Carboxymethyl-phenylalanine as a Replacement for Phosphotyrosine in SH2 Domain Binding*

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Liang Tong‡, Thomas C. Warren, Susan Lukas, Josephine Schembri-King, Raj Betageri, John R. Proudfoot, and Scott Jakes
From Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut 06877

The crystal structure of human p56\(^{\text{src}}\) SH2 domain in complex with an inhibitor containing the singly charged \(p^-\)-(carboxymethyl)phenylalanine residue (cmF) as a phosphotyrosine (Tyr(P) or pY) replacement has been determined at 1.9 \(\AA\) resolution. The binding mode of the acetyl-cmF-Glu-Glu-Ile (cmFEEI) inhibitor is very similar to that of the pYEEI inhibitor, confirming that the cmFEEI inhibitor has a similar mechanism of SH2 domain inhibition despite its significantly reduced potency. Observed conformational differences in the side chain of the cmF residue can be interpreted in terms of maintaining similar interactions with the SH2 domain as the Tyr(P) residue. The crystal structure of the free p56\(^{\text{src}}\) SH2 domain has been determined at 1.9 \(\AA\) resolution and shows an open conformation for the BC loop and an open phosphotyrosine binding pocket, in contrast to earlier studies on the src SH2 domain that showed mostly closed conformation. The structural information presented here suggests that the carboxymethyl-phenylalanine residue may be a viable Tyr(P) replacement and represents an attractive starting point for the design and development of SH2 domain inhibitors with better pharmaceutical profiles.

Protein tyrosine phosphorylation is among the first intracellular events in the transduction of many external signals into the cell. The phosphotyrosyl (Tyr(P) or pY) residues can affect many biological processes by interacting with receptors that recognize the Tyr(P) and its neighboring residues. The src homology 2 (SH2) domain is a specific recognition module for phosphotyrosyl peptides and proteins (1) and has been identified from a variety of enzymes and proteins in general. Individual SH2 domains appear to have a good degree of selectivity toward the sequences of phosphotyrosyl peptides (2). Recognition of a phosphotyrosyl protein by the proper SH2 domain is an important step in many signal transduction processes. Therefore, SH2 domains are attractive targets for the development of therapeutic agents against various human diseases (3).

Phosphotyrosyl peptides containing 4–6 residues can bind SH2 domains with high affinity (2). However, it is unlikely that inhibitors containing the phosphotyrosyl residue can become successful therapeutic agents. This is due both to the liability of the phosphate group toward hydrolysis and to its doubly negative charge at physiological pH, which significantly reduces cell permeability. Therefore, a phosphate replacement that is metabolically stable and possesses lesser (or preferably no) charge at physiological pH may be a crucial component for the development of SH2 domain inhibitors. Most efforts to date have used phosphonates, difluorophosphonates, dicarboxylic acids, and produgs as phosphate replacements (4–10), but these still contain at least two negative charges. The carboxymethyl functionality is an attractive phosphate replacement because it is metabolically stable and contains only a single negative charge. The carboxymethyl-phenylalanine (cmF) residue was used as a mimetic for a sulfated tyrosine residue in cholecystokinin, and the resulting compounds have similar potency to that of the parent peptide in an animal model (11). For SH2 domain binding, however, inhibitors incorporating the cmF residue showed dramatically reduced potency, by about 450-fold, as compared with those containing Tyr(P) (see Fig. 1). A similar degree of loss in potency was observed when the cmF residue was used as a replacement in an inhibitor against the src SH2 domain (5). Therefore, there are serious concerns as to whether the carboxymethyl group truly functions as a phosphate surrogate in SH2 domain binding.

Much structural information has been presented on various SH2 domains in complex with phosphotyrosine-containing inhibitors (12–14), phosphate-containing inhibitors (7), difluorophosphonate-containing inhibitors (10), and dicarboxylate-containing inhibitors (10). We present here the crystal structure of human p56\(^{\text{src}}\) SH2 domain in complex with an inhibitor containing singly charged cmF as the Tyr(P) mimic and show that the cmF residue could be a suitable replacement for Tyr(P).

MATERIALS AND METHODS

**Inhibitor Synthesis and Binding Assay**—The carboxymethyl-phenylalanine residue was synthesized following protocols in the literature (11). The compound thus prepared is enantiomerically pure at the C\(^{\alpha}\) atom (L-form). Details on the chemical synthesis will be presented elsewhere.*

Details on the measurement of the affinity of the inhibitors using surface plasmon resonance have been described elsewhere (15). Briefly, biotinylated EPQpYEEIPIA peptide was immobilized on the surface of a sensor chip. The \(K_d\) of this peptide for the SH2 domain was determined by titration of the SH2 domain over this surface from 100 to 0.31 nM. The affinities of other compounds were determined from competition experiments by preincubating the SH2 domain with varying concentrations of the inhibitors under study. The running buffer for the measurements contained 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, 0.05% P-20, and 0.2 mg/ml bovine serum albumin. The \(K_d\) of the pYEEI inhibitor was monitored frequently to ensure the reproducibility of the experimental measurements.

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† To whom correspondence should be addressed. Present address: Dept. of Biological Sciences, 1212 Amsterdam Ave., Columbia University, New York, NY 10027. Tel.: 212-854-9203; E-mail: tong@como.bio.columbia.edu.

‡ The abbreviations used are: pY, phosphotyrosine; SH2, src homology 2; cmF, carboxymethyl-phenylalanine.
Structure of the SH2 Domain in Complex with the cmFEEI Inhibitor—Crystals of human p56<sup>lck</sup> SH2 domain in complex with the cmFEEI inhibitor were grown at room temperature by the hanging drop vapor diffusion method. The inhibitor stock solution was at 42 mM in Me<sub>2</sub>SO. The SH2 domain (final concentration, 18 mg/ml) was combined with a 1.2-fold molar excess of the inhibitor, giving a final concentration of about 4.5% for Me<sub>2</sub>SO. The reservoir solutions contained 100 mM sodium cacodylate (pH 6.5), 30% PEG 8000, and 200 mM sodium acetate.

X-ray diffraction data to 1.8 Å resolution were collected at cryotemperature on a R-Axis imaging plate system mounted on a Rigaku RU-200 rotating anode generator. The diffraction images were processed with DENOZO (16). Crystals are isomorphic to those in complex with the pYEEI inhibitor (14) and belong to space group <i>P</i><sub>2</sub><i>1</i><i>2</i><i>1</i><i>2</i> (Table I). The structure of the pYEEI complex at 1.8 Å resolution (14) was used as the starting model for the crystallographic refinement with the program X-PLOR (17). Reflections between 6.0 and 1.8 Å resolution (after applying a 2σ cut-off) were used in the refinement. The inhibitor molecule was built into the electron density map with the program FRODO (18) after one cycle of simulated annealing positional refinement. Solvent molecules were identified after the refinement of restrained individual temperature factors from peaks in the difference electron density map that have reasonable hydrogen-bonding interactions. The crystal therefore contained free SH2 domain molecules (see "Results and Discussion").

Structure of the Free SH2 Domain—Crystals of the free p56<sup>lck</sup> SH2 domain were grown in the presence of a weak phosphotyrosyl inhibitor (K<sub>D</sub> = 2.5 μM at pH 7.4). The inhibitor stock solution was at 100 mM in Me<sub>2</sub>SO. The SH2 domain (final concentration, 18 mg/ml) was combined with a 1.2-fold molar excess of the inhibitor, giving a final concentration of about 4.5% for Me<sub>2</sub>SO. The reservoir solutions contained 100 mM sodium citrate (pH 4.5), 30% PEG 4000, and 200 mM ammonium acetate.

X-ray diffraction data to 1.9 Å resolution were collected at cryotemperature on a crystal measuring 0.03 × 0.03 × 0.3 mm<sup>3</sup> (Table I). The crystals belong to space group <i>P</i><sub>4</sub><i>2</i><i>2</i><i>2</i>, with two molecules of the SH2 domain in the asymmetric unit. The structure of the pYEEI complex at 1.8 Å resolution was used as the model for the structure determination by the molecular replacement method with the program Replace (19). The combined molecular replacement protocol (20) successfully located both SH2 molecules for this structure determination, whereas several attempts using traditional molecular replacement methods failed to locate the second molecule. The structure was refined using the slow cooling simulated annealing protocol with the program X-PLOR (17). Residues 155–162 and 170–177 in the two molecules were fitted to the electron density map afteromit refinement. The inhibitor molecule was, however, not visible in the electron density map, and the crystal therefore contained free SH2 domain molecules (see "Results and Discussion").

RESULTS AND DISCUSSION

The crystal structure of the human p56<sup>lck</sup> SH2 domain in complex with an inhibitor containing a phosphotyrosine replacement has been determined at 1.8 Å resolution. The doubly charged phosphotyrosine residue is replaced with a singly charged p-(carboxymethyl)phenylalanine residue (Fig. 1). Despite the large decrease in potency of the carboxylate inhibitor cmFEEI, it binds to the SH2 domain in a conformation very similar to that of the phosphate inhibitor pYEEI (14) (Fig. 2). The conformations of the Ile residue at the pY<sup>-3</sup> position are especially similar between the two inhibitors. Some of the observed differences in the side chains of the Glu residues of the inhibitors, especially those at the pY<sup>-3</sup> position, are likely because of flexibility.

The three nonbridging oxygen atoms (O<sub>1</sub>, O<sub>2</sub>, and O<sub>3</sub>) of the phosphate group (Fig. 2) make different interactions with the SH2 domain (13, 14). Two of them (O<sub>1</sub> and O<sub>2</sub>) participate in tetrahedral hydrogen-bonding interactions with the protein, involving the main chain amide group of Glu-157, and the side chains of Arg-134, Arg-154, and Ser-164 (Fig. 3). The third oxygen atom (O<sub>3</sub>) appears to have weaker interactions with the SH2 domain, making only one hydrogen bond to the side chain.
of Ser-158 in the BC loop (residues 157–161) (Fig. 3). Interestingly, the carboxylate oxygen atoms of the cmF residue in the cmFEEI complex are almost superimposable on the O₁ and O₂ atoms of the phosphate group, maintaining the strongest interactions to the SH2 domain that are observed for the phosphate (Figs. 2 and 3). The apparently weaker interactions from the third oxygen atom of the phosphate appear to be sacrificed by the carboxylate inhibitor.

Small conformational differences are observed between the phenyl ring of the Tyr(P) residue and that of the cmF residue (Fig. 2). Most of these differences can be interpreted to be a consequence of the differences in hybridization between the carboxylate and phosphate groups, and the requirement of placing the carboxylate oxygen atoms near the two oxygens (O₁ and O₂) of the Tyr(P) side chain to maintain optimal interactions with the SH2 domain. The phosphate group is tetrahedral, whereas the carboxylate group is planar. Consequently, a much smaller torsion angle across the CZ–OH bond, which is also expected to be energetically more favorable.

The small torsion angle in the cmF side chain places the carboxylate group almost in the plane of the phenyl ring. Possibly as a consequence of this, a rotation (Fig. 2B) and translation (Fig. 2C) of the phenyl ring of the cmF side chain is observed, so that the carboxylate oxygens are in superposition with the phosphate oxygens. The guanidinium group of the side chain of Arg-134 shows a similar translation as the phenyl ring (Fig. 3), possibly to maintain optimal amino-aromatic interactions (21).

Because the cmFEEI inhibitor lacks any atoms at the O₃ position of the pYEEI inhibitor, it probably has weaker interactions with the BC loop. A small conformational change in this loop is observed, as compared with that of the pYEEI complex structure. Residue Ser-158 moves slightly away from the inhibitor (Fig. 3).

A much larger rearrangement of the BC loop is observed in the crystal structure of the free p56lck SH2 domain, determined at 1.9 Å resolution. The crystals were grown in the presence of a weak pY-containing inhibitor (Kᵣ = 2.5 μM). However, the inhibitor binding regions of the molecules are blocked by residues from neighboring molecules in the crystal. The extremely low pH of the crystallization solution (pH 4.5) is expected to significantly reduce the potency of the inhibitor (Ref. 10 and see discussions below). This might explain the lack of inhibitor binding in these crystals.

Residues Glu-155 (N–C₇ bond) and Ser-162 (C₇–C bond) appear to be the hinge for the conformational change of the BC loop (Fig. 4), which significantly affects the shape of the Tyr(P) binding pocket. In comparison, the BC loop in the free src SH2 domain was found to exist mostly in the closed conformation based on a crystal structure analysis (12). The two molecules in the crystal assume essentially the same conformation for this loop (Fig. 4). Such a “gated” conformational change for the BC loop was first observed in the structure of p56lck SH2 domain in complex with phosphonomethyl-phenylalanine inhibitors (7).

In the crystal lattice of the free SH2 domain, the BC loops in both molecules are involved in crystal packing interactions. However, the details of these interactions are different between the two molecules. Moreover, the BC loops in the phosphonomethyl-phenylalanine inhibitor complexes are essentially not involved in crystal packing (7). The conformations of the BC
loops in these two different crystalline lattices are essentially the same. It is therefore unlikely that the open conformation observed here is due solely to crystal packing effects. The closed conformation of this loop in the pYEEI complex enclose the Tyr(P) residue (Fig. 4). Additional stabilization of this conformation is probably provided by the hydrogen bond between Ser-158 at the tip of this loop and the phosphate group.

The 450-fold loss in potency by the replacement of a phosphate group with a carboxylate group (Fig. 1) appears to be because of several factors. The loss of one negative charge is expected to have the greatest effect in weakening the interactions between the carboxylate inhibitor and the SH2 domain. This is partly reflected by the observed pH dependence of the potency of the pYEEI inhibitor. A 40-fold loss in potency against the src SH2 domain is observed for phosphotyrosine-containing inhibitors when the pH is lowered from 8.0 to 4.6 (10). It is believed that this loss in potency is because of the protonation of the phosphate group in solution, which will make the phosphate a singly charged species.

The replacement of the bridging oxygen atom in the Tyr(P) residue with a methylene in the cmF residue results in the loss of a hydrogen bond to the SH2 domain (12). This replacement leads to roughly a 40-fold loss in potency in the phosphonomethyl-phenylalanyl inhibitors as compared with Tyr(P) inhibitors (4, 5, 7). Most of the potency loss can be recovered by the use of a difluorophosphonomethyl-phenylalanyl group (4, 5). The fluorine atoms lower the $pK_a$ of the phosphonate and can also maintain the same hydrogen bond with the SH2 domain as the bridging oxygen atom of the Tyr(P) residue (10). It might be interesting to try to establish such interactions in the carboxymethyl-phenylalanine inhibitors.

The apparently decreased interactions with the BC loop of the SH2 domain may also be detrimental for the potency of the carboxylate inhibitor, although the BC loop is in a closed conformation in the cmFEEI inhibitor complex. Finally, the bound conformation of the cmFEEI inhibitor may not be the most favorable energetically. The torsion angle of 16 ° across the CZ–CH bond probably would cause steric repulsions between the carboxylate group and the phenyl ring, lowering the potency of the inhibitor.

The observed 450-fold loss in potency of the cmFEEI inhibitor in our study compares favorably with that observed for the
The improvement in the pharmacokinetic properties of a carboxylate inhibitor therefore a reasonable replacement for the phosphate group. The carboxymethyl group has a similar mode of interaction and is a nonphosphate inhibitor showing that the singly charged carboxylate inhibitor versus a phosphate inhibitor probably outweighs the loss of potency in the carboxylate inhibitor. Moreover, the potency of the carboxylate inhibitors may be improved by the establishment of stronger binding to other areas of the protein, for example the BC loop and the pYr3 pocket, and by stabilization of the bound conformation of the inhibitor. A cyclic peptide containing a simple Tyr in place of Tyr(P) has been shown to inhibit Grb2 SH2 domain at 20 μM potency, suggesting that establishment of stronger interactions to other regions of the binding pocket is possible. This crystal structure provides a starting point for the design of such carboxylate-containing inhibitors of the p56lck and other SH2 domains, preferably with nonphosphate moieties at the other positions (9). The carboxyethyl group as a phosphate replacement could also prove useful in the development of inhibitors against other protein targets that normally recognize phosphate groups.

CONCLUSIONS

The crystal structure of p56lck SH2 domain in complex with a nonphosphate inhibitor shows that the singly charged carboxymethyl group has a similar mode of interaction and is therefore a reasonable replacement for the phosphate group. The improvement in the pharmacokinetic properties of a carboxylate inhibitor versus a phosphate inhibitor probably outweighs the loss of potency in the carboxylate inhibitor. Moreover, the potency of the carboxylate inhibitors may be improved by the establishment of stronger binding to other areas of the protein, for example the BC loop and the pYr3 pocket, and by stabilization of the bound conformation of the inhibitor. A cyclic peptide containing a simple Tyr in place of Tyr(P) has been shown to inhibit Grb2 SH2 domain at 20 μM potency, suggesting that establishment of stronger interactions to other regions of the binding pocket is possible. This crystal structure provides a starting point for the design of such carboxylate-containing inhibitors of the p56lck and other SH2 domains, preferably with nonphosphate moieties at the other positions (9). The carboxyethyl group as a phosphate replacement could also prove useful in the development of inhibitors against other protein targets that normally recognize phosphate groups.

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