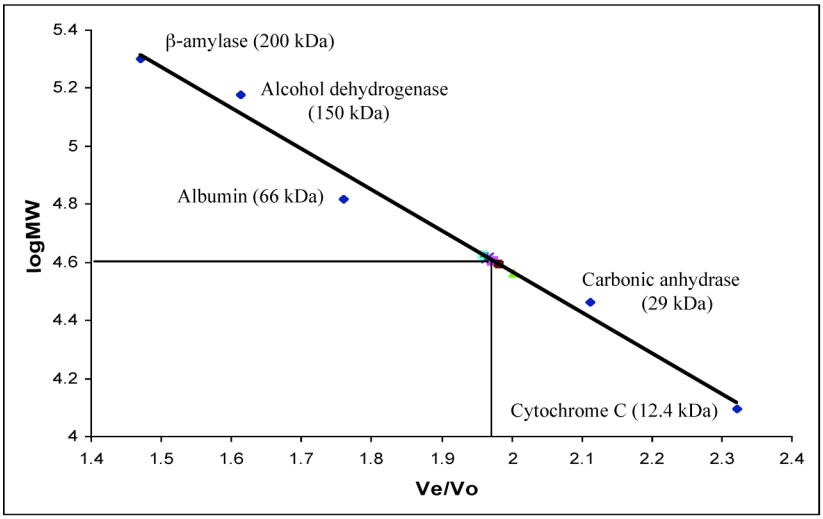
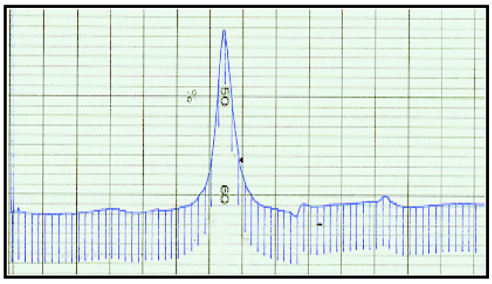
Supplementary Information
Figures S1 and S2 and experimental details.

Supplemental Figure 1.

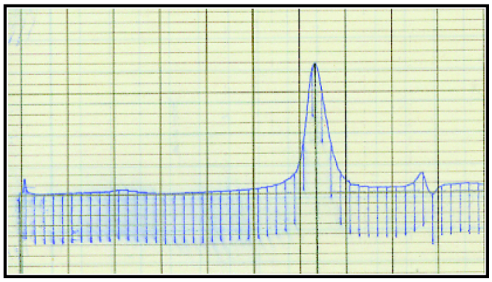


PKA^{P}



63.0mm

PKA^{ATP}

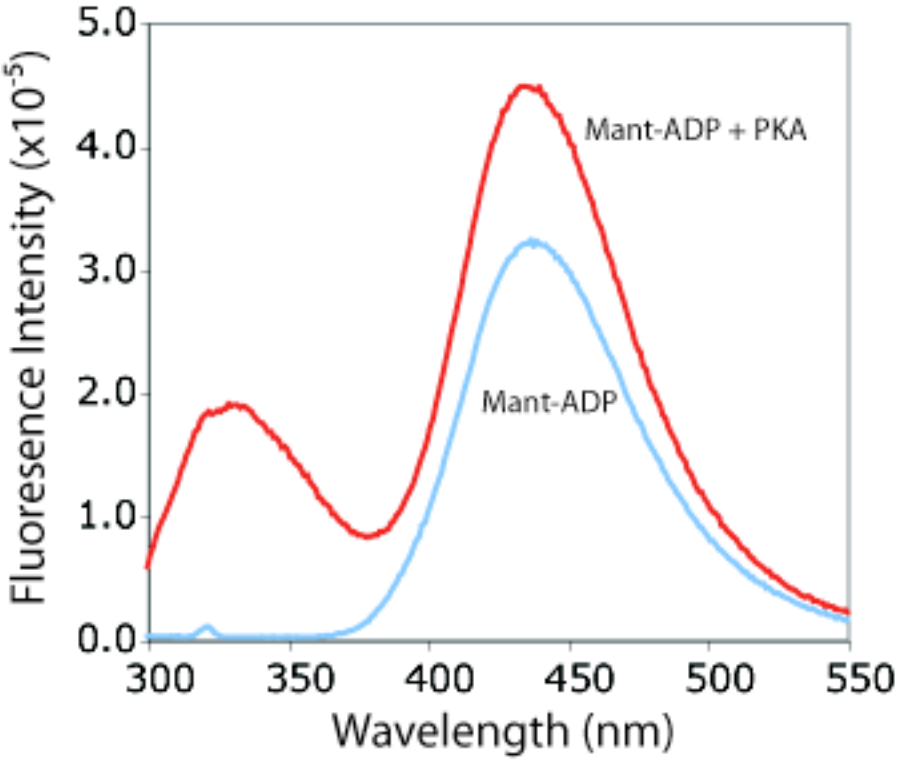


63.6mm

PKA	Calculated MW (g/mol)	Experimental MW (g/mol)
PKA^{ATP}	41 kDa	39 ± 5 kDa
PKA^{P}	40 kDa	41 ± 4 kDa

Supplemental Figure 1. Molecular weight determination of semisynthetic PKAs by size-exclusion chromatography. Mobility on a Superdex S-200 column (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 10 mM $MgCl_2$) was determined. For each sample, retention time was measured and correlated to the molecular weight using log MW vs. V_e/V_o where V_e is the elution volume and V_o is the void volume for the column. The calibration curve with standards is shown. Under these conditions standard recombinant PKA was as follows: calc, 40.5 kDa, found, 41.2 kDa.

Supplemental Figure 2.



Supplemental Figure 2. Fluorescence difference spectra. Representative spectra of Mant-ADP and Mant-ADP in the presence of PKA^{ATP} at 100 μM Mant-ADP shows a measurable difference at 440 nm. Correlation of this difference data at several concentrations allows for the determination of the apparent binding constants by Scatchard analysis.

Materials.
All materials for Fmoc solid phase synthesis were purchased from Novabiochem (Darmstadt, Germay) and used without additional purification. The ATPγS was purchased from Roche Applied Science (Indianapolis, IN), the 2'-O-(N-methylanthraniloyl)adenosine 5'diphosphate, disodium salt (MANT-ADP) was purchased from Invitrogen Molecular Probes (Carlsbad, CA) and the [γ-^{32}P] adenosine triphosphate was purchased from Perkin Elmer (Wellesley, MA). All buffering and bacterial media reagents were purchased from Fisher Scientific, Inc. (Hampton, NH). All additional chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification. All primers for cloning and site directed mutagenesis were ordered from Integrated DNA Technologies (Coralville, IA) and were used without further purification. DNA sequencing and N-terminal sequencing analysis was performed in the Sequencing and Synthesis core facility at Johns Hopkins University (Baltimore, MD).

Synthesis of ATPγS and Thiophosphate PKA Peptides. The synthesis of the ATPγS and thiophosphate conjugated peptides (Fig. 2) were based on syntheses previously described.[S1] All reactions were carried out under an inert atmosphere. In brief, the C-terminal peptide (aa 335-351) NH_2-Cys-Arg-Val-(Xxx)-Ile-Asn-Glu-Lys-Ser-Gly-Lys-Glu-Phe-Thr-Gly-Phe-CO_2H containing an alloc protected diaminopropionic acid (Dap-alloc) at aa 338 was assembled by Fmoc solid phase peptide synthesis (SPPS). The alloc protecting group was removed by gentle mixing of the resin with tetrakis(triphenylphosphine) palladium(0) (10 eq.) in a mixture of $CHCl_3$:NMM:AcOH (37:1:2) for 3 h. The resin was then washed with 0.5% sodium diethyldithiocarbamate in DMF, 10% N-methylmorpholine in DMF, DMF, MeOH, CH_2Cl_2 and dried under vacuum. Bromoacetic anhydride (20 eq.) in DMF was added to the resin and gently mixed for 5 h. The resin was washed with DMF, MeOH and CH_2Cl_2 and dried. The peptide was cleaved from the resin by gentle mixing of the resin with a mixture of 6% phenol, 4% ddH_2O, 90% trifluoroacetic acid for 5 h, and the resin was filtered away. The bromoacetylated peptide was precipitated by the addition of cold diethyl ether and dried under vacuum. The crude peptide was purified by reversed phase HPLC (ddH_2O/ AcCN, 0.5% TFA) and MALDI-TOF confirmed the mass of the peptide (PKA-AcBr, [M+H], calc 2082.19, found 2082.62). For ATP or phosphate, the bromoacetylated peptide was dissolved in 200 mM NH_4Ac (pH 7.5) and 1.5 eq. of ATPγS or thiophosphate was added. The reaction was mixed gently overnight at room temperature. The conjugated peptides were purified by gel filtration (BioRad P-2: Bio-Rad Laboratories, NH_4Ac, pH 7.5). Electrospray mass spectroscopy confirmed the mass of the peptides ($peptide^{ATP}$, [M+H], calc 2522.92, found 2521.35; $peptide^{P}$ [M+H], calc 2115.35, found, 2114.70).

Cloning and Site-Directed Mutagenesis of the PKA Catalytic Subunit. The catalytic subunit of murine PKA was cloned in frame into the Gyr intein containing bacterial expression vector, PTXB1. Full length PKA was amplified from a pRSET vector containing full length PKA using primers containing the Nde I restriction site (5'-AAACATATGGGCAACGCCGCCGCCGCC-3') and the Xho I restriction site (5'-AAACTCGAGAAACTCAGTAAACTCCTTGCC-3'). The amplified DNA was digested with corresponding restriction enzymes and the full length construct for PKA was ligated into the PTXB1 vector using the restrictions sites Nde I and Xho I. Full length PKA in PTXB1 was truncated using Quik-Change (Qiagen) site directed

mutagenesis to create PKA_{1-334} (Forward primer: 5'-GACTATGAGGAGGAAGAGTGCATCACGGGAGATGC-3', Reverse primer: 5'-GCATCTCCCGTGATGCACTCTTCCTCCTCATAGTC-3'). The Quik-Change kit was used as directed by the company and the desired sequence of the insert was confirmed by DNA sequence analysis.

Protein Expression and Semisynthesis of PKA^{ATP} and PKA^{P}. The truncated catalytic subunit of murine PKA was expressed in the *E. coli* strain BL21(DE3).[S2] Cells were cultured in LB media containing 100μg/mL ampicillin at 37°C to an optical density at 600 nm of 0.4-0.5, induced with 1 mM IPTG and grown for an additional 20 h at 15°C. Cells were isolated by centrifugation, resuspended in lysis buffer (10 mL/L culture, 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA) and lysed by french pressure cell. The insoluble material was removed by centrifugation (Beckman JA-10, 15,000 rpm) and the soluble fraction was loaded onto pre-equilibrated chitin resin (5 mL/L culture). The resin was washed with 10 volumes of wash buffer (50 mM HEPES, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) followed by 20 volumes of ligation buffer (50 mM HEPES, pH = 7.5, 150 mM NaCl, 1 mM EDTA). The column was purged with Ar and incubated with 0.6 mM C-terminal peptide and 200 mM p-mercaptophenyl acetic acid (MPAA) in ligation buffer under Ar at room temperature for 45 h. The column was eluted with ligation buffer, dialyzed against storage buffer (50 mM HEPES, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% glycerol) and concentrated by ultrafiltration to approximately 4 mg/mL of protein as determined by Bradford assay. The concentrated eluant was further purified by gel filtration (Sephacryl S-100: GE Healthcare Life Sciences) using a buffer containing: 50 mM HEPES, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% glycerol. Fractions containing the purified semisynthetic proteins were combined and stored at a final concentration of about 1 mg/mL, determined by Bradford assay. SDSPAGE stained with colloidal blue and MALDI MS were consistent with >90% purity of the desired materials.

SDSPAGE and Western Blot Analysis of Semisynthetic PKAs. A 500 ng sample of each of the semisynthetic PKA proteins was loaded onto an SDS-PAGE (12%) gel for comparison with commercially available and full-length recombinant PKA. Colloidal Blue staining of the gel confirmed bands at the appropriate molecular weight of ~40 kD. SDSPAGE gels were transferred by electroelution onto nitrocellulose membrane (BioRad, 0°C, 100 V for 1.5 h). The membrane was removed from transfer, washed, blocked (5% milk, 1 h) and probed with anti-phosphoThr197 antibody (T500P from Santa Cruz Inc., 1:5000) overnight.[S3] The blot was then washed, probed with anti-rabbit antibody (1:10000) for 1 h and visualized by ECL (SuperSignal West Pico Chemiluminescent, Pierce) on film.

Molecular Weight Determination by Size Exclusion Chromatography. The molecular weight of the semisynthetic PKA was determined by gel filtration using a Superdex S-200 column (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 10 mM $MgCl_2$). A series of molecular weight standards were used to determine a calibration curve (Standards: blue dextran, 2000 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa). For each standard as well as semisynthetic and recombinant PKA, a 100 μg protein aliquot was injected in 500 μL of buffer and the elution volume was measured. The elution volume was correlated with the apparent molecular weight (MW) by plotting the log(MW) vs. V_e/V_o where V_e is the elution volume and V_o is the void

volume for the column. The calibration curve allowed for an estimate of the size of the recombinant and semisynthetic PKAs (Fig. S1).

Kinase Activity Assay. The kinase activity of the semisynthetic PKA isoforms was determined as described previously.[S1] In brief, radiometric assays were carried out in 40 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, and 10 μM γ^{32}P-labelled ATP using 30 μM biotinylated kemptide (biotin-Leu-Arg-Arg-Ala-Ser-Leu-Gly-COOH) as the substrate in a 20 μL reaction volume. Reactions were initiated by the addition of 10 nM enzyme, carried out for varying amounts of time (0, 1, 2, and 4 min) at 30°C, and stopped by the addition of 10 μL of 100 mM EDTA. To each sample, 10 μL of 10 mg/mL avidin (Pierce) was added and all samples were transferred to centrifugal filtration units with 30,000 NMWL membranes (Millipore) and washed three times with 100 μL wash solution (0.5 M Phosphate, 0.5 M NaCl, pH 8.5). Substrate turnover was less than 10% for all rate measurements. Duplicate measurements were within 20%. Specific activities were calculated for each enzyme and expressed in min^{-1}. We note that fully recombinant PKA with wt sequence showed kinase activity essentially identical (within 30%) of PKA^P under these conditions. The effects of Ser338 phosphorylation on the kinetics of PKA kinase activity have previously been reported.

Fluorescence Binding Analysis of Semisynthetic PKAs. Fluorescence assays were based on work previously described by Adams and colleagues.[S5] All fluorescence experiments were carried out on a Fluoromax-2 (John-Yvon-SPEX, Instruments S.A., Inc) equipped with a temperature control unit. All measurements were carried out at 25°C. Emission spectra were collected between 300-550 nm after excitation at 290 nm. The slit excitation and emission slit widths were set to 1.25 nm and 3 nm, respectively. In a 150 μL volume, a mixture of 50 mM MOPS (pH 7.0), 1 mM $MnCl_2$ (1 mM free Mn^{2+} final concentration) and Mant-ADP (at varying concentrations) was combined with PKA (1 μM-final concentration). The solutions were incubated at room temperature for 30 min prior to fluorometric readings. For each data series the emission spectrum of a blank containing enzyme in the absence of Mant-ADP was also obtained. Duplicate measurements were obtained and were all within 10%. The fluorescence difference at 440 nm was determined at each concentration by subtracting the individual fluorescence backgrounds of enzyme and Mant-ADP alone at each concentration from the total combined fluorescence (PKA + Mant-ADP). Each value was corrected for inner-filter effects prior to analysis.[S6] The fluorescence difference values were then analyzed by Scatchard analysis to determine the apparent binding constants for both PKA^{ATP} and PKA^P. A representative difference spectrum is shown in Fig. S2.

Limited Proteolysis of PKA^{ATP} and PKA^P and the N-terminal Sequencing of Fragments. Reaction mixtures containing 20 mM Tris-Cl (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT and 5 μg PKA^{ATP} or PKA^P were treated with trypsin (in a final ratio of 1000 ng to 5 μg PKA), and the reactions were allowed to incubate for the designated time (0, 5, 10, 20 min) at 4°C. The reactions were quenched by the addition of 5 μL of 5xSDS loading buffer. A 10 μL aliquot of each time point was then loaded on to a 10-20% gradient SDS-PAGE gel and analyzed by silver stain (BioRad, Silver Stain Plus) to determine the fragmentation pattern that resulted from the digestion at each time point. The results showed the development of a meta-stable fragment at 34 kDa in the PKA^{ATP}. N-terminal sequencing by Edman degradation (Johns Hopkins Protein Core Facility) of this band confirmed it as a portion of the PKA^{ATP} that was consistent with a

proteolytic cleavage between K83 and Q84. (A_{72}MKILDKQKVVKLK // QIEHTLNEKRILQAV$_{100}$) The remaining fragment from the cleavage, $PKA^{ATP}_{85\text{-}351}$, has a calculated molecular weight of ~34 kDa.

Computational Modeling of PKA Autophosphorylation State using Rosetta

The starting structure for loop modeling in Rosetta was the crystallized ternary complex (pdb code 1atp) with residues 314 to 347 removed from the structure and Ser338 placed in the same location as Ala21 of PKI in the same crystal structure. Since a loop length of 34 residues is well outside the range for standard loop modeling protocols, we carried out modeling in three stages. In the first stage we carried out *ab initio* loop modeling[S7] on the N and C-terminal side of the loop, residues 314-338 and 338-346 respectively, to generate 1000 models, or decoys, each. Loop modeling the N-terminal side produced an abundance of closed-loop decoys and the lowest energy decoy was selected for the next stage. Loop modeling on the C-terminal side did not produce any low-energy decoys with closed loops, so the decoy with the smallest chain break and a sufficiently low score (bottom 10%) was selected for the next stage. The two decoys were merged into a single structure that contained a C-terminal tail conformation that placed Ser338 at the active site, but contained a chain break between residues 346 and 347. This structure served as the input for the second stage, where 1000 decoys were generated using *ab initio* loop modeling along residues 338-346 to eliminate the chain break. The lowest energy structure from this stage was free of chain breaks and used in the third and final stage. In the final stage, ATP was added to the active site in the same position as is found in 1atp, and 10 decoys were created by full-atom loop refinement[S7] along residues 328-338 while activating Rosetta's ligand-mode.[S8] The lowest energy structure from this final stage was selected as the proposed structural model of autophosphorylation state.

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