

Chemical Approaches to Reversible Protein Phosphorylation

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ABSTRACT

Protein phosphorylation catalyzed by protein kinases plays a critical role in cellular signaling. Here we review several chemical approaches to understanding protein kinases and the consequences of protein phosphorylation. We discuss the design of bisubstrate analogue inhibitors based on a dissociative transition state, the development of reagents for cross-linking protein kinases with their substrates, the chemical rescue of mutant protein tyrosine kinases, and the application of expressed protein ligation to understanding protein phosphorylation.

It is now well-accepted that the reversible modification of proteins by phosphorylation is a major facet of intracellular signaling networks.¹ Although the importance of protein phosphorylation has been recognized since the 1950s,² until relatively recently, the study of signaling was considered a rather fundamental rather than clinical pursuit. However, over the last 10 years, it has been established that both protein kinases and protein phos-

phatases are attractive drug targets for a wide range of diseases, including leukemias, solid tumors, vascular diseases, diabetes mellitus, and immune/inflammatory disorders.³ This relevance to disease has increased the intensity in the biomedical research community to understand the function of these phosphoryl transfer enzymes and to develop potent and selective inhibitors that might be useful therapeutic agents. Among the continuing challenges in the kinase field are understanding the catalytic mechanism of the protein kinase enzymes for rational inhibitor design, identifying physiologic protein substrates for kinases and physiologic kinases that effect phosphorylation of specific substrates, defining the temporal relationships between kinase action and cellular effect, and characterizing the structural and functional effects of site-specific phosphorylation events. While some of these issues are fairly well-understood for individual kinases and substrates, the functions of the vast majority of the roughly 600 protein kinases and the consequences of phosphorylation of thousands of protein substrates have not been elucidated. To address these issues of kinase function, a number of relatively recent chemical/biochemical approaches have been reported,^{4–6} some of which are described by other authors in this issue. In this Account, we discuss the development and application of several chemical methods pursued in our lab to address the mechanism and function of protein phosphorylation.

Protein Kinase Mechanism

The proteases and glycosidases stand out among enzyme classes as success stories in mechanism-based inhibitor design.^{7,8} Part of the success with these enzymes has stemmed from a detailed understanding of their enzyme mechanisms. In contrast, a detailed understanding of the chemical mechanism of protein kinases has lagged behind. Starting in the early to middle 1990s, a number of dazzling, high-resolution protein serine/threonine and tyrosine kinase X-ray structures were published which gave a new perspective on the mechanism of these enzymes.^{9,10} The amino acid residues composing the active sites of these enzymes were identified and interactions with substrates revealed. In 1994,¹¹ the generally accepted mechanism of phosphoryl transfer for protein kinases based in part on these structures appeared to be “associative” (Figure 1). In an associative mechanism, there is

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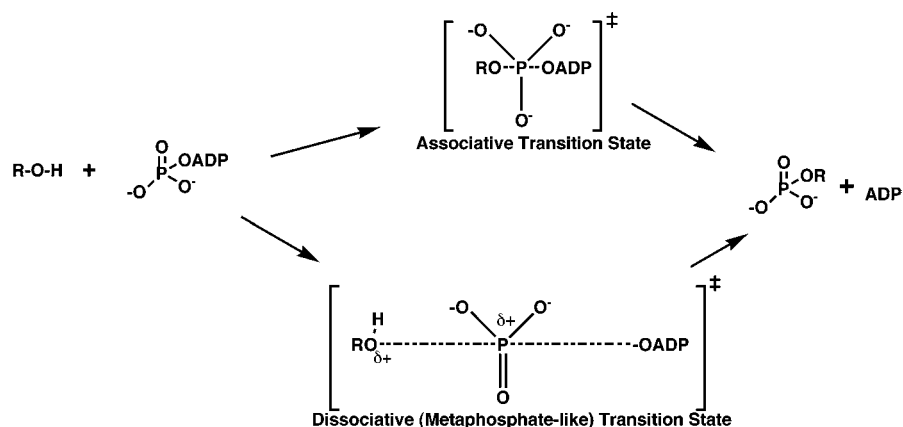


FIGURE 1. Associative vs dissociative transition states for phosphoryl transfer.

substantial bond formation between the entering nucleophile and phosphorus prior to significant bond cleavage between the phosphorus and the leaving group. In this mechanism, a variety of positively charged groups including one or two divalent ions were suggested to interact with the γ -phosphate, converting it into a more reactive electrophile. Furthermore, an active site aspartate (Asp-166 in protein kinase A) appeared to be a general base, enhancing the nucleophilic reactivity of the substrate serine or tyrosine. An associative phosphoryl transfer mechanism is analogous to most proposed acyl transfer mechanisms catalyzed by proteases which are thought to proceed through tetrahedral intermediate states.

However, it has long been known that phosphate monoesters which are dianionic at neutral pH appear to follow dissociative transition states (Figure 1) in nonenzymatic nucleophilic substitution reactions.¹² Thus, these reactions show small Bronsted nucleophile coefficient (β_{nuc}) values, large Bronsted leaving group coefficient (β_{lg}) values, inverse thio effects, small entropies of activation, and ¹⁸O-isotope effects more easily rationalized by a dissociative transition state model.¹² These dissociative transition states are characterized by relatively little participation of the entering group and a rather advanced cleavage of the bond between the phosphorus and the leaving group. While there is consensus among many investigators about the nonenzymatic mechanisms of phosphate monoester reactions, there are dissenting points of view which continue to stimulate discussion.¹³

In contrast to the accessibility of nonenzymatic reactions to experimental investigation, discerning the transition states of enzyme reactions is typically more difficult. Due to enzymes' geometric constraints, narrow solution requirements, and limited stability, many of the techniques used to study nonenzymatic reactions are not applicable. This has certainly been the case with phosphoryl transfer reactions. It is the lack of decisive experimental data that has contributed to the passionate debate regarding the nature of the enzyme-catalyzed phosphoryl transfer reactions. For example, it has been argued that the presence of divalent ions present in the active sites of enzymes that coordinate to the attacked phosphate might cause these reactions to follow associative transition states.¹⁴ However, recent studies have failed to show

changes from dissociative transition states in the presence of divalent ions in the related nonenzymatic reactions.¹⁵

One of the more intuitive and classical approaches to interrogate phosphoryl transfer reactions is the measurement of the Bronsted nucleophile coefficient (β_{nuc}). In this linear free energy relationship, the net effective charge buildup on the entering atom of the nucleophile in the transition state is assessed.¹⁶ A series of homologous nucleophiles which vary in $\text{p}K_{\text{a}}$ are employed as substrates with a given phosphate ester and the log of the rates of the reactions are plotted versus the substrate $\text{p}K_{\text{a}}$ s. If the relationships are roughly linear, the slopes of the plots provide the β_{nuc} value. A large β_{nuc} (close to one) indicates strong dependence on the role of the nucleophile with large charge development on the entering atom, suggestive of an associative transition state. If the β_{nuc} is small (close to zero), there is generally small dependence on the role of the nucleophile, consistent with dissociative character. These relationships have been determined on numerous phosphate esters under nonenzymatic conditions. It is consistently observed that phosphate triesters show relatively large β_{nuc} values (>0.4), diesters intermediate values (0.3–0.6), and monoesters low values (<0.3).^{17,18}

This sort of evaluation is challenging to perform and interpret with many enzymes because of the sensitivity of the enzyme to changes in steric/polar parameters of the substrate in binding and orientation as well as the difficulty of preparation of the homologous substrate class. However, protein tyrosine kinases represent attractive enzymes for such an evaluation because the tyrosine substrate aromatic ring can be readily substituted, and the substrate binding surface on the enzyme is spread out over a relatively large surface of the peptide. Indeed, a relatively early study by Graves and colleagues with a limited analysis of three tyrosine analogues suggested a large β_{nuc} and associative transition state.¹⁹ This effort was handicapped, as it was performed prior to the availability of large quantities of pure, homogeneous tyrosine kinases, and so our lab reinvestigated this with the protein tyrosine kinase Csk.

We prepared the entire set of fluorotyrosine analogues (Figure 2) which allows for broad and systematic $\text{p}K_{\text{a}}$ coverage (5–10).^{20–23} Since fluorine atoms are not much larger than hydrogen atoms but dramatically effect $\text{p}K_{\text{a}}$

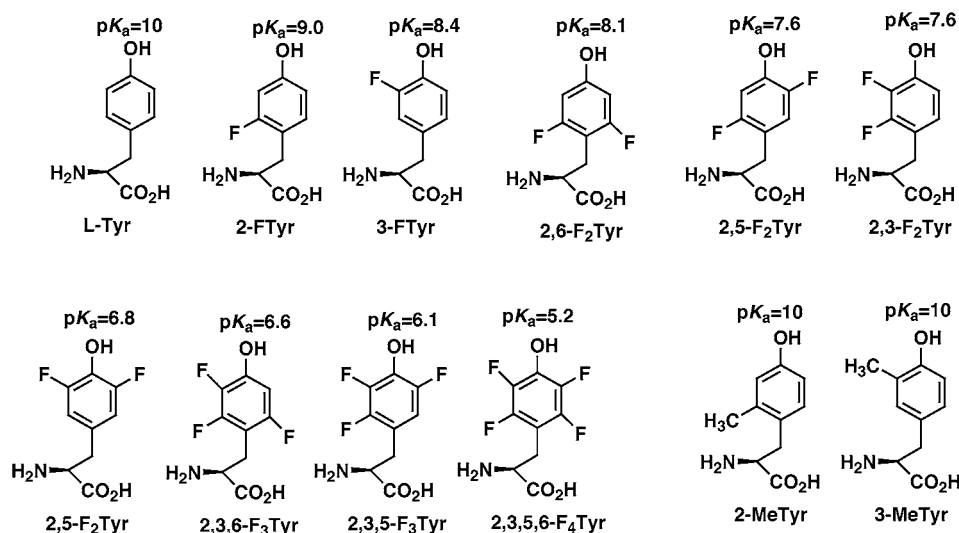


FIGURE 2. Tyrosine analogues used for bronsted nucleophile coefficient (β_{nuc}) measurements.

values, they are commonly employed in enzymology to address mechanistic issues. These fluorotyrosine analogues can be readily synthesized stereospecifically in a single step with tyrosine phenol-lyase.²⁴ We also synthesized several ring alkyl-substituted tyrosine analogues to probe the potential for steric effects by the fluorines.²² Over the course of these studies, we have shown that tyrosine phenol-lyase can be employed to stereospecifically synthesize conformationally restricted β -substituted tyrosine analogues by employing α -ketobutyrate in place of pyruvate.²⁵

With this set of compounds in hand, we showed that the β_{nuc} for these substrates with the tyrosine kinase Csk was near zero.^{20,21} Moreover, complications of steric effects were unlikely to be significant since the methyl-substituted compounds showed rates close to those of tyrosine itself. Interestingly, a noticeable drop-off in rate with the lower pK_a substrates (6.5 and below) was observed. With the use of rate versus pH profiles with several of the fluorotyrosine containing substrates, it was shown that the behavior was due to the inability of the phenoxide anion (tyrosinate) form of the substrate to undergo enzyme-catalyzed phosphorylation.^{20,21} This result was paradoxical for two reasons. First, since the proton of the phenol must ultimately be removed over the course of the reaction, it would be reasonable to expect that early deprotonation would not be problematic. Second, the tyrosine phenoxide anion form of the substrate (pK_a 10) would be expected to be far more reactive as a nucleophile than the neutral phenolic form (pK_a \sim 7). That the enzyme would prefer the neutral form for the kinase reaction would be highly improbable for a highly associative transition state.

In contrast, the combination of a small β_{nuc} and preference for the neutral phenol can nicely be rationalized by a dissociative transition for enzyme catalyzed phosphoryl transfer. Let us assume a β_{nuc} of 0.1 for a nonenzymatic phosphoryl transfer reaction. Comparing two nucleophiles with pK_a differences that are 17 units apart (phenoxide anion and neutral phenol), the relative rate with the more basic nucleophile should be ap-

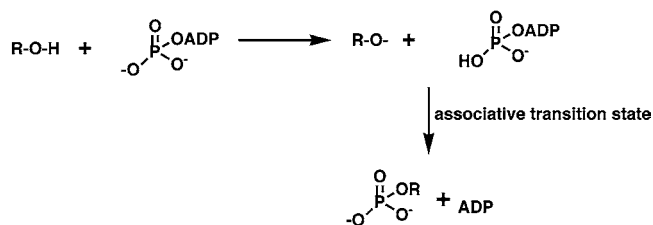


FIGURE 3. Phosphate substrate-assisted proton-transfer model in phosphoryl transfer.

proximately 50-fold greater, neglecting other effects due to solvation, steric hindrance etc. However, in the context of the enzyme with β_{nuc} 0.1, the loss of a hydrogen bond and the potential for electrostatic repulsion generated by the phenoxide anion might well be expected to dominate the modest rate enhancement due solely to basicity. In contrast, if the β_{nuc} were 0.6, the 10 billion-fold rate differential with the more basic nucleophile would be much harder to overcome by hydrogen bonding and electrostatic effects.²¹

An alternative model to account for a low β_{nuc} and requirement of the neutral phenol could still be consistent with an associative transition state has been proposed by Warshel (Figure 3).¹³ In Warshel's model, the substrate phosphate is suggested to act as a general base, directly accepting the proton of the hydroxyl from the other substrate. Once generated, the anionic nucleophile would rapidly react with the diester-like form of the phosphate via an associative transition state.¹³ Thus, more acidic tyrosines would be envisaged to transfer their protons more rapidly to the phosphate, offsetting the expected decrease in reactivity of the less basic anionic species, resulting in small β_{nuc} values overall. This interesting idea is based on quantum mechanical calculations, but it has been cogently challenged by Herschlag and others.²⁶

To experimentally address the phosphate-proton-transfer model, a mutation of the Csk tyrosine kinase (catalytic base) aspartate to asparagine was prepared.²⁷ This mutant, while showing reduced catalytic power, was found to preferentially accept the anionic nucleophilic

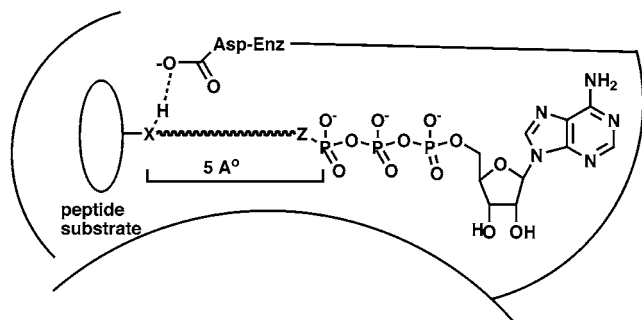


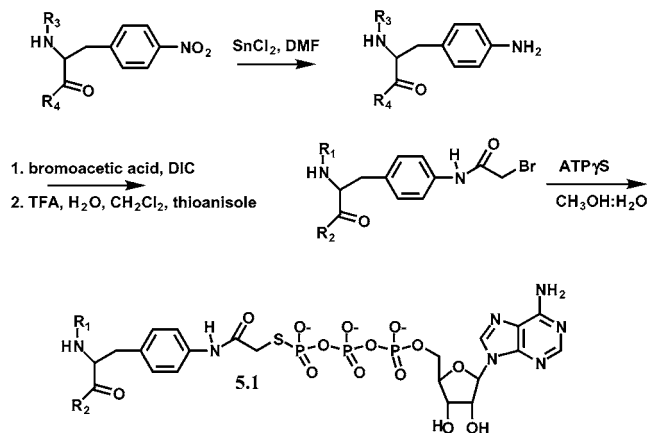
FIGURE 4. Design of a protein kinase bisubstrate analogue inhibitor. The X atom corresponds to the nucleophilic oxygen. The Z atom is linked to the spacer.

form of tyrosine, but still showed a small β_{nuc} , consistent with a dissociative transition state.²⁷ Since the anionic nucleophilic form is incapable of transferring a proton to the phosphate, these data argue against the phosphate proton transfer model and in favor of a dissociative transition state. These results, along with other published kinetic data, have led to the proposal that protein kinases in general are likely to utilize dissociative transition states.

Inhibitor Design

Starting with the premise of a dissociative transition state, it was of interest to design protein kinase inhibitors. On one hand, development of protein kinase inhibitors based on a dissociative transition state would test the mechanism's predictive power. More importantly, the need for selective protein kinase inhibitors as tools and as lead agents for disease treatment provides compelling incentives to generate such compounds. For enzymes utilizing two substrates, covalently attached bisubstrate analogues are often potent and selective inhibitors. As recently reviewed,²⁸ this approach has been attempted in a number of different ways with protein kinases, generally with limited success. One prediction based on the dissociative model is that a relatively large molecular volume might be needed in bisubstrate analogue inhibitors. A quantitative model for the reaction coordinate distances based on simple geometric principles has been proposed by Mildvan.²⁹ The Mildvan concept is that in a dissociative transition state mechanism, the reaction coordinate distance (the distance between the entering oxygen and the phosphorus under attack in the ground state) should be greater than or equal to 4.9 Å. It might be imagined that ideal bisubstrate analogues would be covalently linked ATP-peptide substrate conjugates containing a 5 Å spacer (Figure 4). A second design feature might allow for the tyrosine attacking atom to maintain hydrogen bonding capability to the important active site aspartate.

The initial enzyme targeted by this approach was the insulin receptor tyrosine kinase (IRK).³⁰ IRK was selected because of the following: an efficient peptide substrate had been identified; IRK was independently demonstrated to follow a dissociative mechanism;²³ a crystal structure of IRK bound to nucleotide and peptide had already been accomplished³¹ which encouraged our hope that it might



R₁ = AcNH-Lys-Lys-Lys-Leu-Pro-Ala-Thr-Gly-Asp-
 R₂ = -Met-Asn-Met-Ser-Pro-Val-Gly-Asp-CO₂H
 R₃ = R₁ with side chain protected residues
 R₄ = R₂ with side chain protected residues and Asp linked to Wang resin

FIGURE 5. Synthetic approach to a bisubstrate analogue (5.1) designed against the insulin receptor kinase.

be possible to also determine the structure of the inhibitor with IRK. Although not itself a drug target, IRK is highly similar to the IGF1 tyrosine kinase which shows high activity in many cancers.³² The synthesis of the target compound exploited the chemoselective reaction between ATP- γ S and the α -bromoacetanilido-peptide (Figure 5).³⁰ While acid sensitive, compound 5.1 was shown to be stable at neutral pH for several days at room temperature.

The bisubstrate analogue 5.1 proved to be a potent and selective inhibitor of IRK with a K_i of 370 nM. It displayed competitive inhibition versus ATP and noncompetitive inhibition versus peptide substrate.³⁰ A control compound lacking the peptide amino acid residues was approximately 300-fold worse as an inhibitor. In fact, the free energy of binding of the nucleotide and peptide moieties measured individually approximate the free energy of binding of the bisubstrate analogue inhibitor. This compound appears to be reasonably selective, with approximately 100-fold weaker inhibition against another tyrosine kinase Csk, which recognizes a dissimilar peptide substrate sequence. An X-ray structure of 5.1 in complex with IRK confirmed the design principles.³⁰ Taken together, these results are compatible with the dissociative transition state model for tyrosine kinases and offer a new direction in the development of much needed selective protein kinase inhibitors. While more work will be needed to enhance their pharmacokinetic properties, these bisubstrate analogues have the immediate potential in structural genomics studies on protein kinases, where high-resolution information concerning the recognition of protein substrates is lacking.

Recently, the finding that γ -phosphoryl derivatized compound 6.1 (Figure 6) was not a potent kinase inhibitor was used to design a photoactivatable cross-linking compound 6.2 which has the potential to selectively cross-link protein kinases with protein substrates.³³ A proof of principle experiment with the bis-azide nucleotide analogue 6.2 showed that it could covalently cross-link protein tyrosine kinase Csk with its substrate Src. This

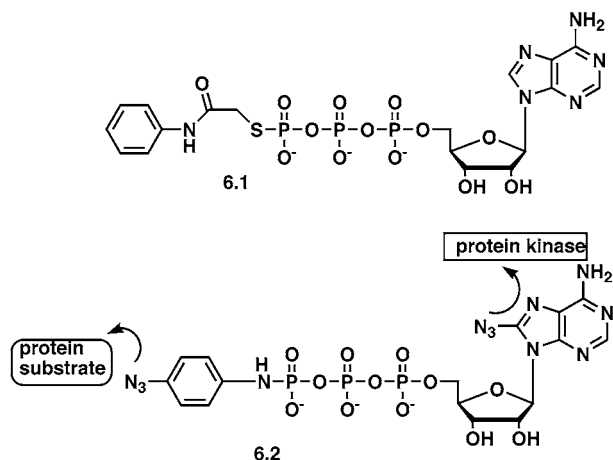


FIGURE 6. Derivatized ATP analogues and an approach to protein kinase-substrate cross-linking.

cross-linking was dependent on UV light and inhibited by ATP. It is hoped that such reagents will be useful in the proteomic analysis of protein phosphorylation. Thus, starting with either a known phosphorylated protein or a protein kinase of interest, cross-linking can be carried out to find its potential partners in cellular extracts.

Chemical Rescue of Mutant Protein Tyrosine Kinase Activity

The discovery that it is possible to rescue mutant enzymes with small molecules dates back to the early years of site-directed mutagenesis.^{34,35} The approach involves mutation of an active site residue of the enzyme to remove a functional group key for catalysis and then supply the missing functional group as an exogenous small molecule or built in to the substrate. The potential advantage of introducing this methodology to protein tyrosine kinases would be to provide a temporal control switch for kinase-dependent signaling pathways. Thus, a small diffusible molecule could provide activation in minutes instead of the hours to days associated with transfecting protein kinases into cells and inducing their transcription. Since many of the most interesting signaling steps are thought to occur over a period of minutes to hours, the chemical rescue of a mutant protein kinase could provide key insights into the timing of important phosphorylation events. Note that such an approach is complementary to the application of small molecule inhibitors which are specific for protein kinases where the small molecule in this case is an off switch.

In considering the requirements of chemical rescue, ideally one would desire a mutation in a highly conserved tyrosine kinase residue so that it could be generally applied. The mutation should be quite deleterious to the kinase activity so that a dynamic range of 100-fold or more would be possible. Finally, the rescuing agent should be cell-permeable so that signaling could be studied in living cells. These are stringent requirements, and it should be stated that to our knowledge, prior to our recent work,^{36,37} chemical rescue had not been applied to mutant enzymes in living cells.

Selected Sequences of Protein Kinases

• CSK	FVHR <u>DLAARN</u> VLV
• IRK	FVHR <u>DLAARN</u> CMV
• LCK	YIHR <u>DLRA</u> NILV
• ABL	FIHR <u>DLAARN</u> CLV
• SRC	YVHR <u>DLRA</u> NILV
• PDGF	CVNR <u>DLAARN</u> VLI
• EGF	LVNR <u>DLAARN</u> VLV
• PKA	LIYR <u>DLK</u> PENLLI
• MKK	VIHR <u>DIK</u> PQNILL

FIGURE 7. Catalytic loop sequences of several protein tyrosine (CSK, IRK, LCK, ABL, SRC, and PDGF, EGF) and a serine kinase (PKA). Underlined residues are the catalytic base (D) and catalytic arginine (R).

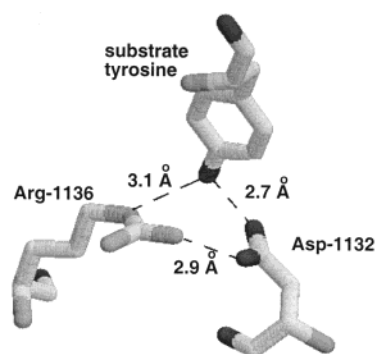


FIGURE 8. Three-dimensional structure of IRK bound to a peptide substrate highlighting the catalytic Asp and Arg residues and the substrate Tyr.

Inspection of the catalytic loop region of protein tyrosine kinases (Figure 7) revealed a highly conserved Arg (Arg-318 in Csk). However, this Arg is found either two or four residues downstream of the absolutely conserved catalytic Asp. It appeared that Nature had identified a residue which could show structural flexibility. High-resolution crystal structure of the insulin receptor kinase showed that the side chain of this Arg is involved in hydrogen bonding to the substrate Tyr as well as the active site Asp (Figure 8). In this way, two of the guanidinium nitrogen atoms appear to be hydrogen bond donors.

To ascertain the function of this Arg, we showed that mutation of the residue to Ala in tyrosine Csk (Arg318Ala) led to a 3000-fold drop in k_{cat} with only small effects on K_m .³⁶ When a double mutant was created which effectively moved this Arg from four to two residues away from the catalytic Asp, much of the catalytic activity was restored.³⁶ This effectively confirmed our suspicion that there is flexibility in positioning the Arg residue along the backbone. We then screened a series of small molecules to test for rescue. We found that a number of compounds (Figure 9) were able to enhance the kinase activity of R318A Csk but not the wild-type enzyme.³⁶ The consistent requirements for strong rescue from over 30 compounds that have been screened against Csk R318A include the presence of two hydrogen bond donating nitrogen atoms and the potential for positive charge. These requirements were readily rationalized from the X-ray structure.

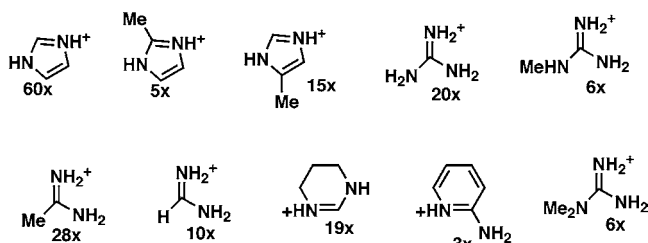


FIGURE 9. Selected small molecules tested to rescue R318A Csk. Fold-activation shown under each structure using 50 mM compound at pH 7.4.

The best of the compounds so far examined was imidazole, likely acting as imidazolium which provides up to 150-fold rescue at pH 6.8, albeit with 50 mM imidazole. This is 7-fold higher than the maximum observed rescue with guanidinium. The concentration of imidazole for half-maximal rescue was 20–40 mM.³⁶

While more work needs to be done to maximize the potency and efficacy of rescue, given that imidazole is a cell permeable molecule and shows low toxicity, it was tested with mutant Csk in intact cells. Mouse embryonic fibroblasts lacking Csk have been shown to be defective in actin stress fiber formation.³⁸ Reintroduction of Csk back into such cells was able to allow for actin stress fiber formation to occur; however, the importance and timing of the kinase activity in this system had not been previously addressed. By use of the R318A Csk protein, it was shown that the kinase activity of Csk was critical for cytoskeletal reorganization, since actin stress fiber formation occurred within 15 minutes of imidazole exposure.³⁷ In contrast, cells containing a D314N Csk mutant which was catalytically defective but not rescuable with imidazole *in vitro* showed no cytoskeletal changes with imidazole. These experiments and others allowed for a temporal connection among G-proteins, Csk, and the small G-protein Rho in this important cellular process.³⁷

Expressed Protein Ligation and Protein Phosphorylation

One of the limitations of the signaling field is precisely defining the structural and functional consequences of phosphorylation events. It is in general difficult to obtain

homogeneous, stoichiometrically labeled phosphoproteins. This is especially difficult when the phosphoprotein linkages are intrinsically unstable such as when they occur within protein phosphatases. Although various techniques have been used to address this problem, all have limitations.

Several years ago, our lab in collaboration with Tom Muir, and independently by a group at New England Biolabs, introduced the method of expressed protein ligation (Figure 10) that substantially overcomes several of the limitations in the generation of site-specifically modified proteins.^{39,40} Expressed protein ligation blends the technique of native chemical ligation with the generation of recombinant protein thioesters via inteins. In EPL, a recombinant protein or protein domain of interest, is generated in frame with an intein, resulting in the generation of a C-terminal thioester. This thioester is reacted with an N-terminal cysteine containing peptide via the native chemical ligation strategy to generate the semisynthetic protein. In this way a host of unnatural entities can be introduced into the synthetic portion of the semisynthetic protein.

EPL, which has now been implemented in a broad array of protein studies by many groups, has been applied to a number of problems in the area of signaling, including TGF β ,⁴¹ Csk-Src,^{39,42} and the tyrosine phosphatases SHP-2⁴³ and SHP-1.⁴⁴ We will highlight the work on SHP-2 and SHP-1 here because it illustrates particularly well the strength in the use of nonhydrolyzable phosphotyrosine derivatives (Figure 11) to address an important issue in signaling, which had proved difficult to attack using standard methods. Whereas phosphoserines and phosphothreonines can often be mimicked with encoded residues Asp or Glu, no such encoded isostere exist for phosphotyrosines making them a particular challenge.

The SHP-2 and SHP-1 are protein tyrosine phosphatases which are composed of two SH2 domains, a catalytic domain, and a flexible C-terminal tail that can be phosphorylated on tyrosines by protein tyrosine kinases (Figure 12).⁴⁵ It is known that these enzymes show reduced catalytic activity in their ground state and deletion of the N-SH2 domain or addition of phosphotyrosine peptides can activate these enzymes.⁴⁵ It has been pro-

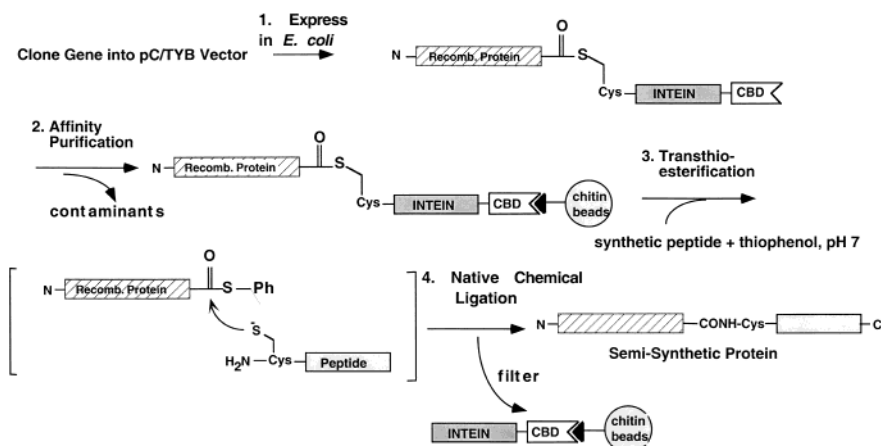


FIGURE 10. The method of expressed protein ligation.

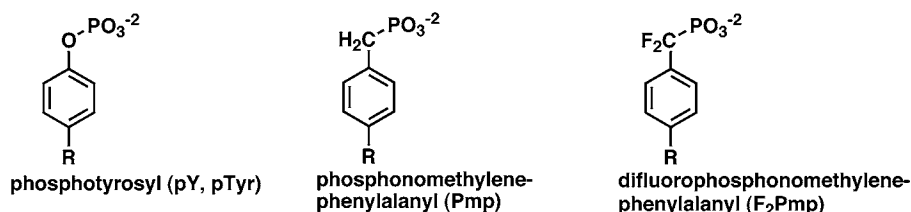


FIGURE 11. Phosphotyrosine and nonhydrolyzable phosphotyrosine analogues. R = alanyl.

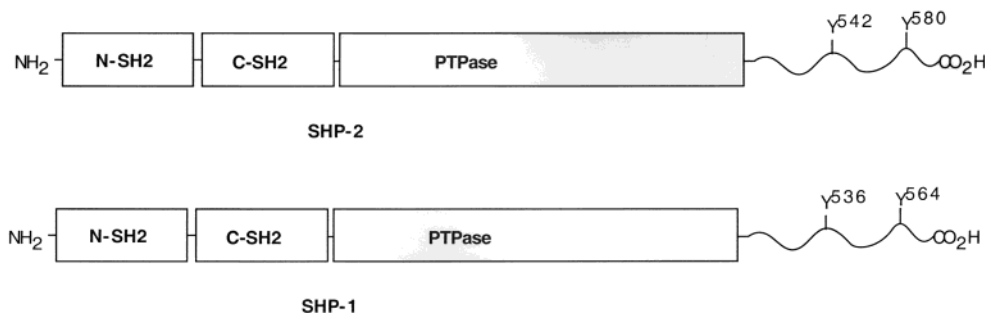


FIGURE 12. Domain organization of the protein tyrosine phosphatases SHP-2 and SHP-1. The C-terminal tyrosine residues highlighted are sites of phosphorylation by various tyrosine kinases.

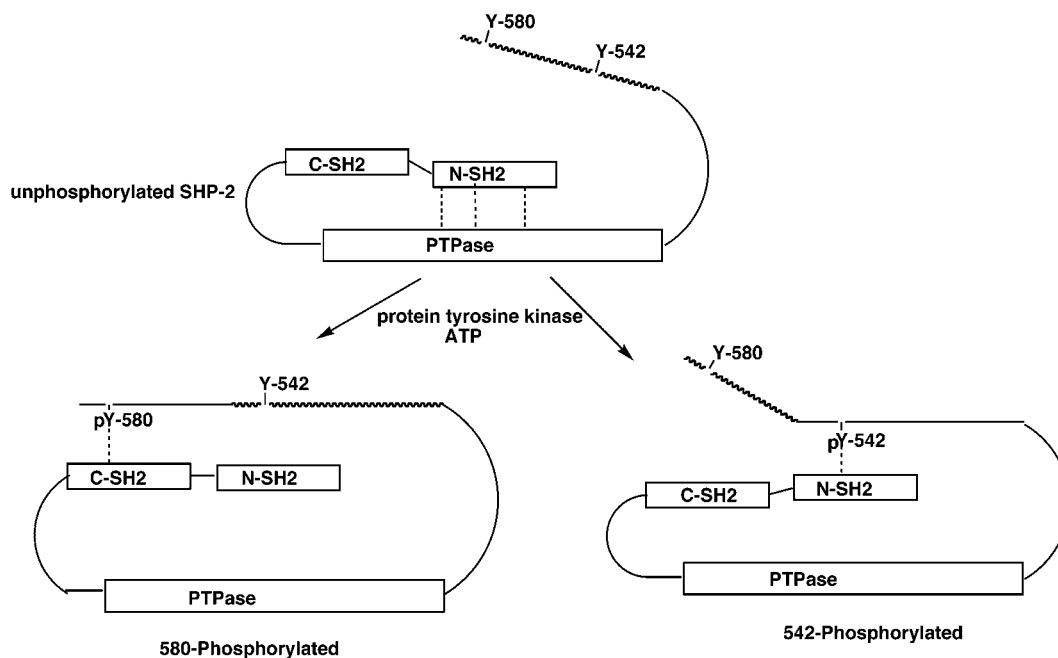


FIGURE 13. Structural model of the consequences of tail tyrosine phosphorylation of SHP-2.

posed that phosphorylation of the C-terminus of these proteins on the two tyrosine residues can function either in adaptor protein recruitment or by intramolecular interaction with the N- or C-terminal domains of the corresponding enzyme. It has been difficult to rigorously address this problem since autodephosphorylation at these sites is relatively rapid.

We realized that EPL would be a potentially useful approach here because it could be employed to replace the C-terminus of the recombinant protein with synthetic peptides containing phosphotyrosine mimetics. We initially explored the Pmp derivative (Figure 12) which is known to be a reasonably good mimic of phosphotyrosine in terms of SH2 domain interaction.^{43,44} EPL worked quite well in this case, and it was possible to generate milligram

quantities of the site-specifically modified Pmp-containing proteins for SHP-2 and SHP-1.^{43,44} For SHP-2 it was found that Pmp modification at either of the two tyrosine phosphorylation sites (542, 580) led to 2–3-fold enhanced phosphatase activity. In contrast, in SHP-1 a 4-fold enhanced activity with Pmp at the 536-position was observed, whereas no stimulation of phosphatase activity at the 564-position was found. A combination of protease sensitivity studies, gel filtration, phosphopeptide binding, and site-directed mutagenesis experiments revealed distinctive interactions between the Pmp modification sites and the SH2 domains in SHP-2 as shown in Figure 13.⁴³

To further evaluate the Pmp interactions in SHP-1, EPL was used to generate the F₂Pmp containing semisynthetic SHP-1 proteins where the fluorophosphonates were in-

roduced as more precise phosphotyrosine mimetics.^{44,46} The pK_a of the phosphate dianion in phosphotyrosine is approximately 1–2 units lower than that of the Pmp derivative. In contrast, F₂Pmp closely matches that of phosphotyrosine. Furthermore, F₂Pmp has the potential to be a hydrogen bond acceptor via its fluorines, possibly mimicking the phosphate bridging oxygen in phosphotyrosine. In detailed comparisons of SH2 domain-phosphopeptide interactions, phosphotyrosine and F₂Pmp show nearly identical behavior, whereas Pmp binds about 5-fold more weakly.⁴⁶ In the studies on SHP-1, the F₂Pmp substituted proteins were subtly different compared to the Pmp substituted proteins. Thus, 536-F₂Pmp induced an 8-fold catalytic stimulation compared with 4-fold with Pmp. 564-F₂Pmp showed a 1.6-fold enhanced effect on catalysis, whereas no effect was seen with Pmp at this site.⁴⁴ Taken together with mutagenesis studies, these experiments suggest a similar model for intramolecular interaction in SHP-2 and SHP-1. Recent studies show that dual phosphorylation of SHP-2 leads to additive effects on activity.⁴⁷

While it is generally challenging to introduce proteins prepared in vitro into cells, Pmp-modified SHP-2 was microinjected into fibroblasts to assess the impact on MAP kinase signaling pathway. These experiments showed a clear ability of the Pmp modification to enhance MAP kinase signaling via increased SHP-2 phosphatase activity.⁴³ These experiments provide an in vivo correlate to the in vitro findings.

Summary

It is clear that to increase understanding of signal transduction pathways in normal and patho-physiology, a wide array of techniques are needed beyond the traditional genetics, biochemistry, and cell biology approaches that have been employed. Organic chemists and enzymologists have important roles to play here. In this Account, we have highlighted how chemical approaches may be used to shed light on issues in signaling and point to new approaches for therapeutic lead discovery. It will be a continuing challenge to refine existing approaches and develop new ones that can be usefully applied to dissect complex biological systems in the postgenomic era.

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References

- Hunter, T. Signaling—2000 and Beyond. *Cell* **2000**, *100*, 113–127.
- Krebs, E. G.; Fischer, E. H. The phosphorylase *b* to *a* converting enzyme of rabbit skeletal muscle. 1956. *Biochim. Biophys. Acta* **1989**, *1000*, 302–309.
- Cohen, P. Protein kinases—the major drug targets of the twenty-first century? *Nat. Rev. Drug Discov.* **2002**, *1*, 309–315.
- Shogren-Knaak, M. A.; Alaimo, P. J.; Shokat, K. M. Recent advances in chemical approaches to the study of biological systems. *Annu. Rev. Cell. Dev. Biol.* **2001**, *17*, 405–433.
- Ghosh, M.; Ichetovkin, I.; Song, X.; Condeelis, J. S.; Lawrence, D. S. A new strategy for caging proteins regulated by kinases. *J. Am. Chem. Soc.* **2002**, *124*, 2440–2441.
- Songyang, Z.; Cantley, L. C. The use of peptide library for the determination of kinase peptide substrates. *Methods Mol. Biol.* **1998**, *87*, 87–98.
- Abdel-Rahman, H. M.; Al-karamany, G. S.; El-Koussi, N. A.; Youssef, A. F.; Kiso, Y. HIV protease inhibitors: peptidomimetic drugs and future perspectives. *Curr. Med. Chem.* **2002**, *9*, 1905–1922.
- Abdel-Magid, A. F.; Maryanoff, C. A.; Mehrman, S. J. Synthesis of influenza neuraminidase inhibitors. *Curr. Opin. Drug Discov. Dev.* **2001**, *4*, 776–791.
- Knighton, D. R.; Zheng, J. H.; Ten Eyck, L. F.; Ashford, V. A.; Xuong, N. H.; Taylor, S. S.; Sowadski, J. M. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **1991**, *253*, 407–414.
- Hubbard, S. R.; Wei, L.; Ellis, L.; Hendrickson, W. A. Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* **1994**, *372*, 746–754.
- Madhusudan; Trafny, E. A.; Xuong, N.-H.; Adams, J. A.; Ten Eyck, L. F.; Taylor, S. S.; Sowadski, J. M. cAMP-dependent protein kinase: crystallographic insights into substrate recognition and phosphotransfer. *Protein Sci.* **1994**, *3*, 176–187.
- Cleland, W. W.; Hengge, A. C. Mechanisms of phosphoryl and acyl transfer. *FASEB J.* **1995**, *9*, 1585–1594.
- Aqvist, J.; Kolmodin, K.; Florian, J.; Warshel, A. Mechanistic alternatives in phosphate monoester hydrolysis: what conclusions can be drawn from available experimental data? *Chem. Biol.* **1999**, *6*, R71–80.
- Yan, H.; Tsai, M. D. Nucleoside monophosphate kinases: structure, mechanism, and substrate specificity. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1999**, *73*, 103–134.
- Admiraal, S. J.; Herschlag, D. Mapping the transition state for ATP hydrolysis: implications for enzymatic catalysis. *Chem. Biol.* **1995**, *2*, 729–739.
- Williams, A. Effective charge and transition state structure in solution. *Adv. Phys. Org. Chem.* **1992**, *27*, 1–55.
- Epstein, J.; Plapinger, R. E.; Michel, H. O.; Cable, J. R.; Stephani, R. A.; Billington, C. A.; West, G. R. Reactions of isopropyl methylphosphonofluoridate with substituted phenols. I. *J. Am. Chem. Soc.* **1964**, *86*, 3075–3084.
- Herschlag, D.; Jencks, W. P. The effect of divalent metal ions on the rate and the transition-state structure of phosphoryl transfer reactions. *J. Am. Chem. Soc.* **1987**, *109*, 4665–4674.
- Yuan, C. J.; Jakes, S.; Elliott, S.; Graves, D. J. A rationale for the design of an inhibitor of tyrosyl kinase. *J. Biol. Chem.* **1990**, *265*, 16205–16209.
- Kim, K.; Cole, P. A. Measurement of a Bronsted nucleophile coefficient and insights into the transition state for a protein tyrosine kinase. *J. Am. Chem. Soc.* **1997**, *119*, 11096–11097.
- Kim, K.; Cole, P. A. Kinetic analysis of a protein tyrosine kinase transition state in the forward and reverse directions. *J. Am. Chem. Soc.* **1998**, *120*, 6851–6858.
- Kim, K.; Parang, K.; Lau, O. D.; Cole, P. A. Tyrosine analogues as alternative substrates for protein tyrosine kinase Csk: insights into substrate selectivity and catalytic mechanism. *Bioorg. Med. Chem.* **2000**, *8*, 1263–1268.
- Ablooglu, A. J.; Till, J. H.; Kim, K.; Parang, K.; Cole, P. A.; Hubbard, S. R.; Kohanski, R. A. Probing the catalytic mechanism of the insulin receptor kinase with a tetrafluorotyrosine-containing peptide substrate. *J. Biol. Chem.* **2000**, *275*, 30394–30398.
- Nagasawa, T.; Utagawa, T.; Goto, J.; Kim, C.-J.; Tani, Y.; Kumagai, H.; Yamada, H. Syntheses of L-tyrosine-related amino acids by tyrosine phenol-lyase of *Citrobacter intermedius*. *Eur. J. Biochem.* **1981**, *117*, 33–40.
- Kim, K.; Cole, P. A. Synthesis of (2S, 3R)-beta-methyltyrosine catalyzed by tyrosine phenol-lyase. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1205–1208.
- Admiraal, S. J.; Herschlag, D. The Substrate-assisted general base catalysis model for phosphate monoester hydrolysis: evaluation using reactivity comparisons. *J. Am. Chem. Soc.* **2000**, *122*, 2145–2148.
- Williams, D. M.; Cole, P. A. Proton demand inversion in a mutant protein kinase reaction. *J. Am. Chem. Soc.* **2002**, *124*, 5956–5957.
- Parang, K.; Cole P. A. Design of bisubstrate analogue inhibitors for protein kinases. *Pharmacol. Ther.* **2002**, *93*, 145–157.
- Mildvan, A. S. Mechanisms of signaling and related enzymes. *Proteins: Struct., Funct., Genet.* **1997**, *29*, 401–416.
- Parang, K.; Till, J. H.; Ablooglu, A. J.; Kohanski, R. A.; Hubbard, S. R.; Cole, P. A. Mechanism-based design of a protein kinase inhibitor. *Nat. Struct. Biol.* **2001**, *8*, 37–41.
- Hubbard, S. R. Crystal Structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analogue. *EMBO J.* **1997**, *16*, 5572–5581.

- (32) De Meyts, P.; Whittaker, J. Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat. Rev. Drug Discov.* **2002**, *1*, 769–783.
- (33) Parang, K.; Kohn, J.; Saldanha, A.; Cole, P. A. Development of Photo-Cross-linking Reagents for Protein Kinase-Substrate Interactions. *FEBS Lett.* **2002**, *520*, 156–160.
- (34) Carter, P.; Wells, J. A. Engineering enzyme specificity by “substrate-assisted catalysis”. *Science* **1987**, *237*, 394–399.
- (35) Toney, M. D.; Kirsch, J. F. Direct Bronsted analysis of the restoration of activity to a mutant enzyme by exogenous amines. *Science* **1989**, *243*, 1485–1488.
- (36) Williams D. M.; Wang, D.; Cole, P. A. Chemical rescue of a mutant protein tyrosine kinase. *J. Biol. Chem.* **2000**, *275*, 38127–38130.
- (37) Lowry, W. E.; Huang, J. J.; Ma, Y.-C.; Ali, S.; Wang, D.; Williams, D. M.; Okada, M.; Cole, P. A.; Huang, X. Y. Csk, A critical link of G protein signals to actin cytoskeletal reorganization. *Dev. Cell* **2002**, *2*, 733–734.
- (38) Nada, S.; Okada, M.; Aizawa, S.; Nakagawa, H. Identification of major tyrosine-phosphorylated proteins in Csk-deficient cells. *Oncogene* **1994**, *9*, 3571–3578.
- (39) Muir, T. W.; Sondhi, D.; Cole, P. A. Expressed protein ligation: A general method for protein engineering. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705–6710.
- (40) Evans, T. C. Jr.; Benner, J.; Xu, M. Q. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci.* **1998**, *7*, 2256–2264.
- (41) Wu, J.; W, Hu, M.; Chai, J.; Seoane, J.; Huse, M.; Li, C.; Rigotti, D. J.; Kyin, S.; Muir T. W.; Fairman, R.; Massague, J.; Shi, Y. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta. *Mol. Cell* **2001**, *8*, 1277–1289.
- (42) Wang, D.; Cole, P. A. Protein tyrosine kinase Csk-catalyzed phosphorylation of Src containing unnatural tyrosine analogues. *J. Am. Chem. Soc.* **2001**, *123*, 8883–8887.
- (43) Lu, W.; Gong, D.; Bar-Sagi, D.; Cole, P. A. Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling. *Mol. Cell* **2001**, *8*, 759–769.
- (44) Zhang, Z.; Shen, K.; Lu, W.; Cole, P. A. The Role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *J. Biol. Chem.* **2003**, *278*, 4668–4674.
- (45) Neel, B. G.; Tonks, N. K. Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol.* **1997**, *9*, 193–204.
- (46) Burke, T. R., Jr.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. Nonhydrolyzable phosphotyrosyl mimetics for the preparation of phosphatase-resistant SH2 domain inhibitors. *Biochemistry* **1994**, *33*, 6490–6494.
- (47) Lu, W.; Shen, K.; Cole, P. A. Chemical Dissection of the effects of tyrosine phosphorylation of SHP-2. *Biochemistry*, in press.

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