

Insights into Aurora-A Kinase Activation Using Unnatural Amino Acids Incorporated by Chemical Modification

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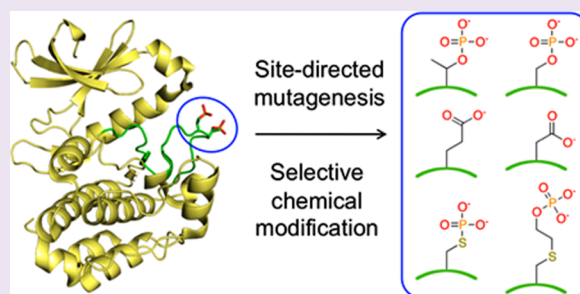
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S Supporting Information

ABSTRACT: Most protein kinases are regulated through activation loop phosphorylation, but the contributions of individual sites are largely unresolved due to insufficient control over sample phosphorylation. Aurora-A is a mitotic Ser/Thr protein kinase that has two regulatory phosphorylation sites on its activation loop, T287 and T288. While phosphorylation of T288 is known to activate the kinase, the function of T287 phosphorylation is unclear. We applied site-directed mutagenesis and selective chemical modification to specifically introduce bioisosteres for phospho-threonine and other unnatural amino acids at these positions. Modified Aurora-A proteins were characterized using a biochemical assay measuring substrate phosphorylation. Replacement of T288 with glutamate and aspartate weakly stimulated activity. Phospho-cysteine, installed by chemical synthesis from a corresponding cysteine residue introduced at position 288, showed catalytic activity approaching that of the comparable phospho-serine protein. Unnatural amino acid residues, with longer side chains, inserted at position 288 were autophosphorylated and supported substrate phosphorylation. Aurora-A activity is enhanced by phosphorylation at position 287 alone but is suppressed when position 288 is also phosphorylated. This is rationalized by competition between phosphorylated T287 and T288 for a binding site composed of arginines, based on a structure of Aurora-A in which phospho-T287 occupies this site. This is, to our knowledge, the first example of a Ser/Thr kinase whose activity is controlled by the phosphorylation state of adjacent residues in its activation loop. Overall we demonstrate an approach that combines mutagenesis and selective chemical modification of selected cysteine residues to investigate otherwise impenetrable aspects of kinase regulation.



Protein kinases catalyze the transfer of phosphate from ATP to an acceptor residue, commonly serine, threonine, or tyrosine, within a substrate protein. They control many cellular pathways, and their activity is tightly regulated by changes to their conformation.¹ Many protein kinases are themselves activated by phosphorylation, either by an upstream kinase or through an autoactivation mechanism. Phosphorylation of serine, threonine, or tyrosine residues in a conserved region known as the activation loop is critical for the activity of many kinases, although phosphorylation at additional sites can also up- or down-regulate activity.^{2,3} Aberrant kinase function is implicated in a range of diseases including cancer,⁴ and mechanistic studies of kinase activation are important to build an understanding of the molecular processes that underpin disease and to facilitate the discovery of small-molecule kinase inhibitors.^{5,6}

To study the functional effects resulting from phosphorylation of specific kinase residues *in vitro*, it is essential to prepare a homogeneous protein sample in which the kinase is

fully and selectively phosphorylated at the relevant sites. This cannot always be achieved by recombinant expression of native proteins, and techniques such as site-directed mutagenesis are often employed.⁷ Most commonly, a serine or threonine residue in the protein of interest is replaced with either a glutamate or aspartate residue.^{8–13} The major advantage of this method is the homogeneity of the resulting protein sample, with all protein molecules containing the same modification at the same site. Because glutamate and aspartate are negatively charged at physiological pH, they are considered to be convenient, suitable substitutes for phospho-serine and phospho-threonine. Indeed, in many cases glutamate and aspartate have successfully been used to functionally mimic these phospho-residues in a variety of systems.^{8,10,13–15}

Received: June 15, 2013

Accepted: July 23, 2013

Published: August 7, 2013

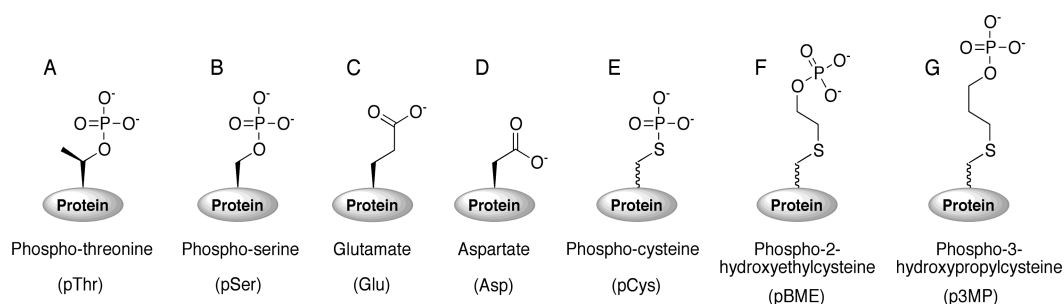


Figure 1. Comparison of naturally phosphorylated residues and corresponding biological and chemical phospho-mimics. (A) Phospho-threonine and (B) phospho-serine occur naturally in many protein kinases, while glutamate (C) and aspartate (D) are often installed as surrogates despite having a different charged state at physiological pH. Phospho-cysteine (E) is accessed using published methodology for the chemical modification of cysteine.²¹ Phospho-2-hydroxyethylcysteine (F) and phospho-3-hydroxypropylcysteine (G) are generated via chemical modification and autophosphorylation.

Nevertheless, this method is not appropriate for all kinases, several of which are rendered inactive by glutamate or aspartate substitution of key phospho-residues.^{9,11,12} This is probably due to the difference in charge and/or shape between these phospho-mimics and the native phosphorylated residues (Figure 1).

An alternative method is to introduce phosphorylation mimics by selective modification of chemically reactive cysteine residues. In some of the earliest examples of this approach, chemical mimics of phospho-aspartate were installed in bacterial chemotaxis signal transduction proteins. These chemical mimics were more stable than natural phospho-aspartate and therefore facilitated functional^{16–18} and structural¹⁹ studies of phosphorylated proteins. More recently, methods have been developed to chemically mimic phospho-serine by selective chemical reaction of cysteine residues.^{20,21} The resulting phospho-cysteine residue is a more similar bioisostere of phospho-serine than glutamate or aspartate by virtue of its matched ionization state at physiological pH and similar distance of the charged motif from the protein backbone; indeed, phospho-cysteine residues are recognized by antibodies directed against phospho-serine.²¹ Phospho-cysteine could also be applied as a phospho-threonine mimic, lacking only the C_{β} methyl group (Figure 1); however, to date there are no studies to indicate whether phospho-cysteine is able to functionally mimic phospho-serine or phospho-threonine on a protein whose activity or conformation is affected by its phosphorylation state, such as a protein kinase.

To investigate whether phospho-cysteine can functionally mimic phosphorylation on a protein kinase and evaluate whether it is a better substitute than glutamate or aspartate, we used the serine/threonine kinase Aurora-A (AurA) to test these hypotheses. AurA functions primarily in G2/M transition and mitotic spindle assembly, although functions in other contexts such as neurite extension have been described.²² AurA is an appropriate model system for technical reasons and because its regulation through phosphorylation is well characterized.^{23,24} Moreover, there is an unanswered biological question regarding the role of adjacent phosphorylation sites T287 and T288 in its activation loop. Phosphorylation at T288 is known to be critical for catalytic activity of AurA;^{12,25} however, it is currently unclear if, or how, phosphorylation at T287 influences kinase activity,²⁵ although it is an essential event in the aPKC-AurA-NDEL1 pathway that leads to neurite elongation and can occur independently of T288 phosphorylation.²⁶ The lack of information on the role of phospho-T287 in kinase activity is

because it has not been possible to generate AurA protein specifically phosphorylated at T287 in the absence of phosphorylation at T288.²⁴ We hypothesized that application of selective chemical modification to AurA would answer this unresolved question.

Here, we present a quantitative *in vitro* biochemical comparison of the catalytic efficiency of AurA with phospho-threonine or phospho-serine at both position 287 and position 288 with traditional phospho-mimics glutamate and aspartate and with chemical mimetics of phospho-serine prepared from the corresponding cysteine residues installed at position 287 or 288.²¹ We show that AurA is activated by insertion of phospho-cysteine in position 288 and is able to autophosphorylate unnatural amino acid residues introduced by chemical modification at position 288. Chemical methods enable specific modification of position 287 for the first time, which reveals a novel role for phosphorylation at this site.

RESULTS AND DISCUSSION

Characterization of Natural Phosphorylated Residues and Phospho-mimics. Although the phospho-sites in AurA are both threonine residues, many other kinases have serine phospho-sites in their activation loops. Phospho-serine differs from phospho-threonine by the absence of the C_{β} methyl group, and we therefore investigated the extent to which phospho-serine could functionally replace phospho-threonine at positions 287 and 288. We found that AurA autophosphorylated serine residues, and LC-MS/MS data indicated similar phosphorylation on the serine and threonine variants (Supplementary Figure S1). However, k_{cat} values for singly and doubly phosphorylated serines at 287 and 288 (3 and 4, Table 1) are 10-fold lower than their equivalent phospho-threonine values (1 and 2, Table 1). As both phospho-threonine and phospho-serine are likely to be doubly deprotonated at the assay pH,²⁷ we hypothesize that the methyl group introduces steric constraints on phospho-threonine that stabilize an optimal orientation, consistent with similar observations in FHA domains that specifically recognize phospho-threonine rather than phospho-serine due to the C_{β} methyl of the former.²⁸

In order to mimic phosphorylation by site-directed mutagenesis, glutamate and aspartate residues were selectively incorporated at positions 287 and 288 in the activation loop of AurA (5–10, Table 1). The best of these mimics, AE (6), has 2.5-fold higher K_m for ATP than wild-type AurA (1), 40-fold lower k_{cat} than wild-type, and only 16-fold higher k_{cat} than a

Table 1. Michaelis–Menten Kinetic Parameters for AurA Kinase Domain Variants

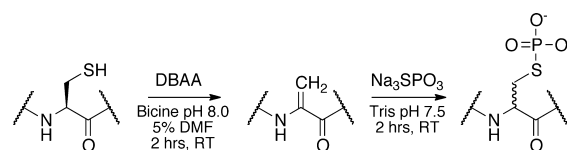
no.	AurA variant ^a	ATP K_m (μM) ^b	k_{cat} (h^{-1}) ^{b,c}	k_{cat}/K_m ($\text{h}^{-1}\mu\text{M}^{-1}$)
1	TT (wt)	59 \pm 9	62.2 \pm 2.5	1.05
2	AT	68 \pm 1	167.1 \pm 0.7	2.46
3	SS	176 \pm 3	6.60 \pm 0.04	0.04
4	AS	88 \pm 3	15.3 \pm 0.2	0.17
5	EE	192 \pm 8	0.91 \pm 0.01	0.005
6	AE	153 \pm 11	1.58 \pm 0.04	0.01
7	EA	271 \pm 10	0.45 \pm 0.01	0.002
8	DD	195 \pm 15	0.64 \pm 0.02	0.003
9	AD	164 \pm 11	0.72 \pm 0.02	0.004
10	DA	230 \pm 15	0.44 \pm 0.01	0.002
11	pseudo TT	84 \pm 1	41.0 \pm 0.2	0.49
12	pseudo AT	70 \pm 3	74.9 \pm 0.9	1.07
13	pseudo AS	101 \pm 2	14.1 \pm 0.09	0.14
14	AC	233 \pm 13	0.45 \pm 0.01	0.002
15	AC ^{Dha}	202 \pm 8	0.45 \pm 0.01	0.002
16	AC ^{pCys}	94 \pm 4	5.46 \pm 0.08	0.06
17	AC ^{pBME}	158 \pm 10	3.06 \pm 0.07	0.02
18	AC ^{p3MP}	325 \pm 14	0.84 \pm 0.02	0.003
19	pseudo AY	344 \pm 5	0.29 \pm 0.00	0.001
20	CA	373 \pm 19	0.24 \pm 0.01	0.001
21	C ^{BME} A	378 \pm 9	0.40 \pm 0.00	0.001
22	C ^{pCys} A	233 \pm 6	0.78 \pm 0.01	0.003
23	AA	204 \pm 44	0.10 \pm 0.01	0.0005

^a(wt) indicates wild-type AurA. “Pseudo” refers to C290A, C393A constructs. Two-letter codes refer to residues at positions 287 and 288, respectively. Constructs 11–22 were made on a pseudo wild-type (C290A, C393A) template. ^bValues are from a single 12-point titration experiment, performed in duplicate. Errors given are \pm standard error of the mean. ^cThe k_{cat} takes into consideration the effects of K_m for substrate peptide, which could not be measured separately.

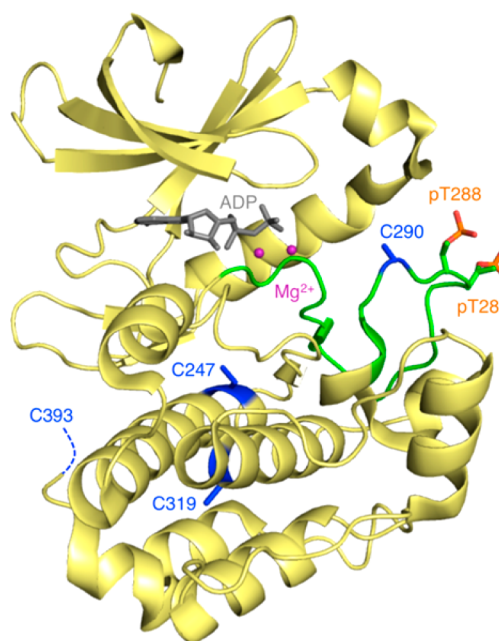
non-phosphorylated double alanine mutant (23). This glutamate mutant (6) also appears to be a poor substitute for phosphorylation when compared to its singly phosphorylated threonine and serine counterparts, AT (2) and AS (4), which have a 100-fold and 10-fold higher k_{cat} , respectively.

Glutamate and aspartate are both singly deprotonated at physiological pH, whereas phospho-threonine is expected to be doubly deprotonated.²⁷ The doubly deprotonated state of the phosphate moiety may therefore be necessary for optimal activity in AurA. Glutamate is a better mimic of phosphorylation than aspartate at position 288 (compare 6 and 9, Table 1), which may be due to the extended glutamate side-chain placing its charged carboxylate moiety in a similar position to the phosphate of wild-type phospho-threonine with respect to the backbone C_α (Figure 1). These results are consistent with literature reports: T288D AurA is more active than kinase-dead or non-phosphorylated AurA,^{25,29} but far less active than the wild-type phosphorylated kinase.^{12,25} Interestingly, the related kinase Aurora-B has been reported to be rendered inactive by an equivalent T232E mutation in its activation loop.³⁰

Chemical Synthesis of a Phospho-serine Mimic. As an alternative to the traditional phospho-mimics glutamate and aspartate, we employed recently developed chemical techniques²¹ to selectively introduce a phospho-mimic at position 288. This method involved the chemical modification of exposed cysteine residues (Figure 2). As a consequence the products resemble phospho-serine, rather than phospho-threonine, as they lack a C_β methyl group (Figure 1). A

**Figure 2.** Reaction scheme for the conversion of L-cysteine to D,L-phospho-cysteine on AurA, adapted from the published literature.²¹

pseudo wild-type template (11) was generated in which surface cysteine residues on AurA were mutated to alanine to prevent chemical modification at additional sites (Figure 3 and

**Figure 3.** Crystal structure of phosphorylated AurA kinase domain (PDB code 1OL7) bound to ADP (gray) and magnesium (purple). The kinase domain contains 4 cysteine residues, colored blue. C247 and C319 are buried within the protein, C290 is on the surface of the protein, and C393 is on the disordered C-terminus and is presumed to be solvent-exposed. In order to prevent undesired chemical modification C290 and C393 were mutated to alanine to generate a “pseudo wild-type” template. The activation loop (green) contains two phosphorylation sites, T287 and T288. Mimics of phosphorylation were installed at these sites by chemical and genetic methods.

Supplementary Figure S2). The pseudo wild-type template (C290A, C393A, 11) was then further mutated to produce singly phosphorylated threonine (12) and serine (13) variants at position 288 as control proteins for *in vitro* biochemical analyses. Characterization of these pseudo wild-type variants revealed they were all catalytically active with activity comparable to that of their wild-type counterparts (compare 11, 12, and 13 with 1, 2 and 4, Table 1), validating their use as controls alongside synthesized phospho-cysteine variants.

Cysteine was introduced at position 288 on the pseudo wild-type template, and T287 was simultaneously mutated to alanine to prevent any phosphorylation at 287, generating the AC variant (14, Table 1). Chemical conversion of this cysteine to phospho-cysteine, via dehydroalanine, was performed following a modified version of the published methodology.²¹ We used mass spectrometric analysis to monitor the reactions. This revealed additional peaks corresponding to mass differences of 178 and 258 Da, which were respectively identified as gluconoyl

and phospho-gluconoyl post-translational modifications of either lysine residues and/or the N-terminal methionine residue (Supplementary Figure S3). These post-translational modifications were independent of the phospho-cysteine chemical reaction and are consistent with frequently observed modifications on recombinant proteins expressed in *E. coli*, particularly within N-terminal hexa-histidine tag regions,^{31–33} but also on lysine residues.³⁴ We optimized the buffer constituents, pH, reaction times, temperature, and molar ratios of reactants in order to achieve a satisfactory level of chemical conversion at C288 on AurA. We found that the cysteine to dehydroalanine reaction proceeded essentially to completion, but we were unable to identify reaction conditions that facilitated complete conversion of dehydroalanine to phospho-cysteine. Nevertheless, the phospho-cysteine species was successfully isolated from the final reaction mixture by high-resolution ion exchange chromatography. Aggregates were removed by size exclusion chromatography, which also served to exchange the protein into a buffer optimized for kinase assays (Supplementary Figure S4). The final yield of product measured by recovery of total protein (determined pure by LC–MS) was 9%. LC–MS analysis of the intact protein confirmed the successful synthesis and purification of the phospho-cysteine AC variant, and LC–MS/MS analysis verified the correct location of the synthesized modification (Supplementary Figures S5 and S6).

Characterization of Phospho-cysteine Mimic of Phospho-serine. The catalytic activities of the AC variants (14, 15, and 16) were determined to assess their influence on AurA activity at each stage of the phospho-cysteine reaction (Table 1). There was no difference between cysteine (14) and dehydroalanine (15) at position 288, both of which resulted in a relatively inactive kinase. However, after reaction to phospho-cysteine (16), the k_{cat} increased 12-fold and was accompanied by a substantial decrease in K_m from >200 to 94 μM . This was a significant improvement over the corresponding glutamate and aspartate phospho-mimics (6 and 9). These observations were due to the presence of phospho-cysteine at position 288 and not artifacts of the reaction conditions, as a pseudo wild-type control construct lacking any surface cysteines was subjected to the same reaction conditions with no effect on K_m or reaction rate (Supplementary Figure S7). Additionally, LC–MS data indicated that the phospho-cysteine modification was stable during the time course of our activity assays (Supplementary Figure S5).

Despite the increased k_{cat} observed for the AC phospho-cysteine mimic at 288 (16), this protein was still 2.5-fold less active than the equivalent phospho-serine variant, pseudo AS (13), and 14-fold less active than its phospho-threonine counterpart, pseudo AT (12). As described earlier, the C_β methyl of phospho-threonine may be essential for optimal activity in AurA, and this cannot be recreated synthetically from AC^{Dha} (15), which lacks an equivalent methyl group. We note that the addition of thiophosphate to AC^{Dha} (15) is likely to result in a mixture of L- and D-phospho-cysteine (Figure 2), which may explain the lower activity of AC^{PCys} (16) versus pseudo AS (13). Phospho-cysteine is, nevertheless, a better surrogate for phospho-serine than the naturally occurring phospho-mimics, glutamate and aspartate.

Autophosphorylation of Unnatural Serine Mimics.

Our results with aspartate and glutamate phospho-mimics suggested that a shorter side chain length reduces AurA catalytic activity. Using naturally occurring amino acids it was

not possible to investigate the effect of longer side chains. Therefore, we used chemical methods to introduce serine mimics with extended aliphatic chains and investigated their autophosphorylation and ability to activate AurA. A sample of AurA AC was chemically modified to produce AC^{Dha} (15), as described above, and subsequently reacted with the thiol nucleophile β -mercaptoethanol (BME) to give a 2-hydroxyethylcysteine variant at position 288, which contains a single hydroxyl moiety three atoms further from the backbone C_α than the equivalent hydroxyl in serine. This unnatural mimic of serine became autophosphorylated in the presence of ATP and Mg^{2+} to generate phosphorylated 2-hydroxyethylcysteine (17), abbreviated to pBME (Figure 4 and Supplementary Figure S8).

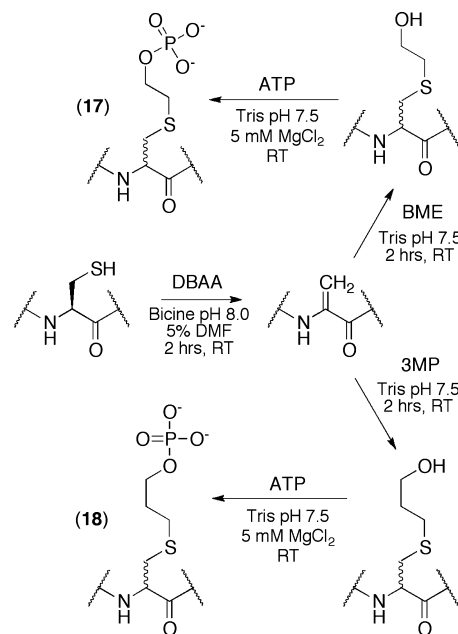


Figure 4. Synthesis of D,L-phospho-2-hydroxyethylcysteine (17) and D,L-phospho-3-hydroxypropylcysteine (18) from L-cysteine. AurA is able to autophosphorylate D,L-2-hydroxyethylcysteine and D,L-3-hydroxypropylcysteine when installed at position 288 in the activation loop.

This modification activated the kinase: AurA AC^{pBME} had a k_{cat} 7-fold higher than the unmodified AC construct (14), was almost as active as AC^{PCys} (16), and was twice as active as its comparable glutamate analogue, AE (6) (Table 1). Moreover, we found that AurA was able to autophosphorylate an even longer unnatural serine mimic, 3-hydroxypropylcysteine, which was generated by reacting AC^{Dha} with 3-mercapto-1-propanol (Figure 4 and Supplementary Figure S8). However, the resulting AC^{p3MP} variant (18) was notably less active than AC^{pBME} (17, Table 1), despite having only one additional atom in the linker between the phosphate group and the backbone C_α (Figure 4). This demonstrates that, at position 288, there is a maximum tolerable length of phospho-residue required to maintain catalytic activity in AurA, whereas the autophosphorylation mechanism is more flexible and can accommodate longer residues at this site. There are, however, limits to the flexibility of the autophosphorylation mechanism, as we observed that a corresponding tyrosine mutant, pseudo AY, was not autophosphorylated (Supplementary Figure S9) and demonstrated weak catalytic activity (19, Table 1) comparable to that of non-phosphorylated AurA variants. It seems that the

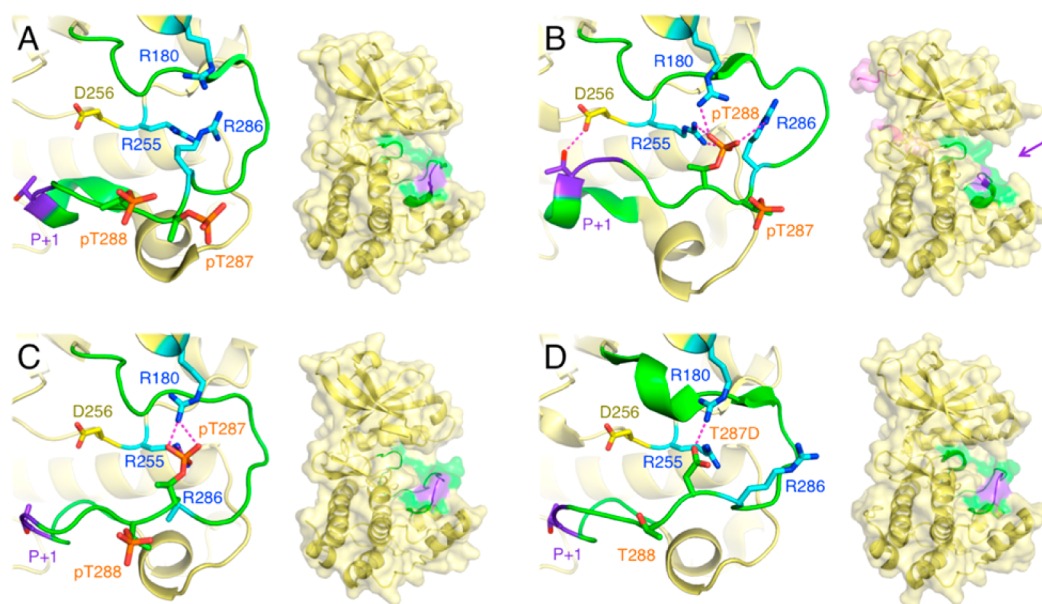


Figure 5. The activation loop of AurA adopts several different conformations, with consequences for substrate binding. In cartoon and surface representations the activation loop is colored green, and the conserved GT motif of the P+1 loop is purple. (A) PDB code 1OL7: the activation loop is ordered in the crystal structure due to crystallographic contacts; however, neither phospho-threonine 287 nor 288 interact with the nearby arginine pocket. (B) PDB code 1OLS: the loop adopts a fully active conformation when bound to residues 1–43 of TPX2 (shown in pale pink in surface representation, although mostly obscured by AurA). Phospho-threonine 288 rotates inward and binds in the arginine pocket formed by R180, R255, and R286. The P+1 loop interacts with a nearby conserved aspartate, D256, which arranges the activation loop in a suitable conformation for substrate binding (indicated by purple arrow on surface representation). (C) PDB code 4BN1: the loop adopts an alternative active conformation in which phospho-T287 is bound in the arginine pocket. (D) PDB code 4DEE: a non-phosphorylated T287D mutant adopts a similar activation loop conformation to 4BN1. In all conformations except panel B, in which phospho-T288 is bound in the arginine pocket, the activation loop is arranged such that substrate binding is obstructed, thereby reducing the catalytic efficiency of AurA.

steric (aliphatic versus aromatic) and electronic (aliphatic alcohol versus phenolic) environment of the hydroxyl group is influential, and our results are consistent with the function of AurA as a serine/threonine, but not a tyrosine, kinase.

Overall, the activity data using unnatural, extended serine mimics highlights the importance of the phosphate group for kinase activity and illustrates that this is a more important determinant of kinase activity than the distance of the phosphomimic group from the protein backbone. However, there are constraints on distance and orientation from the protein backbone, which are more stringent for activity toward substrates than for autophosphorylation.

T287 Phosphorylation. We next used the synthetic approach to investigate the function of phosphorylation on AurA at T287. Wild-type AurA expressed in *E. coli* exhibits high levels of phosphorylation at T287 and T288 (Supplementary Figure S1).^{24,35} However, a T288A mutant does not autophosphorylate at T287.²⁴ In the background of a T288A mutation, AurA modified to include 2-hydroxyethylcysteine at position 287, C^{BME}A, did not significantly autophosphorylate (data not shown) or promote kinase activity (21). We conclude that T287 autophosphorylation is, like phosphorylation of nonself substrates, dependent on T288 phosphorylation. This contrasts with T288, which undergoes intramolecular autophosphorylation.³⁶

To investigate whether phospho-T287 was, in itself, sufficient to stimulate kinase activity, we required a construct that was phosphorylated exclusively at position 287. We therefore synthesized phospho-cysteine at position 287 alongside an alanine substitution at position 288 in order to mimic single T287 phosphorylation. The chemical reaction and purification

conditions were the same as for AC^{P_{Cys}}A, resulting in a pure sample of C^{P_{Cys}}A (22) containing phospho-cysteine at position 287. This variant had a k_{cat} 3-fold higher than that of its unmodified CA precursor (20, Table 1). Additionally, the ATP K_{m} for C^{P_{Cys}}A (22) decreased compared to that of CA and was comparable to having single glutamate or aspartate mimics at position 287 (7 and 10). C^{P_{Cys}}A had nearly 2-fold higher k_{cat} compared to that of EA (7) and DA (10) and was comparable to the poorer phosphomimics at position 288, AD (9) and AC^{p3MP} (18). Thus, using a synthetic chemistry approach, we were able to characterize AurA phosphorylated only on T287, a defined state that is not accessible using other methods.

We noticed that, in all of our variants, a single phosphorylation (or phospho-mimic) at 288 resulted in a more active kinase than double phosphorylation (or phosphomimics) at 287 and 288. This trend was observed for phosphothreonine (1, 2, 11, and 12), phosphoserine (3 and 4), glutamate (5 and 6), and aspartate (8 and 9). This suggested a potential inhibitory role for phospho-T287 when present alongside phospho-T288. In the active conformation of AurA, phospho-T288 is situated in a pocket formed by R180, R255, and R286 (Figure 5B).³⁵ This interaction stabilizes the activation loop and facilitates substrate binding by promoting the formation of the P+1 pocket, formed from residues that follow the activation loop. In this conformation, phospho-T287 is rotated away from the arginine pocket and points away from the protein surface. A second structure of AurA has neither phospho-T287 nor phospho-T288 situated in the arginine pocket, and both point away from the surface (Figure 5A). On the basis of previous structures, it is not clear how T287 phosphorylation might influence the activity of AurA.

We determined the structure of AurA in a new crystal form, in which phospho-T287, rather than phospho-T288, is observed bound in the arginine pocket (Figure 5C). The position of the phosphate group on T287 in this AurA structure is slightly shifted (by ~ 3.7 Å) relative to the position of the phosphate group on T288 in the AurA/TPX2 structure (Figure 5B). Nevertheless, the group is in close proximity (within 3.5 Å) of R180 and R255. Consistent with the low activity observed in phospho-mimics installed at T287, the conformation of the P+1 loop is suboptimal for substrate binding. This could explain why $C^{P_{Cys}}A$ (22) has a 7-fold lower k_{cat} in comparison with that of ACP^{Cys} (16), while still being catalytically active. The structure of non-phosphorylated AurA with a T287D phospho-mimic mutation³⁷ (PDB code 4DEE, Figure 5D) shows a similar conformation of the activation loop with the side chain of T287D in the same position as the side chain of phospho-T287. These structures demonstrate that the activation loop of AurA has sufficient flexibility for phosphate groups and phospho-mimics at T287 to bind the arginine pocket, explaining the observed effects of phosphorylated T287 on AurA activity.

On the basis of the available crystallographic data and the k_{cat} values observed in this work, we propose a model to describe how phosphorylation at positions 287 and 288 function in the regulation of AurA. Single phosphorylation at T288 is the most favorable, stabilizes the activation loop and P+1 loop in the optimal conformation for substrate binding, and results in the highest catalytic activity. Phospho-T287 is able to bind the arginine pocket (Figure 5C) and when in this conformation can stimulate kinase activity, although the activation loop conformation is suboptimal for substrate binding. When both sites are phosphorylated, we propose an equilibrium between two states with either phospho-T288 or phospho-T287 bound in the arginine pocket, resulting in a lower average k_{cat} for substrate phosphorylation compared to AurA bearing phospho-T288 alone. In the cell, T288 phosphorylation is the result of autophosphorylation.³⁸ T287 phosphorylation may be catalyzed by another kinase, such as aPKC,²⁶ providing a mechanism by which other kinases can influence AurA activity: negative regulation if T288 is phosphorylated or positive regulation if T288 is not phosphorylated. We could not find examples of similar proposed mechanisms involving adjacent phospho-sites on serine/threonine kinases. However, there are several examples of tyrosine kinases containing two adjacent phospho-tyrosine residues in their activation loop that differently regulate activity. For example, in ZAP-70, phospho-tyrosine 492 (equivalent to AurA phospho-threonine 287) appears to inhibit kinase activity when present alongside the adjacent phospho-tyrosine 493.³⁹ On the other hand, phospho-tyrosine 981 (equivalent to AurA phospho-threonine 288), rather than phospho-tyrosine 980, seems to be the inhibitory phospho-residue in JAK3.⁴⁰ Further cell biology studies will be required to investigate the physiological context in which the adjacent phospho-threonine sites of AurA are utilized in regulating kinase activity.

Conclusions. Many kinases have several phosphorylation sites that contribute to the regulation of their catalytic activities, but the precise contribution of each site often remains unresolved. Hence, the ability to specify the phosphorylation state of a protein kinase facilitates functional and mechanistic studies. Site-directed mutagenesis is routinely used to genetically encode aspartate or glutamate as phospho-mimics. We found that this approach was not suitable to mimic

phosphorylation at either of two adjacent threonine sites in the activation loop of AurA.

As an alternative approach, we successfully generated mimics of phosphorylation in the activation loop through chemical modification of specific cysteine residues to produce phospho-cysteine. This modification was a better substitute for phosphorylation at position 288 than either glutamate or aspartate and was almost equivalent to phospho-serine. To our knowledge, this is the first time that a synthetic phospho-mimic modification has been demonstrated with functional activity on a protein kinase. Moreover, we demonstrate that this synthetic method is an essential new method for probing the mechanism of multisite kinase activation because it enables the production of homogeneous samples of kinases in specific phospho-states that are otherwise unobtainable. We use this to show that phosphorylation at T287 suppresses kinase activity *in vitro* and propose that this is due to its interaction with an arginine pocket usually occupied by phospho-T288. Consequently, chemical modification of selected sites via mutation to cysteine residues is a valuable method for building our understanding of kinase activation mechanisms.

A key strength of the approach we describe is its flexibility: once the conditions for efficient modification have been established, a diverse set of functional groups can be incorporated into the selected site. We explored the consequence of increasing the distance between the protein backbone and the phospho-acceptor atom. AurA is capable of autophosphorylating longer residues than threonine or serine in its activation loop. Moreover, one of these longer phospho-mimics produces a catalytically active kinase. This observation sheds light on the flexibility of the activation loop of AurA, suggesting that it is able to accommodate significantly longer residues at position 288 during both autophosphorylation and substrate phosphorylation. We anticipate that this methodology will be invaluable in further studies of protein structure–function relationships.

METHODS

AurA Mutagenesis, Expression, and Purification. Site-directed mutagenesis of AurA kinase domain (Supplementary Figure S2) in a pET30-based vector was carried out using a QuikChange protocol (Agilent). Proteins were expressed in *E. coli* BL21-CodonPlus -RIL (Agilent) or Rosetta 2 (Merck) DE3 cells in LB medium, with initial growth at 37 °C followed by overnight incubation at 21 °C after induction with 1 mM isopropyl β -D-1-thiogalactopyranoside. Bacterial pellets were resuspended in lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 40 mM imidazole), supplemented with protease inhibitors and DNase (Roche), lysed by sonication, and clarified by centrifugation. Lysates were filtered and then subjected to Ni²⁺ affinity chromatography (GE Healthcare). Proteins were eluted in lysis buffer containing 250 mM imidazole, purified to homogeneity by S75 size exclusion chromatography (GE Healthcare) (gel filtration buffer: 50 mM Tris pH 7.5, 200 mM NaCl, 5 mM MgCl₂, 10% glycerol, 10 mM BME), then concentrated to ~ 10 mg mL⁻¹, and flash frozen for future use. Protein concentrations were measured in triplicate with a ND-1000 spectrophotometer (NanoDrop) using molecular weights and extinction coefficients calculated by ProtParam (ExPASy).

Kinetic Measurements. Kinase activity assays were performed in duplicate in gel filtration buffer (BME was excluded from the buffer for chemically modified variants that contained dehydroalanine) using a LabChip EZ Reader II system (Caliper Life Sciences) with 1.5 μ M fluorescein-labeled Caliper Peptide 21 (Flu-LRRASLG) as the substrate and 12-point 2-fold serial dilutions of ATP. The concentration of each AurA variant was optimized for individual

assays to give a maximum of 20% substrate phosphorylation after 1 h at the highest concentration of ATP (1 mM). Substrate phosphorylation was measured at 5-min intervals for 1 h, and data analysis (linear regression of ATP titrations and fitting to Michaelis–Menten curves) was performed using GraphPad Prism software.

Chemical Modification of Cysteine Residues. α,α' -Dibromo-adipyl(bis)amide (DBAA) was synthesized and characterized as described.⁴¹ Phospho-cysteine was installed on AurA AC and CA variants as follows: a 500 μ L aliquot of protein at \sim 10 mg mL⁻¹ was shaken for 15 min at room temperature (RT) with 10 mM dithiothreitol (added as a solid) and then exchanged using a PD MiniTrap G-25 column (GE Healthcare) into 50 mM bicine pH 8.0. A 500 \times molar excess of DBAA dissolved in dimethylformamide (DMF) was added to the eluted protein, bicine buffer was added to a final volume of 2.5 mL (final DMF concentration was 5% (v/v)), and the reaction was shaken at RT for 2 h to form dehydroalanine. Protein was desalted and exchanged into 50 mM Tris pH 7.5 over a PD-10 column (GE Healthcare). A 10000 \times molar excess of sodium thiophosphate (Aldrich) was added as a solid and shaken for 2 h at RT. The reaction mixture was exchanged into 20 mM CHES pH 10.0 and then separated on a Mono S 5/50 GL ion exchange column (GE Healthcare). Phospho-cysteine species washed off in unbound fractions, while unreacted cysteine and dehydroalanine species bound to the column until eluted with NaCl. Desired protein fractions were pooled, concentrated to <2 mL, and purified on an S75 10/30 size exclusion column (GE Healthcare) equilibrated with gel filtration buffer. Eluted protein was concentrated to \sim 0.5 mg mL⁻¹ and stored at -80 °C. 2-Hydroxyethylcysteine variants were generated by reacting AC^{Dha} and C^{Dha}A (produced as described above) with 10 mM BME (Aldrich) in 50 mM Tris pH 7.5 for 2 h at RT and then exchanged into gel filtration buffer. AC^{3MP} was generated in the same manner by reacting AC^{Dha} with a 5000 \times molar excess of 3-mercapto-1-propanol (3MP) (Aldrich). AC^{BME} autophosphorylated rapidly during the kinase activity assay (i.e., we did not observe a lag phase for substrate peptide phosphorylation). AC^{3MP} autophosphorylated more slowly (i.e., we observed a lag phase of \sim 30 min; consequently data collected prior to this time were excluded from linear regression calculations). The AC^{Dha} variant was generated by the cysteine to dehydroalanine reaction as described above and then exchanged into gel filtration buffer without BME.

Mass Spectrometry. Detailed LC–MS and LC–MS/MS methods can be found in the Supporting Information.

X-ray Crystallography. Methods for the crystallization and structure determination of AurA^{V174M} can be found in the Supporting Information.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary figures, mass spectrometry methods, and crystallographic methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

F.C.R. produced AurA proteins, performed chemical reactions and kinase activity assays, and wrote the manuscript. M.R. and A.T. carried out mass spectrometry and prepared related figures. R.A.B. obtained the crystal structure of AurA^{V174M}. R.B. and J.B. provided intellectual input and contributed to the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

F.C.R. is supported by The Wellcome Trust Ph.D. program in Mechanism-Based Drug Discovery at The Institute of Cancer Research, grant number 093986/Z/10/Z. M.R. and J.B. are supported by Cancer Research UK grant number C309/A11566. R.B. is a Royal Society Research Fellow and is supported by Cancer Research UK grant C24461/A12772. We thank past and present colleagues at The Institute of Cancer Research: the Hit Discovery and Structural Design team for their assistance with biological assays; in particular R. Burke, J. Schmitt and K. Boxall; C. Dodson for advice on AurA protein production, assay protocols, and input into the manuscript; D. Waalboer for synthesis of DBAA; and M. Widya of the Proteomics Core Facility for analysing LC–MS/MS data and preparing figures. We thank R. van Montfort for critical reading of the manuscript.

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