New Insights from the Structure-Function Analysis of the Catalytic Region of Human Platelet Phosphodiesterase 3A

A ROLE FOR THE UNIQUE 44-AMINO ACID INSERT

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Human phosphodiesterase 3A (PDE3A) degrades cAMP, the major inhibitor of platelet function, thus potentiating platelet function. Of the 11 human PDEs, only PDE3A and 3B have 44-amino acid inserts in the catalytic domain. Their function is not clear. Incubating Sp-adenosine-3',5'-cyclic-S-(4-bromo-2,3-dioxobutyl) monophosphorothioate (Sp-cAMPS-BDB) with PDE3A irreversibly inactivates the enzyme. The high pressure liquid chromatography (HPLC) analysis of a tryptic digest yielded an octapeptide within the insert of PDE3A ((K)T806YNVTDDK813), suggesting that a substrate-binding site exists within the insert. Because Sp-cAMPS-BDB reacts with nucleophilic residues, mutants Y807A, Y807C, and D812A were produced. Sp-cAMPS-BDB inactivates D811A and D812A but not Y807A. A docking model showed that Tyr807 is 3.3 angstroms from the reactive carbon, whereas Asp811 and Asp812 are >15 angstroms away from Sp-cAMPS-BDB. Y807A has an altered $K_m$ but no change in $k_{cat}$. Activity of wild type but not Y807A is inhibited by an anti-insert antibody. These data suggest that Tyr807 is modified by Sp-cAMPS-BDB and involved in substrate binding. Because the homologous amino acid in PDE3B is Cys792, we prepared the mutant Y807C and found that its $K_m$ and $k_{cat}$ were similar to the wild type. Moreover, Sp-cAMPS-BDB irreversibly inactivates Y807C with similar kinetics to wild type, suggesting that the tyrosine may, like the cysteine, serve as a H donor. Kinetic analyses of nine additional insert mutants reveal that H782A, T810A, Y814A, and C816S exhibit an altered $k_{cat}$ but not $K_m$, indicating that catalysis is modulated. We document a new functional role for the insert in which substrate binding may produce a conformational change. This change would allow the substrate to bind to Tyr807 and other amino acids in the insert to interact with residues important for catalysis in the active site cleft.

The anti-platelet drugs aspirin and clopidogrel have proven efficacy in secondary prevention of stroke, myocardial infarction, and peripheral vascular reocclusion (1, 2). Aspirin inhibits cyclooxygenase, thereby decreasing synthesis of thromboxane A2. Clopidogrel, a P2Y12 antagonist, blocks the ability of ADP to inhibit stimulated adenyl cyclase. However, despite prophylaxis with these anti-platelet drugs, reocclusion of coronary arteries occurs in 20–30% of patients after thrombolytic therapy or angioplasty probably because of the inability of these drugs to inhibit thrombin-induced platelet activation (3, 4). At low concentrations of thrombin, platelet aggregation depends in part on ADP and thromboxane A2, which are released by platelets and exert autocrine-mediated enhancement. At high concentrations of thrombin, platelets are aggregated and activated by pathways independent of both ADP and thromboxane A2. In contrast, elevation of intracellular cAMP produces potent inhibition of all pathways of platelet activation including increase in intracellular Ca$^{2+}$, shape change, aggregation, secretion, and the effects of phospholipases A$_2$ and C, as well as their responses of platelets to thrombin.

Cyclic nucleotide PDE3A is the most abundant cAMP PDE in platelets. PDE3A hydrolyzes cAMP resulting in lowering the intracellular cAMP levels, which in turn potentiates platelet activation. Drugs that inhibit PDE3A raise cAMP levels in platelets, thereby increasing the phosphorylation of proteins by cAMP- and cGMP-dependent protein kinases (5). Currently two PDE3A competitive inhibitors cilostazol and milrinone have respectively been used for treating patients with intermittent claudication and acute congestive heart failure (6, 7). Unfortunately cilostazol is contraindicated in patients with congestive heart failure, and milrinone is associated with undesirable cardiac arrhythmias. Examination of the inhibitory mechanism of PDE3A is important to exploit other ways of inhibiting this enzyme to minimize side effects.

The available PDE family crystal structures known to date are those of the catalytic domains cAMP-PDE (PDE4B2B and PDE4D) (8, 9), cGMP-PDE (PDE5A and PDE9A) (10, 11), and dual cAMP/cGMP-PDE (PDE1B and PDE3B) (12, 13). The

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4 The abbreviations used are: PDE, phosphodiesterase; Sp-cAMPS-BDB, Sp-adenosine-3',5'-cyclic-S-(4-bromo-2,3-dioxobutyl) monophosphorothioate; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; 8-BDB-TcAMP, 8-(4-bromo-2,3-dioxobutyl)thiolo-adenosine 3',5'-cyclic monophosphate.
Functional Role of the Unique Insert of PDE3A

Materials—Adenosine 3′,5′-cyclic phosphate ammonium salt [2,8-3H]cAMP was purchased from PerkinElmer Life Sciences. The nonhydrolyzable, reactive cAMP analog, Sp-adenosine-3′,5′-cyclic-S-(4-bromo-2,3-dioxobutyl)monophosphorothioate (Sp-cAMPS-BDB) was synthesized as previously described (15). Sf9 insect cell lines, Sf-902 II SFM medium, BaculoDirect transfection and expression system, the ProBound resin, and Anti-HisG antibody were purchased from Invitrogen. Protease inhibitor mixture set III (PIC III) was purchased from EMD Biosciences (San Diego, CA). A Coomassie Plus protein assay reagent kit was purchased from Pierce. Gentamicin sulfate, cAMP, and N-ethylmaleimide were purchased from Sigma. HPLC grade acetonitrile was obtained from Fisher.

Measurement of the Incorporation of Sp-cAMPS-BDB into PDE3A—PDE3A was incubated with 100 μM Sp-cAMPS-BDB in a 50 mM Hepes buffer at pH 7.3 containing 20 mM MES, 10 mM MgCl₂, and 0.5 mM NaCl. At various times of incubation (0, 20, 30, 40, 60, and 80 min, respectively), the aliquots were removed, and the residual enzyme activity of PDE3A was determined to correlate with the incorporation (see “Enzyme Activity Assay”). At each time interval, 100 mM [3H]NaBH₄ (dissolved in 20 mM NaOH) was added consecutively to reach a final concentration of 2 μM at 4 °C for a total of 1.5 h. [3H]NaBH₄ reduces the two oxygens of the diketo group from Sp-cAMPS-BDB to two [3H] hydroxyl groups. The excess [3H]NaBH₄ and the free Sp-cAMPS-BDB were removed by four consecutive centrifugations using Microcon centrifugal devices (Millipore, Billerica, MA) at 14,000 × g for 20 min. Aliquots were removed from the retentate to measure the protein concentration using the Coomassie Plus protein assay. The amount of Sp-cAMPS-BDB incorporated into PDE3A from reduction of the affinity labeled enzyme by [3H]NaBH₄ was calculated by measuring the radioactive [3H] content by using a Beckman Coulter liquid scintillation analyzer (model LS6500; Fullerton, CA). Control samples were tested using a similar procedure with the pretreatment of cold NaBH₄ with Sp-cAMPS-BDB prior to the addition of enzyme.

Trypsin Digestion of the Sp-cAMPS-BDB-modified Enzyme—PDE3A (0.8 mg) was incubated with 100 μM Sp-cAMPS-BDB at 25 °C for 3 h (~10% residual activity remained). The incubated mixture was treated twice with 100 μM [3H]NaBH₄ for a total of inactivation by Sp-cAMPS-BDB is: Sp-cAMPS (Kᵅ = 24 μM) > Rp-cGMP (1360), Sp-cGMP (1460) > GMP (4250), AMP (10600), Rp-cAMPS (22170 μM). Sp-cAMPS-BDB has proven to be an effective active site-directed affinity label for PDE3A.

In this paper, we describe specific incorporation of PDE3A by a reactive substrate analog, Sp-cAMPS-BDB, isolation of a peptide in the unique insert of PDE3A, and construction of mutant enzymes that identify the amino acid targeted by Sp-cAMPS-BDB. In addition, the role of the insert was further explored by kinetic analyses of nine additional insert mutants. The results define a new functional mechanism by which binding of cAMP to the flexible loop of platelet PDE3A may induce a local conformational change that allows interaction with catalytic residues.

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1.5 h (final concentration, 2 mM), followed by a carboxylation of free SH groups with 10 mM N-ethylmaleimide for 10 min. After removal of the excess reagents by centrifugation using Microcon centrifugal devices, the modified enzyme was digested at 37 °C by 2 consecutive additions of 5% (w/w) tosylphenylalanyl chloromethyl ketone-treated bovine pancreatic trypsin for a total of 2 h.

Purification and Determination of the Sequence of Modified Peptide—The radioactive tryptic digest was hypothesized, redissolved in 250 μl of 0.1% trifluoroacetic acid, and applied to an HPLC system using a reverse phase Vydac (Hesperia, CA) C18 column (0.46 × 25 cm). Separation was conducted at the elution rate of 1 ml/min using solvent A (0.1% trifluoroacetic acid in water) for the first 10 min, followed by a linear gradient from solvent A to 45% solvent B (0.1% trifluoroacetic acid in acetonitrile) for 220 min, a linear gradient from 45% solvent B to 100% Solvent B for 20 min, and solvent B for 10 min, successively. The eluent was monitored at 220 nm. Fractions of 1 ml were collected, from which 400 μl was counted for radioactivity. The amino acid sequence of isolated radioactive peptides was determined using an automated gas phase peptide sequence analyzer from Applied Biosystems (model 470A; Foster City, CA) equipped with an on-line phenylthiohydantoin analyzer (model 120) and computer (model 900A). The sequencing results were used to identify the location of the modified peptide in the active site of the catalytic region of PDE3A. This process was repeated twice with identical results.

Construction and Purification of PDE3A Mutants—A deletion mutant of PDE3A cDNA coding for the amino acid residues 665–1141 (16) was subcloned into a pENTER-TOPO vector (Invitrogen) to produce two sites for linear recombination. PDE3A insert mutants H782A, H796A, H798A, S804A, K805A, Y807A, Y807C, T810A, D811A, D812A, Y814A, G815A, and C816S were constructed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the mutants were confirmed by nucleotide sequence analysis (Sidney Kimmel Nucleic Acid Facility, Thomas Jefferson University, Philadelphia, PA). Recombinant mutant baculoviruses were produced by linear combination using BaculoDirect Transfection kit (Invitrogen). Expression of the catalytic region (residues 665–1141) of PDE3A wild type and mutant enzymes using a baculovirus/insect cell S9 system and protein purification using a ProBond Nickel resin column has been previously described (17, 18).

Protein Concentration Determination—Protein concentration of the purified enzymes and purified anti-insert antibody were determined using Coomassie Plus protein assay reagent using bovine serum albumin as standard. The absorbance at 595 nm was measured using a Bio-Tek automatic microplate reader equipped with KC4 module for data analysis (Bio-Tek Instruments, Inc., Winooski, VT).

Western Blot Analysis—The PDE3A wild type and mutants were separated on 10% Bis-Tris gel electrophoresis purchased from Invitrogen. The proteins were transferred to a polyvinylidene difluoride membrane using the Xcell II module at a constant voltage of 30 volts for 1 h at room temperature for Western blotting. The membranes were processed using the Chromogenic WesternBreeze system and probed with anti-insert PDE3A antibody (see effects of anti-insert antibody) to detect the presence of PDE3A.

Enzyme Activity Assay—PDE3A activity was measured by the amount of cAMP hydrolyzed as previously described (19). Enzyme was added to a buffer containing 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, and 0.8 μM [3H]cAMP. Reaction mixtures both with and without enzymes were incubated at 30 °C for 15 min. Catalysis was terminated by serial addition of 0.2 μl of ZnSO4 and 0.2 μl Ba(OH)2, which precipitates AMP but not cAMP. Samples were vortexed and centrifuged at 10,000 × g for 5 min. The BaSO4 pellets containing the [3H]5′-AMP precipitant were discarded. Aliquots of supernatants containing unreacted [3H]cAMP were removed and counted in a Beckman Coulter liquid scintillation analyzer. Enzyme activity was measured by comparing the amount of cAMP hydrolyzed in PDE3A containing samples to no enzyme controls. These data were then used to calculate enzyme specific activity in nmol of cAMP hydrolyzed per mg of protein per min.

Kinetic Constants Determination—The rates (nmol/s) of cAMP hydrolysis for the PDE3A wild type and mutant enzymes were determined using various concentrations of substrate cAMP from 0.02 to 14 μM. The values of Km and Vmax for each of the enzymes were determined by Michaelis-Menten equation as calculated by Enzyme Kinetics Module 1.1 software (Systat Software, Point Richmond, CA). The kcat (s−1) was obtained by dividing Vmax (nmol/s) by the molar enzyme concentration (nmol).

Reaction of Sp-cAMPS-BDB with Mutant Enzymes—Purified PDE3A mutant enzyme (Y807A, Y807C, D811A, or D812A) was incubated at 25 °C with various concentrations of Sp-cAMPS-BDB in a 50 mM Hepes buffer at pH 7.3 containing 20 mM MES, 10 mM MgCl2, and 0.5 M NaCl. At timed intervals (0, 5, 10, 20, 30, 45, and 60 min), aliquots of the reaction mixture were withdrawn, diluted in a buffer containing 47.5 mM Hepes, pH 7.04, 20 mM MgCl2, 4 mM MES, and assayed in triplicate for residual PDE3A activity. Control samples were performed under identical conditions without the presence of affinity label Sp-cAMPS-BDB.

Effect of Anti-insert Antibody on Enzyme Activity—A rabbit polyclonal antibody against the synthetic peptide S02VFSKTYNVTDKYGCG16, the C-terminal 15 amino acids of the PDE3A insert (Fig. 1), which also contain the octapeptide, was prepared by Sigma Genosys and designated as an anti-insert antibody. PDE3A, and mutants Y807A and Y807C were incubated respectively with various concentrations of the anti-insert antibody to a enzyme to antibody ratio of 1.3, 2.0, or 4.0 for 1 h at 37 °C. After incubation, enzyme activity was determined according to the “Enzyme Activity Assay” procedure. The activity of PDE3A wild type, Y807A, and Y807C without antibody was set as 100% activity. The preimmune IgG was used as a control to compare the activity of wild type, Y807A, and Y807C. All of the experiments were performed in triplicate.

Molecular Modeling—A homology model of PDE3A based on the crystal structure of PDE4B2B has been published (8). However, the model did not contain the additional 44-amino acid insert found in PDE3A. We have now refined the PDE3A model using the recently published PDE3B structures (13) that contain the 44-amino acid insert unique to PDE3. Sybyl 6.9 FlexX
docking module (Tripos) was then used to dock the affinity label Sp-cAMPS-BDB to PDE3A. Because mutant Y807A affected the $K_m$, Tyr$^{807}$ was included in the defined cAMP-binding pocket to construct the model. Residues involved in cAMP binding (17, 18) were used as a defined cAMP binding pocket (Tyr$^{807}$, Asp$^{811}$, Glu$^{866}$, Glu$^{971}$, Phe$^{972}$, and Phe$^{1004}$). This docking model was utilized to illustrate and further evaluate the kinetic results obtained from the mutants of insert amino acids of PDE3A.

RESULTS

Incorporation of Sp-cAMPS-BDB into PDE3A Is Time-dependent—To quantify the amount of the affinity label Sp-cAMPS-BDB incorporated into PDE3A, the enzyme (0.38 mg/ml) was incubated with 100 $\mu$M Sp-cAMPS-BDB at pH 7.3, as described under “Experimental Procedures.” Fig. 2 (left panel) shows that the incorporation of PDE3A by Sp-cAMPS-BDB is linear as a function of time. The addition of $[^3H]$NaBH$_4$ to an incubation mixture of enzyme and Sp-cAMPS-BDB stops the reaction by reducing the diketo group of Sp-cAMPS-BDB to a $[^3H]$diol group. Fig. 2 (right panel) shows that the residual enzymatic activity is inversely proportional to the incorporation. At 80 min, 0.86 mol of Sp-cAMPS-BDB was incorporated for each mol of enzyme which corresponded 19% of residual enzymatic activity or 81% inactivation. Thus, 1.08 mol of Sp-cAMPS-BDB was required to inactivate each mol of enzyme indicating a stoichiometry close to 1.0 of the affinity label and the enzyme.

The Isolated Sp-cAMPS-BDB-modified Peptide in PDE3A Is Located in the Unique 44-Amino Acid Insert—PDE3A (11 nmol) was incubated with 100 $\mu$M Sp-cAMPS-BDB for 3 h and treated with $[^3H]$NaBH$_4$ as described under “Experimental Procedures.” The modified enzyme was digested by trypsin for 2 h as described under “Experimental Procedures.” Fig. 3 (solid line) shows that on the reverse phase HPLC separation of the trypsic digest, most of the peptides elute between 0 and 160 min (0 and 30% solvent B). Two major radioactive peaks were observed as shown in Fig. 3 (dashed line, labeled I and II).

The amino acid sequence of the purified peptides (Fig. 3, peaks I and II) was determined by Edman degradation using an automated gas phase sequencer. Peak I contains small peptides (data not shown). The amino acid sequence of the peptide from peak II exhibits a single octapeptide, assigned as $^{806}$TYNVTDDKK$^{813}$ within the unique 44-amino acid insert of PDE3A (Fig. 1). This peptide results from enzyme cleavage after Lys$^{805}$ and Lys$^{811}$, consistent with the specificity of the trypsin recognition sites. The yield of each phenylthiohydantoin-derivative was recorded and ranged from 40 to 20 pmol (data not shown). As expected, the yield decreases as the cycle number increases. Peptide 806–813 is located C-terminal of the first metal-binding motif, $^{752}$HNRH$^{756}$.

Residue Tyr$^{807}$ in PDE3A Is the Amino Acid Modified by the Affinity Label Sp-cAMPS-BDB—Sp-cAMPS-BDB reacts with nucleophilic amino acids. Thus, Tyr$^{807}$, Asp$^{811}$, and Asp$^{812}$ from peptides 806–813 (determined from the trypptic cleavage study) are candidates for interacting with the affinity label. Lys$^{813}$ was not considered because this is the trypsin cleavage site, and cleavage would not have occurred if that lysine were modified. To identify which amino acid is being modified by Sp-cAMPS-BDB, mutant enzymes Y807A, D811A, and D812A were constructed, expressed, and purified.

To evaluate the effect of mutations on the reaction with Sp-cAMPS-BDB, the mutant enzymes were incubated with the affinity label, and their activity was tested as a function of time. Fig. 4 (A–D) shows the results of reaction of wild type and mutant enzymes. Y807A,
Functional Role of the Unique Insert of PDE3A

FIGURE 4. Sp-cAMPS-BDB reaction plots (A–E) and pseudo-first order rate plots (F–J) of PDE3A wild type, Y807A, D811A, D812A, and Y807C. The enzyme was incubated with Sp-cAMPS-BDB in 50 mM Hepes buffer at pH 7.3. At the indicated time intervals aliquots were removed, diluted, and assayed in duplicate for catalytic activity. A, wild type enzyme is incubated with (○) 0, (△) 12.5, (●) 25, (●) 50, (◇) 75, and (●) 100 μM of Sp-cAMPS-BDB. B, Y807A is incubated with (○) 0, (△) 50, (●) 100, (◇) 200, and (●) 400 μM of Sp-cAMPS-BDB. C, D811A is incubated with (○) 0, (△) 12.5, (●) 25, (●) 50, (◇) 75, and (●) 100, and (○) 125 μM of Sp-cAMPS-BDB. D, D812A is incubated with (○) 0, (△) 25, (●) 50, (◇) 100, (●) 125, and (◇) 150 μM of Sp-cAMPS-BDB. E, Y807C is incubated with (○) 0, (△) 12.5, (●) 25, (●) 50, (◇) 100, and (○) 200 μM of Sp-cAMPS-BDB. F, the pseudo-first order rate constant (k_{obs}) plot of wild type incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 100 μM. G, the k_{obs} plot of Y807A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 400 μM. H, the k_{obs} plot of D811A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 125 μM. I, the k_{obs} plot of D812A incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 150 μM. J, the k_{obs} plot of Y807C incubation with Sp-cAMPS-BDB at concentrations ranging from 0 to 200 μM. These data are the means of three independent experiments. Each experiment was performed in triplicate. The S.E. was not shown because of the multiple lines that would make graphic representation difficult. However, the coefficients of the variance range are within 20%.

D811A, and D812A, respectively, with Sp-cAMPS-BDB. Sp-cAMPS-BDB irreversibly inactivates both mutants D811A and D812A exhibiting saturation kinetics (Fig. 4, H–J). The k_{max} values for D811A and D812A are 0.005 ± 0.002 and 0.003 ± 0.0001 min^{-1}, and the K_{i} values are 29.9 ± 2.9 and 24.9 ± 2.5 μM, respectively. The K_{i} values of both D811A and D812A is 2.5–3-fold larger than that of wild type (K_{i} = 10.1 ± 1.7 μM; Fig. 4F). The k_{max} values of D811A and D812A are one-half and one-third, respectively, that of wild type (k_{max} = 0.0116 ± 0.0004 min^{-1}; Fig. 4F). These relatively minor changes in kinetics indicate that residues Asp^{811} and Asp^{812} are not the modified amino acid of the wild type enzyme that reacts with Sp-cAMPS-BDB. In contrast, Y807A is not inactivated by Sp-cAMPS-BDB (50–400 μM; Fig. 4, B and G), identifying Tyr^{807} as the amino acid modified by Sp-cAMPS-BDB.

Docking Model of Sp-cAMPS-BDB into PDE3A Supports Tyr^{807} as the Amino Acid Modified by the Affinity Label—The catalytic domain of PDE3A, including the unique 44-amino acid “insert,” was modeled using Sybyl Composer based on the crystal structure of PDE3B (1SO2 and 1SOJ) (13). FlexX docking module (Sybyl 6.91) was then used to dock Sp-cAMPS-BDB into the PDE3A model with a defined active site pocket of Tyr^{807}, Asn^{845}, Glu^{866}, Glu^{971}, Phe^{972}, and Phe^{1004}. Molecular modeling of the “insert” region, based on the crystal structure of PDE3B, suggests that this region is a flexible loop (Fig. 5). Based on the docking model of Sp-cAMPS-BDB into the PDE3A model, Tyr^{807} (green) is most likely to be the amino acid modified by Sp-cAMPS-BDB, because the reactive carbon C-9 of the affinity label is 3.3 Å from the hydroxyl oxygen of Tyr^{807}, whereas the carboxyl oxygens of Asp^{811} and Asp^{812} are more than 15 Å away from the reactive carbon of the affinity label (Fig. 5). These results further support the inactivation data that Tyr^{807} is the amino acid modified by Sp-cAMPS-BDB.

Residue Tyr^{807} in PDE3A Is Involved in Substrate cAMP Binding—Table 1 shows the kinetic characteristics of the mutant enzymes D811A and D812A. The K_{m} values for both D811A and D812A are similar to that of the wild type. The k_{cat} values of D811A and D812A are similar to that of wild type and suggest that single alanine mutation of the residues, Asp^{811} and Asp^{812} does not affect the enzyme catalytic activity. We further studied the mutant Y807A. The K_{m} of Y807A is 6.79 ± 0.83 μM, which is 30-fold greater than that of the wild type PDE3A. This indicates that Tyr^{807} is involved in cAMP binding. The k_{cat} value of the mutant Y807A was similar to the wild type (Table 1).

Mutant Y807C Mimics the Wild Type PDE3A—The amino acid corresponding to Tyr^{807} in the second member of the PDE3 gene family PDE3B is Cys^{792} (20). We hypothesized that the cysteine 792 might serve as a hydrogen donor similar to tyrosine 807. Therefore, we produced the mutant Y807C in the PDE3A mutant Y807C were similar to the wild type. To test the hypothesis that the thiol group mimics the phenolic group, we performed the inactivation studies of the mutant Y807C using Sp-cAMPS-BDB. This mutant, Y807C, is irreversibly inactivated by the affinity label Sp-cAMPS-BDB in a time-dependent manner exhibiting a K_{i} of 18.0 ± 2.7 μM and k_{max} of 0.004 ± 0.0002 min^{-1} (Fig. 4, E and J). These values of Y807C are very close to the wild type.

Mutant Y807A Is Not Inhibited by the Anti-insert Antibody—The anti-insert antibody was raised against the 15 amino acids (RSKTVYNTDDKYGCG^{115}) located at the C-terminal end of the insert, within which the octapeptide at positions 806–813 is identified by the affinity label. When the anti-insert antibody was added prior to the PDE3A activity assay, the wild type decreased in activity to 84, 55, and 32% proportional to antibody concentration (Fig. 6A). Under the same conditions, PDE3B, the other gene product of the PDE3 gene family does not decrease in enzymatic activity when the anti-insert antibody is added (data not shown), indicating the specificity of the neutralization by antibody to PDE3A.
Functional Role of the Unique Insert of PDE3A

Two mutants were made at residue 807 position to compare with the wild type tyrosine: Y807A eliminates the phenolic group, and Y807C substitutes a thiol group mimicking the corresponding finding that residue Tyr\(^{807}\) is the amino acid modified by the affinity label Sp-cAMPS-BDB and is consistent with the markedly increased \(K_m\) of the mutant Y807A.

Four of the Nine Additional Insert Mutants Are Involved in Catalytic Activity—Based on the conservation of the amino acid sequence (Fig. 1) and molecular model of PDE3A (Fig. 5), eight additional amino acid residues His\(^{782}\), His\(^{796}\), His\(^{798}\), Ser\(^{804}\), Lys\(^{805}\), Thr\(^{810}\), Tyr\(^{814}\), and Gly\(^{815}\) at the unique 44-amino acid insert were chosen to mutate to alanine. In addition, Cys\(^{816}\) was mutated to a serine, because as previously reported mutant C816A completely abrogated the enzyme activity (21). Each of the mutants exhibited a single band on SDS gel electrophoresis (data not shown). Table 1 shows that mutants H796A, H798A, S804A, K805A, and G815A have no marked changes in kinetic constants compared with wild type PDE3A. In contrast, mutants H782A, T810A, Y814A, and C816S show a significant 5–29-fold decrease in \(k_{cat}\) but no major changes in \(K_m\) as wild type PDE3A. Because these four amino acid residues are distant from those involved in catalysis in the enzyme, the insert amino acid residues His\(^{782}\), Thr\(^{810}\), Tyr\(^{814}\), and Cys\(^{816}\) are important for PDE3A catalytic activity only after substrate binding.

### Table 1

Kinetic parameters of PDE3A insert mutants

| Enzyme   | \(K_m\) (mM) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}\)mM\(^{-1}\)) |
|----------|-------------|-------------------|-------------------------------|
| PDE3A    | 0.23 ± 0.15 | 178.0             | 7.78                          |
| H782A    | 0.45 ± 0.10 | 22.3              | 0.50                          |
| H796A    | 0.20 ± 0.03 | 165.6             | 8.28                          |
| H798A    | 0.25 ± 0.01 | 101.3             | 4.14                          |
| S804A    | 0.28 ± 0.01 | 69.0              | 2.51                          |
| K805A    | 0.27 ± 0.05 | 76.3              | 2.88                          |
| Y807A    | 6.79 ± 0.83 | 84.0              | 0.12                          |
| Y807C    | 0.16 ± 0.01 | 105.0             | 6.58                          |
| T810A    | 0.53 ± 0.21 | 36.9              | 0.69                          |
| D811A    | 0.17 ± 0.02 | 136.0             | 8.02                          |
| D812A    | 0.20 ± 0.01 | 50.0              | 2.50                          |
| Y814A    | 0.65 ± 0.11 | 23.1              | 0.36                          |
| G815A    | 0.26 ± 0.01 | 86.7              | 3.40                          |
| C816S    | 0.34 ± 0.12 | 8.5               | 0.25                          |

The data shown in bold indicate statistically significant changes (Student’s \(t\) test, \(p < 0.01\)) in kinetic parameters of the mutant enzyme when compared with the wild type tyrosine: Y807A eliminates the phenolic group, and Y807C substitutes a thiol group mimicking the corresponding residue Cys\(^{792}\) of PDE3B. When the anti-insert antibody was preincubated with the enzyme prior to the activity assay, the mutant Y807A did not decrease in activity at any of the antibody concentrations (Fig. 6B). Similarly, when the Y807C was preincubated with varying concentrations of anti-insert antibody, the enzyme activity did not decrease as a function of antibody concentrations (Fig. 6C). The failure of the anti-insert antibody to inhibit both the inactive mutant Y807A and the active mutant Y807C indicates that tyrosine residue is a critical part of the epitope of the antibody. Aromatic amino acids are frequently highly antigenic. The results indicate that the concentration of the antibody used did not block the active site.

The \(K_m\) of the wild type enzyme was similar in the presence or absence of the anti-insert antibody (\(K_m = 0.203\) versus 0.197 µM). The \(k_{cat}\) is decreased 2.2-fold (\(k_{cat} = 158\) versus 70.6 s\(^{-1}\), respectively), which is consistent with the decrease of 3.6-fold in enzymatic activity in the presence of 0.053 µM anti-insert IgG (Fig. 6A). The preimmune IgG, which does not contain the epitope to interact with the enzyme, did not inhibit the activity of wild type, Y807A, and Y807C (data not shown). These data support the

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**Figure 5. Molecular model of PDE3A.** PDE3A was modeled using Sybyl Composer with 16 chains of PDE3B (1SO2 and 1SOJ) shown as quintuple red lines. The 44-amino acid insert is depicted as a solid red ribbon. FlexX (Sybyl) was then used to dock Sp-cAMPS-BDB into the PDE3A model with a defined cAMP-binding pocket of Tyr\(^{782}\) (green) and Asn\(^{845}\), Glu\(^{866}\), Glu\(^{971}\), Phe\(^{972}\), and Phe\(^{1004}\) labeled in black. Mutants in the insert that affect the \(k_{cat}\) are labeled in blue.

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### Notes

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Y807A are virtually superimposable and display a negative trough at 209 nm ([θ]WT = −5040 and [θ]Y807A = −4890 deg cm² dmol⁻¹, respectively (data not shown). The overall similarity in the far-UV CD spectra of the mutant Y807A and wild type PDE3A indicates that the mutant Y807A maintains a native secondary structure. The Western blot results and the CD spectral data suggest that all of the mutants have overall conformation similar to that of the wild type PDE3A.

DISCUSSION

Reactive purine nucleotide analogs have been used as affinity labels to probe nucleotide binding sites (22–24). We have described the use of the cAMP affinity analog 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate (8-BDB-TcAMP) in studies to identify important amino acids within the active site of PDEs. 8-BDB-TcAMP irreversibly inactivated PDE2A (25), PDE3A (26), and PDE4A (27). In the case of PDE4A, a peptide containing the residue modified by 8-BDB-TcAMP was isolated, and the amino acid sequence was identified. However, the utility of 8-BDB-TcAMP was limited because it inactivates PDEs only at millimolar concentrations, because of continuous hydrolysis to the 5′-AMP derivative by the enzymes under investigation. We reported the synthesis of a new nonhydrolyzable reactive cAMP derivative, Sp-cAMPS-BDB, which contains both reactive bromoketo and dioxo groups (15). The bromoketo group can form covalent bonds with the nucleophilic side chains of many amino acids including cysteine, aspartate, glutamate, histidine, tyrosine, and lysine, whereas the dioxo provides the ability to react with arginine residues.

We here demonstrate that Sp-cAMPS-BDB acts as an affinity label of PDE3A. The Sp-cAMPS-BDB is a substrate analog of cAMP with the reactive bromodioxobutyl group at the phosphorothioate ester. An octapeptide (806TYNVTDDK813) in PDE3A has been identified by tryptic digest, peptide isolation, and N-terminal amino acid sequencing. Three nucleophilic amino acid residues in the octapeptide were selected to produce mutants (Y807A, D811A, and D812A) to identify the target residue reacting with Sp-cAMPS-BDB. Both mutants D811A and D812A were inactivated by Sp-cAMPS-BDB, whereas Y807A is not inactivated by the affinity label. Furthermore, we showed that Tyr807 exhibits a large change in Km and that this amino acid is close (3.3 Å) to the reactive carbon of the affinity label in a docking model based on the crystal structure of PDE3B. Tyr807, although it is present in the 44-amino acid insert, is functionally part of the cAMP-binding site. The other two amino acids, D811A and D812A, in the octapeptide capable of reacting with the affinity label are more than 15 Å from the reactive carbon of Sp-cAMPS-BDB and have similar kcat/Km to...
Figu 1. Although the unique insert lies in the first conserved domain, a flexible loop exposed on the surface of the enzyme. The kinetic constants $K_m$ and $k_{cat}$ of $Y_807C$ are similar to those of the wild type. It is likely that the phenolic hydroxy group reacts similarly to Tyr. The kinetic constants $K_m$ and $k_{cat}$ of $Y_807C$ are similar to those of the wild type. Therefore, $Y_807C$ is a participant in the substrate-binding site. Because the $K_m$ is increased 30-fold compared with the wild type, it was especially important to test whether a major conformational change had occurred in $Y_807C$. The CD spectra of both wild type and mutant $Y_807C$ are almost superimposable, indicating that the mutant $Y_807C$ maintains a native conformation (data not shown).

When we assessed $Y_807C$ with the anti-insert antibodies, we found no inhibition at any of the concentrations tested in contrast to the behavior of the wild type enzyme. These results indicate that although $Y_807C$ does not affect the $k_{cat}$, it regulates the catalytic activity directly, presumably because of conformational change upon substrate binding, and directly by influencing the substrate binding. However, this is not related to the loss of activity because the active mutant $Y_807C$ is also not inhibited by the antibody. These results suggest that $Y_807C$ is a critical part of the epitope of antibody, but other epitopes exist in the loops.

We speculate that $Cys^{792}$ in PDE3B might play the same role as $Y_807C$ in PDE3A. The corresponding PDE3A mutant and its functional group reacts similarly to Tyr. The kinetic constants $K_m$ and $k_{cat}$ of $Y_807C$ were similar to that of wild type. Furthermore, $Y_807C$ is irreversibly inactivated by the affinity label Sp-cAMPS-BDB in a time-dependent manner to a similar extent when compared with the wild type. It is likely that the phenolic group of PDE3A and the thiol group of PDE3B both function as hydrogen donors in the interaction with substrate cAMP.

The 44-amino acid insert shown in the molecular model constitutes a flexible loop exposed on the surface of the enzyme (Fig. 5). Although the unique insert lies in the first conserved metal-binding motif $\text{HNRH}\_7^{24}\_24\_26\_6^{825}$ in the active site cleft from the primary structure of the enzyme, the homology model based on crystalline PDE3A indicates that the flexible loop of the insert is distant from the active site cleft in the model. These kinetic analyses and molecular modeling data imply that upon substrate binding, this surface flexible insert may undergo substantial local conformational change. We hypothesize that the flexible insert flips into the active site cleft to regulate the substrate binding and catalytic activity. Further studies are underway to document any conformational change associated with substrate binding.

A precedent for local conformational change of a loop has been shown in the reaction of trypsin with $\alpha_1$ antitrypsin (28, 29). When trypsin cleaves the reactive center loop of $\alpha_1$ antitrypsin, the cleaved reactive center loop undergoes a large local conformational change and zinc into a groove of $\beta$-sheet of the molecule with the translocation of trypsin to the other pole of $\alpha_1$ antitrypsin.

In the case of aspartic peptidases, the variation in flap conformations observed in X-ray studies of free and inhibitor-bound enzymes indicates that the flaps in the free enzyme are flexible in solution. For example, binding of pepstatin to cathepsin D induced small structural changes in the flap region that contains the $\beta$-hairpin structure from residues 72–87 (30). Residues 79 and 80 at the tip of the flap moved in toward the inhibitor by about 1.7 Å, and the flexibility of this $\beta$-bend decreases because of electrostatic interaction of His$^{77}$ of the flap with the C terminus of the inhibitor. Similar changes in conformation upon inhibitor binding have also been shown in the pepstatin-bound form of both rhizopuspepsin and penicillopepsin (31, 32). The closing of the flap over the inhibitor substrate serves to remove the peptide bond of the substrate from effective contact with solvent.

In conclusion, use of the nonhydrolyzable affinity label Sp-cAMPS-BDB and structural analysis have allowed us to identify a new cAMP-binding amino acid ($Y_807C$) in the 44-amino acid insert that forms a flexible loop unique for the PDE3 gene family. These results challenged us to produce nine additional insert mutants that defined the role of His$^{782}$, Thr$^{810}$, Tyr$^{814}$, and Cys$^{816}$ as amino acids interacting with the residues involved in catalysis and/or metal binding. The identical behavior of the mutant $Y_807C$ to the wild type suggests that this tyrosine residue may be functioning as a H donor. The presence of a similar loop in PDE3B with a cysteine instead of tyrosine in PDE3A at the homologous position suggests that a similar mechanism may be involved with PDE3B substrate binding. The affinity labeling results and the kinetic data from the mutants suggest a functional role of the insert and provide a new strategy for structure-based inhibitor design to develop new specific inhibitors for PDE3A.

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