Chemical Rescue of a Mutant Protein-tyrosine Kinase*

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Protein-tyrosine kinases contain a catalytic loop Arg residue located either two or four positions downstream of a highly conserved Asp residue. In this study, the role of this Arg (Arg-318) in the protein-tyrosine kinase C-terminal Src kinase (Csk) was investigated. The observed $k_{cat}$ for phosphorylation of the random copolymer poly(Glu,Tyr) substrate by Csk R318A is ~3000-fold smaller compared with that of wild type Csk, whereas the $K_{m}$ values for ATP and poly(Glu,Tyr) are only mildly affected. The $k_{cat}$ value for poly(Glu,Tyr) phosphorylation by the Csk double mutant A316R,R318A is 100-fold greater than the $k_{cat}$ value for the single R318A mutant, suggesting that an Arg positioned at the alternative location fulfills a similar function as in wild type. Csk R318A kinase activity can also be partially recovered by several exogenous small molecules including guanidinium and imidazole. These molecules contain key features whose roles in catalysis can be rationalized from a known x-ray structure of the insulin receptor tyrosine kinase. Imidazole is the best of these activators, enhancing phosphorylation rates by Csk R318A up to 100-fold for poly(Glu,Tyr) and significantly stimulating Csk R318A phosphorylation of the physiologic substrate Src. This chemical rescue of mutant protein kinase activity might find applications in cell signal transduction experiments.

Protein-serine/threonine and -tyrosine kinases are important catalysts in cell signal transduction (1–2). As a consequence, there has been considerable interest in developing novel tools to enhance our current understanding of protein kinase function (3–11). One particularly interesting method to study the function of protein kinases has been developed by McMahon and co-workers (3). In this approach, the kinase domain is genetically fused to the estrogen receptor ligand binding domain. It has been found that the catalytic activity of the fused kinase is stimulated if estrogen ligands are added to the cell culture media. This powerful technology has been used to characterize the targets and functions of raf kinase (3, 4). However, the potentially unwanted biological impact of the additional estrogen receptor domain suggests that the development of alternative, complementary approaches is desirable.

There are a number of reported examples where mutant, catalytically defective enzymes have been reactivated by introducing small molecules that have the requisite properties of the altered residues (12–23). By making slight structural changes in the small molecule activators, it is possible to learn more about the specific molecular contributions of the altered amino acid residue toward catalysis. Our goal was to apply this chemical rescue technique to a mutant, catalytically impaired protein kinase for eventual use in signaling studies.

The catalytic loop of protein-tyrosine and -serine/threonine kinases is highly conserved among each of these enzyme families. In serine/threonine kinases, a lysine residue is found at the $n + 2$ position, where $n$ is a critical catalytic loop aspartate residue. In protein-tyrosine kinases, an arginine residue is instead present in the catalytic loop at either the $n + 2$ position or $n + 4$ position (Fig. 1). X-ray crystallography of a peptide substrate and ATP analogue complexed to the insulin receptor-protein-tyrosine kinase catalytic domain has shown that this catalytic loop arginine residue is within hydrogen bonding distance of the substrate tyrosine phenol, as well as the highly conserved aspartate (Fig. 2) (24). To date, the functional role and contributions of this arginine to protein-tyrosine kinase catalysis have not been investigated in detail but we hypothesized that it might be a good candidate for external replacement given its variable site location in protein-tyrosine kinases.

In this study, we examined the role of the protein-tyrosine kinase Csk (1 catalytic loop arginine in substrate phosphorylation. Csk is a protein-tyrosine kinase with importance in inhibiting Src family members, because it catalyzes the site-specific phosphorylation of Src protein tails (25–26). Csk has proven to be a particularly useful system for exploring mechanistic and substrate selectivity issues in protein-tyrosine kinase function (27–32), because it does not undergo autophosphorylation to any significant extent.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents were purchased from commercially available sources unless otherwise indicated. Appropriate primers for the QuickChange® mutagenesis were purchased from Integrated Data Technologies and were purified by either preparative PAGE or standard desalting techniques prior to use. Catalytically inactive chicken Src K295M protein was expressed and purified from Escherichia coli. ¹

**Preparation of GST-Csk and Its Mutant**—The Csk gene was cloned into the pGEX-3Xb vector (Amerham Pharmacia Biotech) via N-terminal NdeI and C-terminal HindIII restriction sites. All GST-Csk mutants were prepared using the Quick-Change® procedure by Stratagene. For all mutants, DNA sequencing confirmed the presence of only the desired mutation.

¹ The abbreviations used are: Csk, C-terminal Src kinase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; BSA, bovine serum albumin.

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and its mutants were estimated to be; yields ranged from between 2–5 mg/liter of SDS-PAGE stained with Coomassie Blue (Fig. 3). The protein Rutgers University, New Brunswick, NJ (Protein Data Bank number pertinent interatomic distances. The crystal structure was viewed using the insulin receptor protein-tyrosine kinase complexed to peptide substrate and ATP analogue (24).

The proteins were overexpressed and purified analogously to previously described methods (27) with the exception that the DH5α strain of E. coli cells were grown at 37 °C to A595 = 0.6 prior to induction with 0.4 mM IPTG at 16 °C for 22 h. GST-Csk was used in place of Mn (30). The GST-Csk R318K mutant showed only about a 180-fold cat compared with that of wild type enzyme GST-Csk. The GST-Csk R318K, and GST-Csk R318H, actually less catalytically active than R318A or R318Q mutants. Interestingly, the optimal manganese concentration for the mutant GST-Csk-catalyzed reactions (12 mM) was higher than that for the wild type reactions (2 mM). A reasonable explanation for this change in Mn dependence is a change in the rate-determining step for the wild type versus the mutant Csk-catalyzed reactions.

**RESULTS AND DISCUSSION**

As in the insulin receptor tyrosine kinase, the catalytic loop arginine of Csk is present at the n + 4 position as referenced to the catalytic loop aspartate. To dissect the role of this arginine (Arg-318) in Cak action, this residue was initially replaced by site-directed mutagenesis with Ala, Gln, Ile, and Lys residues. To simplify the preparation of the mutant proteins, these enzymes were expressed and purified as GST fusion enzymes in E. coli. Previously, it was shown that the kinetic parameters obtained from bacterially expressed Csk are in reasonable agreement with those from rat-derived enzyme (30). Contrary to an earlier report (33), the GST moiety did not significantly affect the K_m of ATP or k_cat value using the artificial substrate poly(Glu,Tyr), although it did result in a modest (4- to 5-fold) drop in K_m for poly(Glu,Tyr) compared with that of the GST-free material (Table I).

Each of the above mutants exhibited greatly decreased catalytic efficiency compared with wild type GST-Csk. For GST-Csk R318A and R318Q, the k_cat values were reduced 2000- to 3000-fold (Table I). The effects on K_m for substrate poly (Glu,Tyr) were relatively minor, with 8- to 10-fold elevations compared with wild type GST-Csk. Interestingly, the optimal manganese concentration for the mutant GST-Csk-catalyzed reactions (12 mM) was higher than that for the wild type reactions (2 mM). A reasonable explanation for this change in Mn dependence is a change in the rate-determining step for the wild type versus the mutant Csk-catalyzed reactions. The higher Mn concentration may impede ADP release, which is likely the rate-limiting step for the wild type enzyme-catalyzed reaction (29), yet augment the rate of the chemical step, which is likely rate-determining for the mutant Csk-catalyzed reactions. Similar behavior was seen in kinetic analyses with a previous Csk mutant (D314E) and in studies in which Mg was used in place of Mn (30).

The GST-Csk R318K mutant showed only about a 180-fold drop in K_cat with that of wild type enzyme GST-Csk (Table I). The greater activity of R318K compared with R318A and R318Q suggests that the positive charge of the residue side chain could be important in facilitating catalysis. An alternative possibility for the enhanced activity of R318K compared with R318A and R318Q is the greater hydrophobicity of the lysine side chain. Arguing against this hypothesis however was the discovery that the hydrophobic GST-Csk mutant R318I was actually less catalytically active than R318A or R318Q mutants (data not shown).

Given the great importance of the Arg residue in catalysis and its conserved location in the n + 2 or n + 4 positions of...
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TABLE I

| Protein          | $k_{\text{cat}}$ | $K_m$ poly(Glu, Tyr) | $K_m$ ATP |
|------------------|------------------|----------------------|-----------|
| GST-Csk          | 25 ± 3           | 10 ± 1               | 15 ± 1    |
| Csk              | 25 ± 3           | 40 ± 4               | 9 ± 1     |
| GST-Csk R318A    | 0.008 ± 0.002    | 80 ± 10              | 13 ± 2    |
| GST-Csk R318K    | 0.14 ± 0.04      | 40 ± 6               | 7 ± 1     |
| GST-Csk R318Q    | 0.018 ± 0.004    | 90 ± 9               | 14 ± 2    |
| GST-Csk A316R,R318A | 1.2 ± 0.2     | 11 ± 2               | 12 ± 2    |
| GST-Csk R318A 100 | 0.8 ± 0.1       | 310 ± 40             | 18 ± 2    |

* The elevated $K_m$ value for poly(Glu, Tyr) is likely because of the extreme sensitivity of this copolymer to ionic strength changes and does not predict a similar increase for protein substrates (29, 34).

TABLE II

| Activator                  | Relative velocity |
|----------------------------|-------------------|
| none                       | 1                 |
| imidazole                  | 60                |
| acetamidinium              | 28                |
| guanidinium                | 20                |
| 1,4,5,6-tetrahydroxypuridin| 19                |
| 4-methylimidazole          | 15                |
| formamidinium              | 10                |
| 2-methylimidazole          | 5                 |
| 1,2,3-triazole             | 3                 |
| 2-aminopyridine            | 3                 |
| L-arginine                 | 2                 |
| ethyl, methyl, or 1,1-dimethylguanidin | 5–7               |
| urea, thiazole, 2-methylxanthine | 1            |
| pyrimidine, ethylamine, ammonium, benzimidazole, 2-methyl-2-imidazole | 5–7 |

* Only 10 mM benzimidazole was used because of solubility.

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Steady-state kinetic parameters for Csk, GST-Csk, and its mutants. Assays were carried out as described under “Experimental Procedures.” Relative velocities of GST-Csk R318A phosphorylation of poly(Glu, Tyr) (1 mg/mL) by ATP (100 μM) in the presence of 50 mM activator. Assays were carried out as described under “Experimental Procedures.” Error from duplicate runs was less than 10% for each assay.

The overall recovery of the activity lost with the single Csk mutant (R318A). We were intrigued to find that this second mutation (A316R) led to substantial rescue of kinase activity at around 50 mM guanidinium for 100-fold activation was observed with imidazole at 100 mM concentration, yielding a $k_{\text{cat}}$ value that is only ~30-fold below wild type and similar to that of the Csk A316R,R318A double mutant (see Fig. 5A and Table I). As in the case of guanidinium, imidazole did not significantly activate wild type GST-Csk (~2-fold activation). Other analogs showed a range of rescue activities, but the general rule was upheld that two hydrogen bond donating nitrogens with one carrying a positive charge were necessary for activation. The above results are consistent with the x-ray structure of the insulin receptor protein-tyrosine kinase where two nitrogens of Arg-1136 are within hydrogen bonding distance of (~3 Å from) the substrate tyrosine-phenol hydroxyl and the highly conserved, catalytically important aspartate (24, 30) (Fig. 2).

The enhanced rescue activity of imidazole versus guanidinium was unexpected and to our knowledge is the first example in enzymology of a chemical rescue of an arginine mutant with imidazole. In the case of the protein-tyrosine kinase rescue, the enhanced action of imidazole over guanidinium is rationalized as likely resulting from 1) the planar, aromatic backbone and still subserve a salutary effect on kinase action.

Based on the guanidinium rescue, a number of analogs were observed with imidazole at 100 mM concentration, yielding a $k_{\text{cat}}$ value that is only ~30-fold below wild type and similar to that of the Csk A316R,R318A double mutant (see Fig. 5A and Table I). As in the case of guanidinium, imidazole did not significantly activate wild type GST-Csk (~2-fold activation). Other analogs showed a range of rescue activities, but the general rule was upheld that two hydrogen bond donating nitrogens with one carrying a positive charge were necessary for activation. The above results are consistent with the x-ray structure of the insulin receptor protein-tyrosine kinase where two nitrogens of Arg-1136 are within hydrogen bonding distance of (~3 Å from) the substrate tyrosine-phenol hydroxyl and the highly conserved, catalytically important aspartate (24, 30) (Fig. 2).

The enhanced rescue activity of imidazole versus guanidinium was unexpected and to our knowledge is the first example in enzymology of a chemical rescue of an arginine mutant with imidazole. In the case of the protein-tyrosine kinase rescue, the enhanced action of imidazole over guanidinium is rationalized as likely resulting from 1) the planar, aromatic 5-membered ring with its extra ethylene group having favorable intermolecular interactions with the enzyme and/or substrates or 2) the lower $pK_a$ value of the imidazolium ion versus the guanidinium ion increasing hydrogen bond strength with the phenol oxygen or Asp carbonyl. Further experimentation will be needed to reveal which structural features are important for rescue.

Interestingly, GST-Csk R318H is a very poor kinase compared with wild type GST-Csk (observed velocity similar to unactivated GST-Csk R318A). This suggests that the geometry of the covalently attached imidazole side chain in R318H is not optimal for forming key interactions with the enzymes or substrates and is consistent with the absence of catalytic loop histidines in place of arginines in protein-tyrosine kinases in nature.

Because imidazole and its analogs should be cell permeable, and significant activation of poly(Glu,Tyr) phosphorylation at concentrations as low as 10 mM imidazole is observed, the use of this activator as a rescue agent for mutant protein-tyrosine kinase activity may hold promise for cell biology applications. As a precursor to examining activation within cells, we felt it was important to examine the ability of imidazole to rescue the phosphorylation of a physiologic substrate by GST-Csk R318A in vitro. To function as an in vitro substrate of GST-Csk and
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Csk R318A versus wild type GST-Csk. This diminished rate of Src K295M phosphorylation by GST-Csk R318A was stimulated ~20-fold by 100 mM imidazole (Fig. 5B), whereas imidazole showed no effect on the phosphorylation rate catalyzed by wild type GST-Csk. Therefore the arginine rescue approach also extends to a physiologic protein substrate for the protein-tyrosine kinase Csk. It is our hope that the imidazole rescue results shown here may ultimately be exploited in providing direct, kinetically controlled information about kinase function in cell signaling pathways.

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