Synergistic Binding of Nucleotides and Inhibitors to cAMP-dependent Protein Kinase Examined by Acrylodan Fluorescence Spectroscopy*

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We have engineered an acrylodan-modified derivative of the catalytic subunit of cyclic AMP-dependent protein kinase (cAPK) whose fluorescence emission signal has allowed the synergistic binding between nucleotides and physiological inhibitors of cAPK to be examined (Whitehouse, S., and Walsh, D. A. (1983) J. Biol. Chem. 258, 3682-3692). In the presence of the regulatory subunit, R², the affinity of cAPK for adenosine, ADP, AMP-PNP (adenosine 5'-([β,γ-imino]triphosphate), or ATP was 5-, 55-, 420-, and 15,000-fold enhanced, while in the presence of the heat-stable inhibitor protein of cAPK (PKI), there was a 3-, 20-, 33-, and 2000-fold enhancement in the binding of these nucleotides, respectively. A short inhibitor peptide, PKI-(14–22), enhanced the binding of ADP to the same degree as did full-length PKI (20-fold) but, in contrast, did not significantly enhance the binding of ATP or AMP-PNP. The full binding synergism between PKI and either ATP (2000-fold) or AMP-PNP (33-fold) to cAPK could, however, be mimicked by a longer peptide, PKI-(5–24), suggesting that the PKI NH₂ terminus (residues 5–13) is most likely critical. Since this region is remote from the ATP γ-phosphate, the binding synergism must arise through an extended network communication mechanism between the PKI NH₂ terminus and the ATP binding site.

Protein kinases catalyze the transfer of phosphate from ATP to protein substrates and are central regulatory elements of all known pathways of signal transduction. While the deduced amino acid sequences of several hundred protein kinases are now known (1), the best characterized member of this family at the physiological, biochemical, and structural level is, by far, cyclic AMP-dependent protein kinase (cAPK) (2). cAPK was discovered as a component in the regulation of glycogen and intermediary metabolism (3), and more recently has been demonstrated to be directly involved in cell cycle control (4, 5) and the phosphorylation and activation of transcription factors (6, 7), emphasizing the diversity of cellular processes under this enzyme’s control. In vivo, cAPK exists as an inactive tetrameric holoenzyme which, upon binding cAMP, undergoes dissociation to a single dimeric regulatory subunit and two free, active catalytic subunits (8). While the inhibitory role of the regulatory subunit is well defined in vivo, the catalytic subunit can, in addition, be potently inhibited by the heat-stable inhibitor protein, PKI. Although its biochemical properties have been well characterized, the physiological significance of inhibition by PKI remains enigmatic (9).

The crystal structures of several complexes of recombinant cAPK or cAPK purified from porcine heart have been solved (10). The enzyme displays a globular fold consisting of two lobes defining a catalytic core which is conserved among all protein kinases whose three-dimensional structures are known. The active site is defined by an interlobal cleft in which ATP is deeply buried; at the mouth of the cleft is the binding site for peptide substrates, which is localized primarily to the surface of the larger lobe. The catalytic core is flanked by an amino-terminal α helix and carboxyl-terminal tail of 50 amino acids, both of which traverse the two lobes of the core. The COOH terminus is well ordered in structures displaying a closed conformation but displays considerable disorder in a recently solved structure of an open conformer (11), suggesting that this region may undergo differences in mobility during opening or closing of the active site cleft.

The equilibrium dissociation constants of both ATP and ADP for the free catalytic subunit lie between 10 and 20 μM (12, 13) and are not influenced by the binding of a short synthetic peptide substrate (LRRASLG) or substrate analogue (LRR-RALG) (14). However, ATP, but not other nucleotides, show dramatically increased affinity (~1000-fold) toward both the C-PKI and R²C₂ holoenzyme complexes (15, 16). The recent elucidation of two x-ray crystal structures containing the cAPK catalytic subunit, a 20-residue inhibitor peptide (PKI-(5–24)) and either MnATP (17) or MnAMPNP (18) reveal the apparent structural basis for the binding synergism, which is attributed to an observed network of hydrogen bonds directly interlinking the nucleotide γ-phosphate, the enzyme’s glycine-rich loop, and the P-site Ala of the inhibitor peptide.

The binding of substrates, products, and other ligands to proteins is commonly monitored by ligand-induced changes in protein fluorescence emission owing to a direct interaction of the bound ligand with endogenous tryptophan residues or, alternatively, to ligand-induced conformational changes. The primary advantages of fluorescence spectroscopy are its high sensitivity, the ability for measurements to be carried out at equilibrium, and convenience of assay. The principal limitation in many cases, however, is the lack of a fluorescent signal upon

*This study was supported by National Institutes of Health Grant GM19301 (to S. S. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: cAPK, cyclic adenosine 5'-3-monophosphate-dependent protein kinase; C, catalytic subunit of cAPK; R, regulatory subunit of cAPK; PKI, heat-stable inhibitor protein of cAPK; Acyr-cAPK, acrylodan-labeled cAPK; wt-cAPK, wild type cAPK; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; AMP-PNP, adenosine 5'-([β,γ-imino]triphosphate).
complex formation, as has been found in the case with cAPK. The catalytic subunit of cAPK contains six tryptophan residues. However, its intrinsic fluorescence emission is perturbed little, or not at all, upon binding of nucleotides, PKI, or substrate peptides.

To circumvent this problem, we have engineered a fluorescently tagged derivative of cAPK by substitution of Asn326 with Cys, which in turn was selectively modified with the environmentally sensitive, fluorescent dye, acrylodan (Ac-cAPK). The biochemical and fluorescent properties of the Ac-cAPK molecule reveal it to be one of the most sensitive and versatile probes yet described for quantitative measurements of nucleotide binding. Using this technique, we have measured the equilibrium dissociation constants of several cAPK-inhibitor complexes toward various nucleotides, and describe the relative contributions of specific structural determinants within ATP and PKI to their mutual interaction with the catalytic subunit of cAPK.

**MATERIALS AND METHODS**

**Mutagenesis and Expression of Proteins—**cDNA corresponding to the wild-type PEF subunit of cAMK was subcloned into plasmid pYES2 by standard procedures in plasmid pYES2Tb mutated to encode Cys instead of Asn at position 326, as described previously (19). Wild type cDNA (in plasmid pLWS-3) and mutant cDNA were overexpressed in Escherichia coli strain BL21-DE3 and purified by the protocol described below. Bacteria were grown in 1-liter shaker flasks at 37 °C to an optical density of 0.8 and with 0.5 mm isopropyl-1-thio-β-galactopyranoside for 6 h at 23 °C. Bacteria were harvested by centrifugation (5000 rpm × 15 min, Sorvall H4000 rotor) and frozen at −20 °C.

**Protein Purification—**Bacterial pellets from 4 liters of culture were thawed, resuspended in buffer A (30 mM MES, 50 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 6.5) (20 ml/pellet), and homogenized by two passes through a French press cell at 900-1200 p.s.i. The homogenate was cleared by centrifugation at 15,000 rpm for 40 min (Beckman JA-20 rotor). The supernatant was diluted 4-fold with cold distilled water to a salt conductivity ≤1.2 millisiemens/cm, adjusted to pH 6.5, and batchwise incubated with 30 ml of packed P-11 phenylcellulose resin (Whatman) for 2–3 h at 4 °C. The slurry was centrifuged and the resin batch washed with 250 ml of buffer A containing 90 mM potassium phosphate, then washed with an additional 400 ml of the same buffer in a column. Enzyme was eluted with 250 ml of 0.1 M potassium phosphate at a flow rate of 10–15 ml/hr. The P-11 eluate (30 ml) was dialyzed against 1 liter of buffer B (20 mM potassium phosphate, 1 mM DTT, pH 6.5) overnight, cleared by centrifugation (15,000 rpm for 30 min), and applied to a Mono S 5/5 FPLC column at a flow rate of 1 ml/min pre-equilibrated in the same buffer. The column was eluted with a linear NaCl gradient (0–300 mM/50 ml), which resulted in three peaks of eluting protein for both the wild type and mutant enzyme. The earliest peak was pooled and stored at 4 °C. From 4 liters of original culture, approximately 5–10 mg of protein are routinely recovered.

The heat-stable protein kinase inhibitor, PKI (α isoform), was expressed in E. coli strain BL21 DE3 and purified as described previously (20). Briefly, cells were lysed by French press, the lysate centrifuged (15,000 × g for 40 min, Beckman JA-20 rotor). The supernatant was dialyzed against 90 mM potassium phosphate, then washed with an additional 400 ml of the same buffer in a column. Enzyme was further purified by buffer A containing 250 mM potassium phosphate at a flow rate of 10–15 ml/hr. The P-11 eluate (30 ml) was dialyzed against 1 liter of buffer B (20 mM potassium phosphate, 1 mM DTT, pH 6.5) overnight, cleared by centrifugation (15,000 rpm for 30 min), and applied to a Mono S 5/5 FPLC column at a flow rate of 1 ml/min pre-equilibrated in the same buffer. The column was eluted with a linear NaCl gradient (0–300 mM/50 ml), which resulted in three peaks of eluting protein for both the wild type and mutant enzyme. The earliest peak was pooled and stored at 4 °C. From 4 liters of original culture, approximately 5–10 mg of protein are routinely recovered.

The heat-stable protein kinase inhibitor, PKI (α isoform), was expressed in E. coli strain BL21 DE3 and purified as described previously (20). Briefly, cells were lysed by French press, the lysate centrifuged (15,000 × g for 40 min, Beckman JA-20 rotor) and the supernatant heated to 95 °C for 5 min. After centrifugation the supernatant was acidified to pH 5.0 for 30 min, centrifuged, and supernatant applied to DE-52. The column was eluted with a gradient of 10–300 mM sodium acetate. The peak corresponding to PKI was chromatographed through Sephax G-75 resin equilibrated in 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA.

**R′ subunit (R209K) was expressed in XL-1 Blue R and attached via its C-terminal cysteine residue to DE-52 and eluted with a 0–150 mM potassium phosphate gradient. The peak corresponding to the R′ subunit was chromatographed through Superdex 200.**

**PKI-(5–24) and PKI-(14–22) peptides were synthesized by the Department of Biology Peptide Synthesis Facility (UCSD, La Jolla, CA).**

**Acrylodan Labeling—**Acrylodan (Molecular Probes) was dissolved in dimethylformamide to a stock concentration of 100 mM (measured by absorbance at 387 nm. ε = 16,400 M⁻¹ cm⁻¹ in ethanol) (22). Enzyme (1.5 mg in 1 ml) was reduced with 3 mM DTT in the presence of 1 mM EDTA, pH 7.0, for 2 h at room temperature. Free DTT was removed by chromatography through NAP-10 (Pharmacia Biotech Inc.) that had been pre-equilibrated in 20 mM MOPS, 50 mM NaCl, pH 8.0. The column was eluted (1.4 ml) with 10 mM MgCl₂ and 1 mM ATP. Two μl of the acrylodan stock was slowly added to the reduced enzyme (final molar ratio of acrylodan:protein = 5:1) with gentle vortexing, and labeling was allowed to proceed for 14 h at 4 °C. The reaction was quenched with 5 mM DTT, and the unreacted acrylodan was removed by chromatography through NAP-10 which was pre-equilibrated in 20 mM MOPS, 50 mM NaCl, pH 7.0. The labeled protein was resolved by replacing SDS-PAGE gel electrophoresis and was visualized by UV prior to staining. Total protein was visualized by staining with Coomassie Brilliant Blue R.

The stoichiometry of acrylodan labeling was quantitated by electrospray mass spectrometry. Protein (~10 μg) was desalted prior to analysis by reverse-phase liquid chromatography (Michrom Inc. Auburn, CA) on a polymeric, 1 mm diameter column (Polymer Laboratories Inc.) A rapid, 2-min gradient of increasing acetonitrile from aqueous 0.05% v/v trifluoroacetic acid was used. The protein-containing fraction was flow-injected into a 50% v/v water/methanol with 1% v/v acetic acid solution, which was continuously flowing into the electrospray interface of the mass spectrometer (Hewlett-Packard Inc., Falo Alto, CA).

The site of labeling by tryptic digestion of the labeled enzyme was determined by reverse phase HPLC chromatography and microsequence analysis of peptides. Briefly, protein (300 μg, in 20 mM MOPS, pH 7, 50 mM NaCl, 10 mM MgCl₂) was dried by Speed-Vac, resuspended in 1 ml of 50 mM ammonium bicarbonate, and digested with trypsin (1:100) at 37 °C for 18 h. The digest was analyzed by HPLC (C-18) at pH 6.8 eluted with a 5–40% gradient of acetonitrile for 80 min, followed by a 40–75% gradient for 20 min. Detection of peptides was monitored by absorbance at 219 nm. Fluorescence was detected by excitation at 390 nm and emission at 520 nm. Fluorescent peaks were purified to homogeneity by re-chromatography at pH 2.0. The eluted samples were neutralized by addition of 50 μl of 50 mM ammonium bicarbonate, and fluorescence was detected by a hand-held UV lamp. Sequencing of fluorescently labeled peptides was performed by automated Edman degradation.

**Enzyme Assay—**Enzyme activity was measured by a coupled-enzyme spectrophotometric assay (12). Concentrations of the coupling reagents in a 0.5-ml assay volume were as follows: 1 mM phosphoenolpyruvate, 100 μM NADH, 6 units of lactate dehydrogenase, and 2 units of pyruvate kinase. All reactions were performed in buffer containing 20 mM MOPS, 50 mM NaCl, 9 mM free MgCl₂, pH 7.0 at 23 °C. Typically, enzyme was preincubated with ATP in the assay mixture and the reaction initiated by the addition of Kemptide. Progress of the reaction was monitored continuously by a decrease in absorbance at 340 nm in a Hewlett-Packard 1587 diode array spectrophotometer. Reaction velocity was constant over 60 s. Values for Kₐ and Vₐmax were determined from plots of initial velocity versus substrate concentration fit to Equation 1 using the computer program, KALEIDODAGPH (Synergy).

**Equation 1**

\[ v = \frac{V_{\text{max}} [S]/K_{\text{a}} + [S]}{1} \]  

v is the initial velocity, S is the variable substrate, Vₐmax is the maximum velocity, and Kₐ is the Michaelis constant. The wt-cAPK concentration was determined by absorbance at 340 nm in a Hewlett Packard 1587 diode array spectrophotometer. Reaction velocity was constant over 60 s. Values for Kₐ and Vₐmax were determined from plots of initial velocity versus substrate concentration fit to Equation 2 using the computer program, KALEIDODAGPH (Synergy).

**Equation 2**

\[ I_{\text{f}} = \frac{I[1] + K_{\text{f}}}{1 + [S]/K_{\text{f}}} \]  

Iₐ is the fraction of inhibitor bound, being equal to: 1 − (1/(1 + [S]/K_{\text{f}})) when the concentration of inhibitor and K_{\text{f}} was determined by fitting of the data to Equation 2. Iₐ is the fraction of inhibitor bound, being equal to: 1 − (1/(1 + [S]/K_{\text{f}})) when the concentration of inhibitor and K_{\text{f}} was determined by fitting of the data to Equation 2.
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(5–24) crystal structure. The partial charge of acrylodan was calculated using MOPAC. A comprehensive conformational search for allowed positions for acrylodan was performed using the program RÖTOR. Briefly, the acrylodan-Cys

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tide. All assays were performed in 1 ml of buffer containing 20 mM indicated inhibitor molecule with increasing concentrations of nucleotide for cAPK were obtained by titrating a fixed quantity of Acr-cAPK in the absence or presence of saturating concentrations of the enzymatic activity on temperature. Reaction mixtures (20 µl) containing 20 mM MOPS, 50 mM NaCl, 9 mM free MgCl₂, 2 µM enzyme, pH 7.0, were kept on ice, rapidly heated to the target temperature for 3 min in a thermocycler (MJ Research, model PTC-100), then rapidly cooled back on ice. Enzyme activity versus temperature was plotted, and the Tm, corresponding to the temperature resulting in 50% loss of enzyme activity, was determined.

RESULTS

Expression, Purification, and Fluorescence Labeling—Asn

326

in the catalytic subunit of mouse α cAPK (Fig. 1) was mutated to cysteine as described under “Materials and Methods.” The mutant enzyme was overexpressed in a non-fusion protein in E. coli, and purified to homogeneity by conventional chromatography (see “Materials and Methods”). Three isoforms of the mutant were resolved by FPLC Mono S chromatography (data not shown). In the case of the wild type enzyme, these isoforms differ in their phosphorylation state at Ser10, and Ser139, while Cys326. A small amount (3%) of labeling occurred on Cys199 distant structure was fixed. ROTOR calculations revealed five distinct families of final conformers. The minimum energy conformer from each family was selected and subsequently minimized in water through 1000 iterations of steepest descent (the entire cAPK molecule with the exception of the acrylodan-Cys

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tide was fixed). From the set of 5 minimal families a single conformer was found to possess a dramatically lower energy level than the next most stable structure. The position of acrylodan in this conformer is shown in Fig. 4.

Spectrofluorometric Assays—The equilibrium dissociation constants of nucleotides for cAPK were obtained by titrating a fixed quantity of Acr-cAPK in the absence or presence of saturating concentrations of the indicated inhibitor molecule with increasing concentrations of nucleotide. All assays were performed in 1 ml of buffer containing 20 mM MOPS, 50 mM NaCl, 9 mM free MgCl₂, pH 7.0 at 23 °C in a Hitachi 4100 fluorometer. The concentration of Acr-cAPK varied between 30–300 nM for all experiments. Emission and excitation slits were both set at 5 nm. Excitation of acrylodan was at 395 nm and emission was scanned from 420 to 600 nm. Data was collected at 120 nm/min. Sample dilution due to ligand addition (typically less than 2% of the total volume) was corrected for all Kd measurements. Spectra were analyzed using the computer program SPECTRACALC (Galactic Ind. Corp.). Kd values were obtained by plotting the relative fluorescence values at fixed emission wavelengths, shift in emission wavelength maxima, or integration of the peak areas between 420 and 600 nm of each spectra, versus nucleotide concentration. Data were fit to Equation 3 using the computer program, KALEIDOGRAPH (Synergy).

∆F = [Fmax - (E + L + Kd)] - [Fmax - E] = [E(L)(E)](Fmax) (Eq. 3) ∆F and ∆Fmax are the change and maximum change in fluorescence, respectively, E is the total enzyme concentration, L is the total nucleotide concentration, and Kd is the equilibrium dissociation constant.

Thermal Inactivation Studies—The structural stability of wt-cAPK and Acr-cAPK was evaluated by examining the dependence of catalytic activity on temperature. Reaction mixtures (20 µl) containing 20 mM MOPS, 50 mM NaCl, 9 mM free MgCl₂, 2 µM enzyme, pH 7.0, were kept on ice, rapidly heated to the target temperature for 3 min in a thermocycler (MJ Research, model PTC-100), then rapidly cooled back on ice. Aliquots (10 µl) were assayed for enzyme activity as described above. Enzyme activity versus temperature was plotted, and the Tm, corresponding to the temperature resulting in 50% loss of enzyme activity, was determined.

To generate a potential fluorescent probe for monitoring ligand binding to cAPK, Cys326 in the mutant enzyme was specifically labeled with the sulfhydryl-reactive, environmentally sensitive fluorescent dye, acrylodan. The two endogenous cysteine residues, Cys399 and Cys443 were protected from chemical modification by preincubation with the enzyme with saturating concentrations of Mg-ATP prior to acrylodan labeling. As shown in Fig. 2a, upon preincubation with 1 mM MgATP, the mutant enzyme underwent extensive acrylodan labeling, while an equivalent amount of the wild type enzyme was completely protected. Analysis by mass spectroscopy revealed that the labeled protein had a molecular mass of 40,900 atomic mass units, consistent with the mutant displaying three phosphate groups, and being labeled to a stoichiometry of one acrylodan molecule per molecule of enzyme. Digestion with trypsin and sequence analysis of the proteolytic peptides showed that this protein was labeled nearly exclusively on Cys326. A small amount (3%) of labeling occurred on Cys399 (Fig. 2b).

The effect of the mutation and chemical modification on various physical and kinetic properties of the enzyme was tested. Analysis of steady state kinetic parameters for the phosphorylation of a synthetic peptide substrate, LRRASLG (Kemptide), showed that the Kcat and K<sub>Michaelis</sub> for Acr-cAPK both differed by only 1.5-fold from wild type, while K<sub>ATP</sub> was approximately 2.5-fold higher (Table I). These small but reproducible differences were not attributable to acrylodan labeling but, rather, were a direct consequence of the mutation itself, as both the labeled and unlabeled mutant enzymes displayed identical steady state kinetic parameter values.

To evaluate the effect of the mutation on the equilibrium binding of nucleotide and peptide substrates, the affinities of competitive inhibitors of either ATP or Kemptide were measured in a steady state kinetic competition assay. The K<sub>1</sub> of adenosine for Acr-cAPK differed by less than 2-fold in comparison to wt-cAPK, while the K<sub>1</sub> of a 7-residue inhibitor analogue of Kemptide (LRLASLG) was virtually identical (Table I). These data show that the affinities of ligands for the active site are largely unperturbed in Acr-cAPK.

The x-ray crystal structure of cAPK reveals that the side chain nitrogen atom of Asn326 is within hydrogen bonding distance (3.3 Å) of the backbone carboxyl oxygen of Ala324 in the interlokal linker region of the enzyme. We tested whether the consequent disruption of this hydrogen bond by replacement of Asn326 with Cys would manifest as a decrease in the enzyme’s thermal stability. Thermal denaturation was monitored by the loss in catalytic activity as a function of increasing temperature. The T<sub>m</sub> of Acr-cAPK proved to be identical to that of wt-cAPK (48 °C) (data not shown), suggesting either that
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Fluorescence Emission Spectra—Excitation of Acr-cAPK resulted in a broad emission spectrum displaying a $\lambda_{max}$ of 498 nm. Addition of ATP, ADP, adenosine or AMPNP all resulted in both quenching of fluorescence emission and a shift in the emission $\lambda_{max}$ to longer wavelengths (Fig. 3). Addition of 20 mM EDTA (2-fold molar excess over MgCl$_2$) reversed the quenching induced by ATP and ADP, suggesting that quenching was the result of specific Mg$^{2+}$-dependent nucleotide binding (data not shown). The red shift associated with ATP binding was consistently greater than that for AMPNP, ADP, or adenosine. The fluorescence emission spectra of Acr-cAPK complexed with any of the inhibitor molecules tested were similar to the spectrum of the free catalytic subunit, and were equally sensitive to nucleotide binding (data not shown).

Structural Basis for Fluorescence Change—In wt-cAPK, the side chain of Asn$_{326}$ lies proximal to the active site nucleotide-binding pocket (Fig. 1). To speculate on whether fluorescence changes were the result of a direct or indirect effect of nucleotide binding, we carried out computer modeling studies to determine the position of the acrylodan molecule in cAPK in solution. Based on steric allowance only, five favorable positions for the acrylodan molecule were found. When the energy of each of these structures was minimized in water, it was found that all but one displayed large hydrophobic surface areas exposed to solvent. In the one exception, the acrylodan molecule was well packed and exhibited good hydrophobic interaction with the enzyme core, suggesting that this conformer was by far the most populated in solution (Fig. 4). The acrylodan moiety in this structure is more than 12 Å away from the ATP molecule, indicating that fluorescence quenching is an indirect effect of nucleotide binding, and must be due to a nucleotide-induced conformational change.

Synergistic Binding of Nucleotides to cAPK by Inhibitor Molecules—$K_d$ values were determined either by fluorescence quenching or by the shift in emission wavelength maximum, as a function of increasing nucleotide concentration, both of which gave similar results (Fig. 3). In determining the binding affinity of the free cAPK for ATP, we were concerned that ATPase activity may complicate estimation of the true $K_d$ value for this nucleotide. However, the rate of ATPase activity is more than 1300-fold lower than the rate of Kemptide phosphorylation (24), and estimates of initial ATPase rates at the given enzyme and ATP concentrations used in the fluorescence measurements suggested that less than 0.5% of the ATP was turned over during the time of assay. The $K_d$ values of adenosine, ADP, AMPNP, and ATP for the free catalytic subunit are shown in Table II.

We also determined the dissociation constants of ATP, ADP, adenosine and AMPNP toward the acrylodan-labeled C-PKI and R$^{2}_{C2}$ holoenzyme complexes. Since the binding of neither PKI nor R$^2$ subunit to Acr-cAPK significantly altered the enzyme’s fluorescence properties, measurements of nucleotide binding could be carried out as with the free Acr-cAPK but in the presence of saturating concentrations of either inhibitor ($K_d$ of both PKI and R$^2$ for the free cAPK in the absence of nucleotide is $\leq$100 nM; Ref. 25). In the presence of 1 µM PKI, the $K_d$ of the C-PKI complex for ATP was 13 nM. When the total PKI concentration was increased to 10 µM, the $K_d$ value for ATP did not change (11 nM), suggesting that 1 µM PKI was sufficient for complete saturation. A similar control was carried out with measurements of R$^2$. The measured $K_d$ values of nucleotides toward C-PKI or R$^{2}_{C2}$ holoenzyme are shown in Table II.

ATP displayed dramatic binding synergism to cAPK in re-

TABLE I

|               | $K_{ATP}$ | $K_{Kemph}$ | $k_{cat}$ | $K_{adenosine}$ | $K_{(A+Kemph)}$ |
|---------------|-----------|-------------|-----------|-----------------|-----------------|
|               | $\mu M$   | $\mu M$    | $s^{-1}$  | $\mu M$         | $\mu M$         |
| wt-cAPK       | 17.2 ± 1.8| 24.7 ± 2.7  | 25.6 ± 1.2| 65 ± 3.5        | 1.0 ± 0.12      |
| N326C-cAPK    | 43.2 ± 6  | 39.0 ± 5.8  | 42.0 ± 0.54| 42.0 ± 2.1      | 120 ± 7.5       |
| Acr-N326C-cAPK| 42.0 ± 4.7| 41.0 ± 3.4  | 38.6 ± 2.1| 120 ± 7.5       | 1.2 ± 0.18      |

$^a$ $K_i$ (adenosine).

$^b$ $K_i$ (Ala-Kemptide).
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FIG. 3. Titration of Acr-cAPK with nucleotides: fluorescence emission spectra and binding isotherms. Fluorescence measurements were carried out as described under "Materials and Methods." a, c, e, and g, fluorescence emission spectra of Acr-cAPK. Fluorescence is quenched and red shifted with increasing nucleotide concentration. b, d, f, and h, corresponding binding isotherms. a and b, ATP; c and d, ADP; e and f, adenosine; g and h, AMPPNP. $K_d$ values are reported in Table II.

response to the binding of either PKI or R1 (2000- and 15,000-fold lower $K_d$ values, respectively) (Table II), equal to or greater than that previously reported (15, 16). In the case of ADP or AMPPNP the binding synergism was markedly less (20- and 50-fold, respectively). However, it was significantly higher than that observed for adenosine, whose interaction was only minimally enhanced (3- and 6-fold, respectively) by either inhibitor. These results demonstrate that the phenomenon of synergistic binding between nucleotides and the inhibitor molecules to cAPK is not unique to ATP, but is apparent with other nucleotides.

The binding of ATP to the free catalytic subunit is not influenced by Kemptide concentration during normal catalytic cycling (12), nor has binding synergism between ATP and the Ala-Kemptide peptide inhibitor been observed in equilibrium binding assays (14). Since both Kemptide and Ala-Kemptide bind poorly to the cAPK-ATP complex ($K_d = 0.2–1$ nM for both) in comparison to PKI ($K_d = 0.2$ nM), it is reasonable to speculate that the structural determinants necessary for synergistic binding with ATP have been altered in these short peptides. We tested two active fragments of PKI for their ability to stabilize nucleotide binding to cAPK. PKI-(14–22) is only slightly larger than Ala-Kemptide but displays markedly increased affinity ($\approx 1$ $\mu$M) for the C-ATP complex. PKI-(5–24) was originally identified as a high affinity ($K_d = 2$ nM), inhibitory fragment of PKI (9, 26, 27), and is the peptide with which the cAPK catalytic subunit has been co-crystallized in several x-ray structures (Fig. 5).

In comparison to the free catalytic subunit, the affinities of ATP, AMPPNP, and ADP for C-PKI-(14–22) were increased 6, 5, and 20-fold, respectively (Table III). In the case of ADP, this represents 100% of the of the affinity enhancement observed with full-length PKI. In contrast, the affinities of AMPPNP and ATP for the C-PKI-(14–22) complex were only 13.5% and 0.33%, respectively, of that for the C-PKI complex. The full synergism of AMPPNP and ATP binding could be mimicked only by PKI-(5–24). These results suggest that virtually all of the binding synergism observed between full-length PKI and ATP depends on a domain between residues 5–13 in PKI. In sharp contrast, the binding synergism observed with ADP is supported completely by residues 14–22. In the case of AMP-PPN, the two respective domains in PKI contribute nearly equally to the binding enhancement of this nucleotide.

DISCUSSION

We have generated a simple and sensitive fluorescence technique for measuring nucleotide binding to cAPK by engineering an acrylodan-labeled derivative of the catalytic subunit (Acr-cAPK). The rationale for targeting acrylodan to position 326 in the COOH-terminal tail of cAPK derives from the crystal structure, which shows that Asn-326 in wt-cAPK is proximal to the interlobal linker peptide segment (Fig. 1), a region that we speculate may be sensitive to ligand-induced conformational changes. The labeled enzyme is effectively wild type in steady state kinetic parameters, substrate binding, and thermal stability. The fluorescence emission spectrum of Acr-cAPK is not sensitive to PKI or R1 subunit binding, but is both red-shifted and quenched by the binding of all nucleotides tested. Molecular modeling predicts that the acrylodan moiety is more than 12 Å away from the nucleotide binding pocket, suggesting that the observed changes in fluorescence emission is not the result of a direct interaction between the nucleotide and the fluorophore.

Nucleotide binding to the free catalytic subunit of cAPK has

\begin{table}
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\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Nucleotide} & \textbf{ATP} & \textbf{ADP} & \textbf{Adenosine} & \textbf{AMPPNP} \\
\hline
Free C & 25 ± 1.3 & 24 ± 1.3 & 171 ± 36 & 182 ± 15 \\
C-PKI & 0.013 ± 0.0026 & 1.2 ± 0.14 & 51 ± 7.8 & 5.4 ± 0.6 \\
R1, C-PKI & 0.0016 ± 0.0015 & 0.5 ± 0.065 & 37 ± 4.5 & 1.5 ± 0.25 \\
\hline
\end{tabular}
\caption{Dissociation constants (\mu M) of free or inhibitor-bound cAPK and nucleotides}
\end{table}

2 B. Grant and S. S. Taylor, unpublished data.
previously been measured by steady state kinetic methods (12, 28, 29) and by displacement of lin-benzoadenosine 5'-diphosphate monitored by fluorescence anisotropy (13). Gel filtration and filter binding assays have been employed to detect the high affinity interaction between ATP and various cAPK-inhibitor complexes (15, 16, 30). While the utility of these techniques has long been established, each possess inherent limitations. Kinetic methods cannot be used with inactive enzyme species. The studies using lin-benzoadenosine 5'-diphosphate require micromolar amounts of enzyme (13, 31) precluding the measurement of this peptide. Instead, the full synergism of ATP binding was mimicked only by PKI-(5–24), suggesting that the NH₄⁺ terminal portion of PKI (residues 5–13) was critical. While a possible role for residues 23 and 24 in PKI cannot be ruled out, these amino acids do not affect the binding of PKI-(5–24) to cAPK (32).

The observation that the mutual binding between PKI-(5–24) and ATP is profoundly greater than that between PKI-(5–24) and ADP proves that the γ-phosphate is a key determinant (see Whitehouse et al., Ref. 16). The absence of the γ-phosphate results in a decrease in nucleotide affinity by 2 orders of magnitude. While the terminal phosphate in AMPPNP partially suffices for the γ-phosphate of ATP, the reduced binding synergism in comparison to ATP (Table III) indicates that the position of the terminal phosphate in AMPPNP must be suboptimal.

A correlation between the affinity of inhibitor binding and the degree of binding synergism with ATP is apparent. The affinity of PKI-(5–24) for the C-ATP complex (Kᵦ = 2 nM) is approximately 500-fold higher than that of PKI-(14–22) (Kᵦ = 1 μM), which, within error, is the same extent to which the affinity of ATP is increased by the longer peptide. Furthermore, the affinity of Kleptide or Ala-Kleptide (Kᵦ = 1 μM) is, in turn, lower than that of PKI-(14–22) and, accordingly, these peptides display no binding synergism with ATP (14). We speculate that the hydrogen bonding network linking the inhibitor peptide, ATP, and the enzyme is significantly weaker in complexes containing PKI-(14–22) compared to those containing PKI-(5–24). The major role of residues 5–13, therefore, must be to properly position the critical determinants within PKI-(14–22) for tight interaction.

Previously, we have shown that substitution of Arg¹³⁵ for Ala in the cAPK catalytic subunit results in an approximate 500-fold decrease in its affinity for PKI (30). This is attributed to the contribution of its methylene side chain in forming a critical hydrophobic pocket into which the Phe¹⁰ (P-site) side chain of PKI inserts, as well as to hydrogen bonding between a guanidinium nitrogen and the P(−7) backbone carbonyl oxygen in PKI (33, 34). Notably, the difference in affinity between PKI-(5–24) and PKI-(14–22) for wt-cAPK-ATP is also 500-fold, consistent with the possibility that Arg¹³⁵ may participate directly in the communication between the NH₂ terminus of PKI and ATP. The idea that a communication network exists between the enzyme’s active site and the P(−11) position in PKI is supported by the finding that substitution of the P site Ala with Ser abolishes the ability of the P(−11) Phe to confer high affinity binding of PKI-(5–24) to cAPK-ATP (35).

The structural basis for the moderate enhancement of ADP binding to PKI-(5–24) is provided by the hydrogen bond network between ATP γ-phosphate, glycine-rich loop, and P-site of PKI-(5–24) (17, 18, 36). Dotted lines indicate potential hydrogen bonds, with the exception of R134:D328 which form an ion pair. In the crystal structure, the interatomic distance between this ion pair is 7 Å (17), while it is predicted to be 3–5 Å by molecular dynamics simulation (see Footnote 2). The greater distance observed in the crystal structure is possibly the result of an intermolecular contact between Arg¹³⁵ and Trp¹⁰⁰ from a neighboring molecule (37). P site is Ala, P-3 is Arg, and P-11 is Phe. Single-letter amino acid code is used.

![Hydrogen bond network between ATP, γ-phosphate, glycine-rich loop, and P-site of PKI-(5–24)](image_url)

**Fig. 6.** Hydrogen bond network between ATP γ-phosphate, glycine-rich loop, and P-site of PKI-(5–24) (17, 18, 36). Dotted lines indicate potential hydrogen bonds, with the exception of R134:D328 which form an ion pair. In the crystal structure, the interatomic distance between this ion pair is 7 Å (17), while it is predicted to be 3–5 Å by molecular dynamics simulation (see Footnote 2). The greater distance observed in the crystal structure is possibly the result of an intermolecular contact between Arg¹³⁵ and Trp¹⁰⁰ from a neighboring molecule (37). P site is Ala, P-3 is Arg, and P-11 is Phe. Single-letter amino acid code is used.
affinity (20-fold) by PKI-(14–22) is unclear. It is unlikely due to the interaction of the nucleotide 3'-OH with the P-3 Arg in the peptide, since this hydrogen bond is present in the C-PKI-(5–24) adenosine structure. While hydrogen bonding from the main chain amidates of both Gly and Phe to a nucleotide β-phosphate oxygen are apparent in the C-PKI-(5–24)-ATP/AMPNP structures (17, 18), it is not clear if they exist with bound ADP alone. A binary structure containing ADP alone has not been crystallized, and in the ternary C-PKI-(5–24)-ADP structure, the nucleotide β-phosphate is not visible (36).

The distance between the acrylodan and the nucleotide adenosine ring (12 Å) predicted from molecular modeling suggests that the fluorophore senses the binding of nucleotides by an indirect mechanism. The comparison of two crystal structures of cAPK co-crystallized with (17) or without ATP (34) demonstrates that ATP binding results in contraction of the active site cleft as well as a decrease in the mobility of adjacent structural subdomains. It is unclear exactly how these structural changes are transmitted to the acrylodan. However, the final Acr-cAPK structure from modeling studies suggests that the acrylodan molecule senses the polarity of an electrostatic field generated by an adjacent arginine:aspartic acid ion pair (Arg123-Asp328) (Fig. 4). Molecular dynamics simulations show that the distance between this ion pair fluctuates during the normal breathing of the molecule in solution, and we speculate that nucleotide binding stabilizes a conformer in which the distance between this ion pair is perturbed, resulting in altered local electric field polarity.

In summary, the site-specific labeling with acrylodan has permitted measurement of the equilibrium dissociation constants of various nucleotides toward several inhibitor complexes of cAPK. Owing to the sensitivity of this probe, we have been able to dissect the binding synergy between ATP and PKI into component parts. A minimal degree of binding synergy (3-fold) arises from the nucleotide adenosine moiety, while the addition of two phosphate groups results in an additional 6-fold increase in affinity. This synergy (−20-fold) can be mimicked completely by a short synthetic peptide inhibitor, PKI-(14–22). An additional −100-fold increase in nucleotide affinity is directly attributable to the ATP γ-phosphate. This effect of the γ-phosphate, however, is observed only with the longer peptide, PKI-(5–24), which mimics the synergy observed with the full-length PKI, completely. We propose that there is communication between residues 5–13 in PKI and the ATP γ-phosphate, in which Arg323 plays a critical role. A clearer understanding of the synergistic mechanism awaits crystal structures of cAPK containing various nucleotides and truncated peptides, and studies of site directed mutants of cAPK. Such knowledge will provide deeper insight into the structural mechanism of other protein kinases, and will be valuable in guiding the rational design of peptide-based inhibitors targeted to these enzymes.

Acknowledgments—We thank Dr. Lynn Ten Eyck for critical discussion of the structural and modeling data, Dr. Larry Gross for mass spectroscopy, Dr. Elizbieta Radzio-Andzel for graphics, and Drs. Bruce Grant, Sarah Cox, and Joe Adams for extensive and critical discussion.

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