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Increasing the Kinase Specificity of K252a by Protein Surface Recognition

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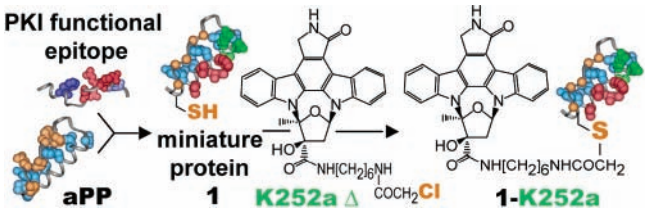
ABSTRACT



Here we describe a miniature protein (1) that presents the cAMP-dependent protein kinase (PKA) recognition epitope found within the heat-stable Protein Kinase Inhibitor protein (PKI) and a miniature protein conjugate (1-K252a) in which 1 is joined covalently to the high-affinity but nonselective kinase inhibitor K252a. Miniature protein 1 recognizes PKA with an affinity that rivals that of PKI and, in the context of 1-K252a, leads to a dramatic increase in kinase specificity.

Protein kinases play fundamental roles defining and maintaining signal transduction pathways, and aberrant kinase activity is linked to a plethora of human disease. Despite intense interest in this area, the design of highly selective protein kinase inhibitors remains a challenge[1,2] due to the large number of kinases encoded by the human genome[3] and the striking similarity of their active sites.[1,4] We have previously described a strategy for DNA and protein surface recognition in which the α-helix in the small, well-folded protein avian pancreatic polypeptide (aPP) presents recognition epitopes found within other, larger proteins.[5−13] The miniature proteins designed in this manner recognize even shallow clefts on protein surfaces with (in many cases) nanomolar affinities and high specificity and inhibit protein−protein interactions.[14,15] Here we describe a miniature protein (**1**) that presents the cAMP-dependent protein kinase (PKA) specificity epitope found within the heat-stable Protein Kinase Inhibitor protein (PKI) and a miniature protein conjugate (**1-K252a**) in which **1** is joined covalently to the high-affinity but minimally selective kinase inhibitor K252a. The indolocarbazole natural product K252a is a potent, active-site-directed inhibitor of many tyrosine and serine/threonine kinases[16−19] and a common starting point for the

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discovery of specific kinase inhibitors.[1,20] Miniature protein **1** recognizes PKA selectively with an affinity that rivals that of PKI and, in the context of **1-K252a**, leads to a dramatic increase in kinase specificity. These results suggest that molecules that combine a potent active-site-directed inhibitor with a miniature protein specificity element could represent viable tools to selectively explore kinase function.[21−27]

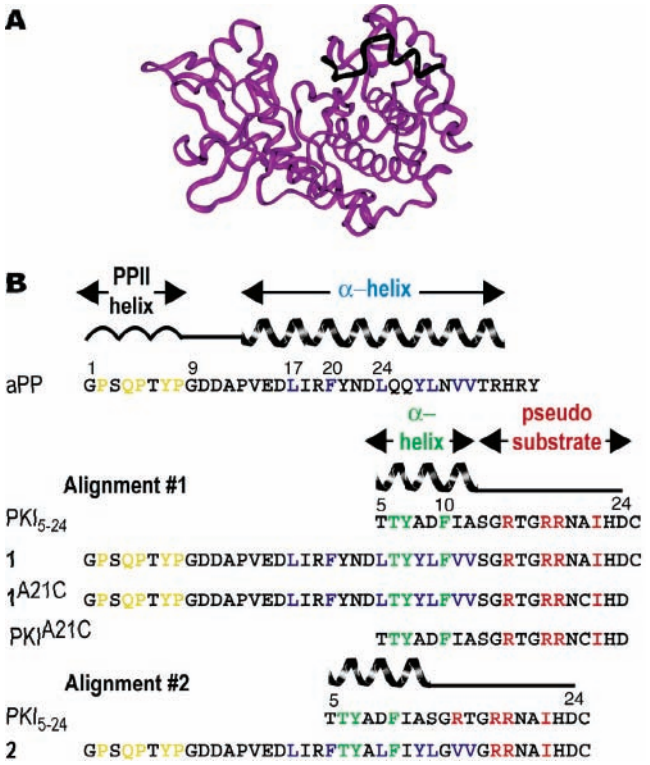

Figure 1. (A) Structure of the catalytic subunit of PKA (purple) in complex with PKI_{5-24}[28] (black). (B) Strategy for miniature protein design. The PPII and α-helix regions within aPP are identified, as are the α-helix and pseudosubstrate regions of PKI_{5-24}. Residues within aPP that contribute to folding are in blue (α-helix) or yellow (PPII helix). Residues that comprise the PKI_{5-24} pseudosubstrate are in red; residues that comprise the PKI_{5-24} α-helical specificity element are in green.

Our design of miniature protein **1** began with the structure of the catalytic subunit of PKA in complex with the active portion of PKI (PKI_{5-24}, Figure 1A), a molecule that selectively recognizes PKA and inhibits its function. In this complex, the N-terminal α-helix of PKI_{5-24} (residues 5−13) nestles in a shallow hydrophobic groove outside the substrate-binding site of PKA with contacts from F10 and perhaps T6 and Y7 (residues in green in Figure 1B). Located C-terminal to this specificity element is the nonhelical pseudosubstrate region (residues 17−24), which occupies the peptide substrate-binding site of PKA with energetically significant contacts from R18, R19, I22, and R15 (residues in red in Figure 1B). Miniature protein **1** was designed to contain both the PKI_{5-24} N-terminal specificity element (Thr at position 25, Tyr at position 26, and Phe at position 30) and the C-terminal pseudosubstrate (Arg at positions 35, 38, and 39 and Ile at position 42), as well as all residues required to maintain the aPP fold. In miniature protein **2**, the specificity and pseudosubstrate elements of PKI_{5-24} are located one helical turn closer to the N-terminus compared to their location in miniature protein **1**. Miniature proteins **1** and **2** were synthesized using standard solid-phase methodology; the cysteine residue at the C-terminus of each molecule was modified with 5-iodo-acetamidofluorescein to facilitate fluorescence polarization analysis of PKA affinity.

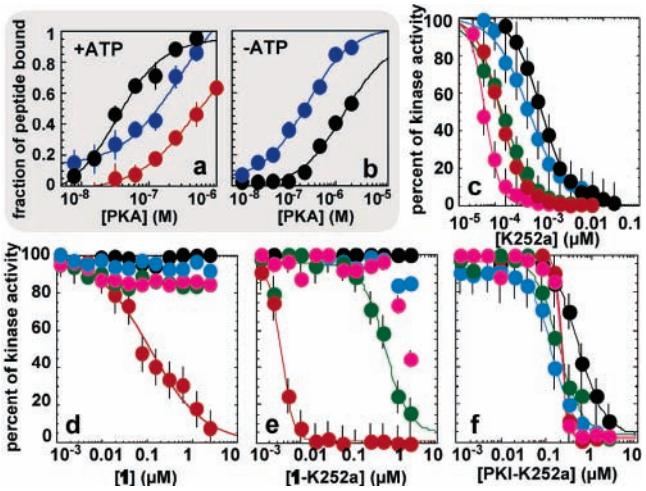

Figure 2. Affinity and inhibitory potency of PKA ligands. Fluorescence polarization analysis of the equilibrium affinity of $\mathbf{PKI}^{Flu}$ (black), $\mathbf{1}^{Flu}$ (blue), and $\mathbf{2}^{Flu}$ (orange) for PKA in the presence (A) and absence (B) of 100 μM ATP. Inhibition of the phosphotransferase activity of PKA (red), PKB (black), PKCα (blue), PKG (green), and CamKII (pink) by (C) K252a, (D) **1**, (E) **1-K252a**, and (F) **PKI-K252a**.

The relative affinities of $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, and $\mathbf{PKI}_{5-24}^{Flu}$ for the catalytic subunit of PKA were measured by fluorescence polarization analysis in the presence and absence of ATP (Figure 2). In the presence of 100 μM ATP, the complex between PKA and $\mathbf{1}^{Flu}$ was characterized by an equilibrium

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dissociation constant (K_d) of 99 ± 39 nM. The stability of PKA·**1**^{Flu} was only 3-fold lower than that of PKA·**PKI**$_{5-24}$^{Flu} under identical conditions (K_d = 31 ± 8 nM). **2**^{Flu} bound PKA with much lower affinity (K_d = 570 ± 123 nM), perhaps because it lacked R15, and was not considered further (Figure 2A). Surprisingly, **1**^{Flu} retained significant affinity for PKA in the absence of ATP (K_d = 230 ± 34 nM). By contrast, **PKI**$_{5-24}$^{Flu} bound PKA far more poorly in the absence of ATP, as expected,[29] undergoing a 50-fold decrease in affinity (K_d = 1.6 ± 0.4 μM) (Figure 2B). Structural and biochemical studies have documented the dramatic change in PKA conformation induced by the binding of ATP and substrate.[30] Whereas the PKA apoenzyme exists in an open conformation[31] that maximizes solvent accessibility but binds peptide substrate poorly, coordination of ATP rotates the large and small enzyme lobes closer together to produce an intermediate conformation that is partially preorganized for substrate binding.[31,32] Binding of peptide substrate completes the conformational change to produce the catalytically active, closed conformation seen in several crystal structures.[28,31,33,34] Our results suggest that **1** may recognize the open and intermediate conformations of PKA with similar affinities or, alternatively, that the binding of **1** inhibits the conformational changes associated with ATP binding.[35]

The miniature protein conjugate **1-K252a** was designed after examination of the ternary complex of PKA with PKI$_{5-24}$ and the related indolocarbazole natural product staurosporine.[36] This examination suggested that an octamethylene chain would appropriately link a C3′ amide derivative of K252a to the side chain of residue 40 within **1**. K252a analogues with conservative substitutions at C3′ retain potency against a range of kinases,[37] suggesting that an octamethylene chain at this position would be tolerated. Moreover, the PKA−PKI$_{5-24}$ structure shows the side chain of the corresponding residue of PKI$_{5-24}$, A21, pointing directly into the ATP/staurosporine binding pocket.[28] Accordingly, we synthesized chloroacetamide K252aΔ (Figure 3A) and a derivative of **1** with a cysteine residue in place of alanine at position 40 (Figure 1B). **1**^{A40C} was alkylated with K252aΔ in the presence of NaI, yielding **1-K252a** (Figure 3B). K252aΔ was also used to alkylate PKI^{A21C} to produce PKI−K252a.[38]

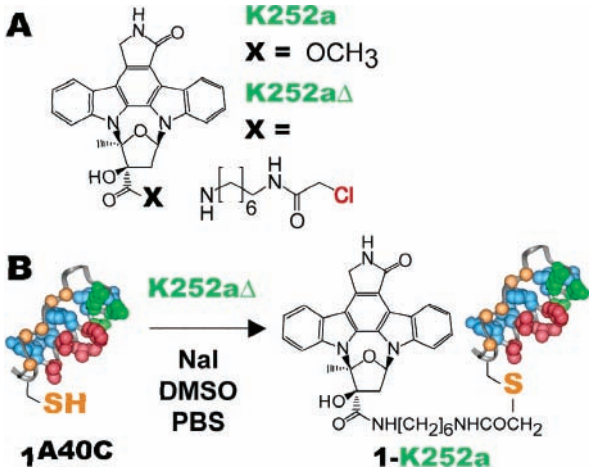

Figure 3. (A) Structure of the natural product K252a and K252aΔ. (B) Reaction of K252aΔ with **1**^{A40C} to produce **1-K252a.** Similar conditions were used to produce **PKI-K252a**.

The relative abilities of **1**, **1-K252a**, **PKI-K252a**, and K252a itself to inhibit the catalytic activity of PKA were measured using an assay based on streptavidin-matrix capture of biotinylated, [^{32}P]-phosphorylated substrates in which ATP and peptide substrate concentrations were fixed below their respective K_M values.[38,39] As expected, K252a was an excellent PKA inhibitor (IC_{50} = 0.140 ± 0.003 nM) (Figure 2C) and the inhibitory potency of **1** was similar to its PKA affinity (IC_{50} = 117 ± 14 nM) (Figure 2D). The miniature protein conjugate **1-K252a** was 30-fold more active as an inhibitor (IC_{50} = 3.65 ± 0.13 nM) than was **1** alone (Figure 2E) and only 26-fold less active than K252a. Interestingly, the analogous molecule **PKI-K252a** was 60-fold less active as an inhibitor of PKA (IC_{50} = 221 ± 2 nM) than was **1-K252a** (Figure 2F) and far less active than PKI (K_I = 2.3 nM).[40] Both **1-K252a** and **PKI-K252a** were significantly better inhibitors than were variants of **1**^{A40C} or **PKI**^{A21C} alkylated with bromoacetamide in place of K252aΔ (IC_{50} > 1 μM, data not shown). The differential activities of **1-K252a** and **PKI-K252a** may arise from differences in the affinity of **1** and PKI$_{5-24}$ for the unique conformation of PKA observed in the ternary complex with PKI$_{5-24}$ and staurosporine.[39] Further work will be necessary to characterize the conformational changes induced in PKA upon the binding of **1-K252a**.[41,42]

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To evaluate the extent to which **1** alters the kinase specificity of K252a, the phosphotransferase assay described above was reconfigured to assay the activities of four distinct but related protein kinases. Akt kinase (PKB), protein kinase Cα (PKC-α), Ca^{++}/calmodulin kinase II (CamKII), and cGMP-dependent protein kinase (PKG) are all inhibited by K252a [18] (Figure 2C) but not by PKI_{5-24}.[40] By contrast, both **1** and **1-K252a** showed remarkable specificity for PKA, inhibiting no other kinase tested at concentrations as high as 100 nM (**1-K252a**) or 5 μM (**1**) (Figure 2D,E). The only other kinase inhibited by **1-K252a** was PKG ($IC_{50} = 679 \pm 202$ nM), the kinase most similar to PKA.[43] By contrast, **PKI-K252a** displayed low specificity, inhibiting all kinases tested with IC_{50} values within a 4-fold range (Figure 2F). In summary, the PKI_{5-24} conjugate **PKI-K252a** displayed lower activity than K252a and lower specificity than PKI_{5-24}, whereas the miniature protein conjugate **1-K252a** displayed higher specificity than K252a and higher potency than **1**. It is possible that the 26-fold lower inhibitory potency of **1-K252a** compared to K252a itself could be overcome (and perhaps reversed) using in vitro selection methods.[44] Our results suggest that molecules such as **1-K252a** that embody elements of protein surface recognition, in addition to bisubstrate inhibition,[26] could represent a general strategy to selectively explore kinase function.[21−26,45−47]

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Supporting Information Available: Synthesis and purification of **1**, **2**, PKI_{5-24}, $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, $\mathbf{PKI_{5-24}}^{Flu}$, $\mathbf{1}^{A40C}$, $\mathbf{PKI_{5-24}}^{A21C}$, K252aΔ, **1**−K252a, and PKI−K252a; analysis of $\mathbf{1}^{Flu}$, $\mathbf{2}^{Flu}$, and $\mathbf{PKI_{5-24}}^{Flu}$ for PKA affinity; inhibition and specificity of **1**, K252a, **1-K252a**, and **PKI-K252**. This material is available free of charge via the Internet at http://pubs.acs.org.

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