ± 0.2. These pKₐ values are approximately 4 units below that of standard tyrosine. Although they are marginally higher than the pKₐ measured for tetrafluorotyrosine (pKₐ = 5.4) (19) used for study with the insulin receptor tyrosine kinase, the pKₐ of 6.5 implies that approximately 90% of EDNE(F₃Y)TA exists as the phenoxide anion at pH 7.4 (the enzyme assay pH).

Initially, EDNE(F₃Y)TA was tested as a Csk competitive inhibitor with poly(Glu,Tyr) as the tyrosine-containing substrate. As shown, EDNE(F₃Y)TA exhibited similar inhibitory properties to EDNEYTA (see Fig. 2B). This similarity was surprising given the potency reported for the tetrafluorotyrosine-containing peptide toward insulin receptor kinase (Kᵢ = 4 μM; Kₓ = 400 μM) (12). A careful examination of the reported data in this paper reveals that the o- and l-tetrafluorotyrosine analog-containing peptides show very similar inhibitory properties (12) when plotted against varying lysozyme substrate concentrations. This lack of stereospecificity, as well as the lack of reported data comparing the corresponding tyrosine and tetrafluorotyrosine-containing peptides as inhibitors of lysozyme phosphorylation (12), led us to question the Kᵢ determinations and mechanistic interpretations.

The peptide EDNE(F₃Y)TA was next investigated as a Csk substrate. As mentioned, the tetrafluorotyrosine-containing peptide was reported not to be a substrate for insulin receptor tyrosine kinase (12). Much to our surprise, EDNE(F₃Y)TA was found to be a substrate for Csk by monitoring ADP formation using a spectrophotometric coupled assay (15). A large scale reaction with EDNE(F₃Y)TA was followed by ¹⁹F NMR. As shown in Fig. 2C, there is nearly complete conversion of unphosphorylated EDNE(F₃Y)TA to phosphorylated EDNE(F₃Y)TA catalyzed by Csk as evidenced by the downfield shifting of the three fluorine signals. Note that the signals at -141 and -160 ppm of EDNE(F₃Y)TA represent the fluorines closest to the hydroxyl because their chemical shifts are moved most downfield by phosphorylation.

Remarkably, EDNE(F₃Y)TA was a very efficient Csk substrate, with a Kᵢ (12 ± 1 mW) and kₓ (18 ± 0.9 min⁻¹) quite...
EDNE(F3Y)TA, the reaction is driven toward phosphorylation similar (within 1 kcal/mol) to the free energy of hydrolysis of adenylyl-(favoring ATP). Note that the free energy of hydrolysis of the equilibrium constant for the reaction may lie to the left (insulin receptor) tyrosine kinase was being studied, several kinasesubstratymayhavebeenduetothefactthatadifferent rafluorotyrosine-containing gastrin peptidetoactasatyrosine receptor tyrosine kinase (12). Although the lack of the tetrafluorotyrosine-containing gastrin peptide and the insulin is particularly striking in light of the reported results with the fluorocontaining substrate. In this case, the nucleophilicity of the trifluorophenoxide anion could be expected to be comparable with the largely protonated phenol.

As the above studies led us to question the importance of catalytic base function in the Csk kinase reaction, the site-directed Csk mutant D314E was generated. Aspartate 314 is one of 4 invariant protein kinase amino acid residues (22). In protein kinase A, the corresponding aspartate (Asp-166) has been identified near the substrate serine hydroxyl and predicted to be the catalytic base for this enzyme from x-ray crystallographic analysis (23), although additional roles for this residue have been considered (3). Note that although Csk DNA constructs for D314N and D314A were also generated, protein expression was not detectable with these mutants.

The Csk mutant D314E had greatly reduced enzyme activity vs. the wild-type enzyme. *K*ₘ and *k*ₐₐₕ values were measured for D314E Csk. The *K*ₘ values for ATP (35 ± 8 μM) and poly(Glu,Tyr) (88 ± 12 μM) are rather similar to those of wild-type Csk (ATP *K*₂ = 12 ± 1 μM; poly(Glu,Tyr) *K*₂ = 48 ± 2 μM/μM), but the *k*ₐₐₕ is reduced by a factor of approximately 10⁴ (*k*ₐₐₕ for wild-type Csk ~40 min⁻¹; *k*ₐₐₕ for D314E Csk ~0.005 min⁻¹) (10). That this enzyme activity in D314E Csk was not due to trace wild-type contamination was most convincingly demonstrated by examining the sucrose microviscosity effect. Behaving similar to the microviscosity effect on the ATP*ψ*, wild-type Csk reaction (slope = −0.22 ± 0.03) (10), there is a modest fall in *k*ₐₐₕ-control/*k*ₐₐₕ-visco- gen with increasing levels of sucrose for the ATP, D314E Csk reaction (slope = −0.28 ± 0.06) (see Fig. 3). This is clearly different from the sucrose microviscosity effect on the ATP Csk reaction (slope = −0.42 ± 0.04) (see Fig. 3). It further suggests that as expected the chemical step is fully rate-determining in both the Csk mutant D314E, ATP reaction, and the ATP*ψ*, wild-type Csk reaction.

The substantial rate loss associated with D314E Csk compared with wild-type Csk was very surprising given the trifluorotyrosine results described above. That large structural perturbations in D314E Csk could account for the larger than anticipated *k*ₐₐₕ effect cannot be ruled out. The rather minor *K*₂ changes for both ATP and poly(Glu,Tyr) with D314E Csk as well as a similar sucrose microviscosity effect to the wild-type Csk ATP⁴S reaction weaken such an argument, however. Further support for the structural integrity of D314E Csk comes from data using phospho-Ultrogel affinity chromatography (11) and native polyacrylamide gel electrophoresis, where behavior proved to be identical to wild-type Csk (data not shown).
As the trifluorotyrosine studies argued against early tyrosine deprotonation in the Csk catalytic mechanism, it was not surprising that, like the apparent $k_{cat}$ of the wild type Csk reaction, $k_{cat}$ is unaffected by increasing the pH of the D314E Csk kinase reaction from 7.4 to 8.8. Furthermore, the deuterium solvent kinetic isotope effect on $k_{cat}$ measured with D314E Csk (at pH 7.4) is actually modestly inverse ($k_{cat}$/$k_{cat}$D = 0.55 ± 0.08), arguing against a proton transfer step becoming dominant in the transition state of the mutant. A significant, standard kinetic isotope effect ($k_{cat}$/$k_{cat}$D > 2) might have been expected with disruption of catalytic base function.

At this point, we were unable to fully explain the rather dramatic 10^4-fold rate reduction in the D314E Csk mutant. Clearly, aspartate 314 could be important in hydrogen bonding and orienting the tyrosine hydroxyl toward the γ-phosphate. Results of the D314E Csk mutant with ATPγS suggested another important function, however. The thio effect ($k_{cat}$-ATP/$k_{cat}$-ATPγS) (24) in the wild-type reaction is 16.9 ± 0.6 (corrected = 34 assuming that the rate of ADP release is equal to the chemical step) (10). In contrast, the thio effect in the mutant reaction is 4.8 ± 0.6. Recently, it has been pointed out that the thio effect for non-enzymatic (non-acid catalyzed) phosphor transfer is different for phosphotriesters versus phosphodiesters (24). For phosphotriester reactions, the usual thio effect is in the range of 10–200 (25), whereas for phosphodiester reactions, the range is 4–11 (26). While enzymatic thio effects can be more complex to interpret because of subtle changes in substrate-active site interactions, it is tempting to rationalize the difference in thio effects for wild-type and mutant Csk as being related to a change in transition state from triester-like to diester-like.

Triesters typically react much faster (10^3–10^5-fold) under non-acidic conditions with nucleophiles than diesters, probably because of the enhanced electrophilicity of the phosphorus in the former (27, 28). Thus one might anticipate a large rate reduction to be associated with rendering the γ-phosphate of ATP more diester-like in the enzyme active site. For example, one hydrogen bond donor to one of the oxyanions on the γ-phosphorus could be disrupted in Csk D314E. Extending this proposal, Asp-314 could actually serve as the γ-phosphate in the wild-type enzyme, but because of its altered side chain, Glu-314 is unable to fulfill this role. An alternative possibility is that the wild-type aspartate 314 enforces a structure that allows another active site functional group to hydrogen bond to the γ-phosphate. In this scenario, D314E mutation indirectly destabilizes a hydrogen bond to the γ-phosphate by altering the active site organization.

Although a three-dimensional structure of a catalytically active tyrosine kinase with bound substrates or inhibitors remains to be reported (29), it seems reasonable to suggest that the "catalytic base" in Csk and other tyrosine kinases may have a dual role. The "catalytic base" in Csk may still be important in hydrogen bonding the tyrosine phenol and orienting it in the active site (3). Concomitantly, it could serve to render the γ-phosphate more acidic by hydrogen bonding either directly ($X = H^+$) or indirectly ($X = metal$, amino acid side chain) (see Fig. 4). ATP activation may be particularly important in tyrosine kinase reactions where the nucleophilicity of the attacking oxygen may be reduced by aryl conjugation compared with alkyl alcohols of serine/threonine kinases.

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