Dual Specificity and Novel Structural Folding of Yeast Phosphodiesterase-1 for Hydrolysis of Second Messengers Cyclic Adenosine and Guanosine 3′,5′-Monophosphate

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ABSTRACT: Cyclic nucleotide phosphodiesterases (PDEs) decompose second messengers cAMP and cGMP that play critical roles in many physiological processes. PDE1 of Saccharomyces cerevisiae has been subcloned and expressed in Escherichia coli. Recombinant yPDE1 has a $K_M$ of 110 μM and a $k_{cat}$ of 16.9 s⁻¹ for cAMP and a $K_M$ of 105 μM and a $k_{cat}$ of 11.8 s⁻¹ for cGMP. Thus, the specificity constant $(k_{cat}/K_M)^{cAMP}/(k_{cat}/K_M)^{cGMP}$ of 1.4 indicates a dual specificity of yPDE1 for hydrolysis of both cAMP and cGMP. The crystal structures of unliganded yPDE1 and its complex with GMP at 1.31 Å resolution reveal a new structural folding that is different from those of human PDEs but is partially similar to that of some other metalloenzymes such as metallo-β-lactamase. In spite of their different structures and divalent metals, yPDE1 and human PDEs may share a common mechanism for hydrolysis of cAMP and cGMP.

Efficient integration of extracellular and intracellular signals is required to maintain adaptive cellular functions. The signaling systems of cyclic adenosine and guanosine 3′,5′-monophosphate (cAMP and cGMP, respectively) represent the earliest identified signaling pathways in the regulation of a vast number of critical physiological processes, including visual transduction, cell proliferation and differentiation, gene expression, inflammation, apoptosis, steroidogenesis, insulin secretion, glycogen synthesis, glycogenolysis, and lipolysis.¹⁻³ Cyclic nucleotide phosphodiesterases (PDEs) play important roles in these physiological processes with their ability to decompose cellular cAMP and cGMP.⁴⁻⁶ Inhibitors of human PDEs have been widely studied as therapeutics for the treatment of diseases, such as central nerve system diseases and cardiomyocyte hypertrophy.⁷⁻⁹ A well-known example of this drug class is the PDE5 inhibitor sildenafil (Viagra) that has been approved for the treatment of male erectile dysfunction and pulmonary hypertension.¹⁰¹¹

The superfamily of PDE can be categorized into two main classes of nonhomologous proteins that have been known to specifically hydrolyze cAMP and cGMP, although a third class of PDE was mentioned but not fully characterized.¹² All PDEs found in mammals and flies belong to class I, while yeast and protozoans contain classes I and II PDEs. The conserved catalytic domain of class I PDEs consists of ~300 amino acids and contains two divalent metal ions, typically zinc and magnesium/manganese, to facilitate the catalysis.¹³ The yeast genome contains two types of PDEs: yPDE1 and yPDE2. Yeast PDE2 is a member of the class I PDE superfamily and shares a similar folding of its catalytic domain with human PDEs, whereas yPDE1 belongs to the class II PDE superfamily and uses two zinc ions for its catalysis.¹⁴ The sequence alignment suggests that yPDE1 has a structural folding different from that of class I PDEs but shares a partially common pattern with other metalloproteins such as metallo-β-lactamase (MBL).¹⁵ Because no three-dimensional structure is available for any member of class II PDEs, how yPDE1 is evolutionarily related to class I PDEs and other metalloenzymes remains a puzzle.

The signaling of cAMP in yeast has been shown to play critical roles in physiological processes, including metabolism, cell wall biosynthesis, cell growth, and mating.¹⁶⁻²³ Over-expression of the PDE2 gene enhances the tolerance of yeast to
oxidative and ethanol stress for survival.24–26 Both yPDE1 and yPDE2 appear to participate in the cAMP signaling pathway, although yPDE2 shows high affinity with a $K_M$ of 0.17–10 μM for cAMP,27–30 and yPDE1 has a low affinity with a $K_M$ of 100–150 μM. In comparison with numerous publications about the roles of cAMP in the physiological processes of yeast, the cGMP signaling pathway has not been well studied, although it was reported that cGMP activated cAMP-depend-ent protein kinase of Candida albicans.23 There is only one report showing that yPDE1 from Candida albicans has weak cGMP activity with a $K_M$ of 250 μM and a $V_{max}$ of 0.044 μmol mg$^{-1}$ min$^{-1}$, in comparison with a $K_M$ of 490 μM and a $V_{max}$ of 1.17 μmol mg$^{-1}$ min$^{-1}$ for cAMP.34 Thus, physiological roles of the cGMP signaling pathway in yeast remain illusive. This paper reports the enzymatic properties of full length yPDE1 and shows that yPDE1 has a dual activity on hydrolysis of both cAMP and cGMP with similar enzymatic efficacies. The crystal structure of yPDE1 at 1.31 Å resolution reveals a new topological folding that is different from those of human PDEs.

## EXPERIMENTAL PROCEDURES

### Subcloning and Protein Purification of Yeast PDE1.

The cDNA of full length yPDE1 (residues 1–369) from bakers’ yeast Saccharomyces cerevisiae was subcloned into vector pET28a for expression. Oligonucleotide primers containing the NheI and EcoRI sites were designed and synthesized for amplification of yPDE1 by polymerase chain reaction. Amplified yPDE1 cDNA and expression vector pET28a were separately digested by restriction enzymes NheI and EcoRI, purified on an agarose gel, and then ligated by T4 DNA ligase. The resultant pET28-yPDE1 plasmid was confirmed by DNA sequencing and then transferred into Escherichia coli strain BL21 (CodonPlus) for expression. When the cells were grown in 2×YT culture medium at 37 °C to an OD$_{600}$ of 0.7, 0.1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to induce the overexpression of wild-type yPDE1 at 15 °C for 2 days.

The selenomethionyl mutant of yPDE1 was expressed in M9 minimal medium (6 g of Na$_2$HPO$_4$, 3 g of KH$_2$PO$_4$, 1 g of NH$_4$Cl, 0.5 g of NaCl, 2 mM MgSO$_4$, 4 g of glucose, 100 mg of ampicillin, 50 mg of selenomethionine, 100 mg of lysine, 100 mg of threonine, 100 mg of phenylalanine, 50 mg of leucine, 50 mg of isoleucine, and 50 mg of valine per liter). A colony of E. coli transferred with pET28-yPDE1 was grown in 10 mL of 2×YT medium at 37 °C overnight, centrifuged at 5000 rpm at room temperature, and then resuspended in 500 mL of prewarmed M9 medium at 37 °C. After the cells had been cultured at 37 °C to an OD$_{600}$ of 0.4–0.5, 0.1 mM IPTG was added to induce the overexpression at 12 °C for ~2 days.

The harvested cells were suspended in an extraction buffer of 20 mM Tris base (pH 8.0), 0.3 M NaCl, 15 mM imidazole, and 1 mM β-mercaptoethanol (β-ME) and lysed by being passed once through a Nano DeBee homogenizer. After centrifugation, the supernatant was loaded onto a 2.5 cm column containing 100 mL of 20 mM β-ME. After digestion with thrombin at room temperature for 2 h, the digested sample was loaded on a Q-Sepharose column (2.5 cm × 8 cm). The column was washed with ~100 mL of 20 mM

### Table 1. Data Collection and Structural Refinement Statistics

|                | unliganded yPDE1 | yPDE1–GMP | selenomethionyl yPDE1 |
|----------------|-------------------|-----------|-----------------------|
| **Data Collection** |                   |           |                       |
| space group    | C222$_1$          | C222$_1$  | P2$_1$                |
| unit cell      |                   |           |                       |
| $a$ (Å)        | 73.5              | 73.7      | 68.2                  |
| $b$ (Å)        | 85.2              | 85.2      | 56.9                  |
| $c$ (Å)        | 130.6             | 130.8     | 102.3                 |
| $\beta$ (deg)  |                   |           | 106.7                 |
| wavelength (Å) | 1.075             | 1.075     | 0.97920               |
| resolution (Å) | 50–1.31 (1.33–1.31) | 50–1.31 (1.33–1.31) | 50–2.3 (2.38–2.3) |
| total no. of measurements | 1272718 | 1398700 | 241585               |
| no. of unique reflections | 96740 | 98268 | 31820               |
| completeness (%) | 98.4 (79.8) | 99.3 (88.0) | 95.1 (96.1) |
| average $I/\sigma$ | 12.6 (4.9) | 10.1 (2.3) | 10.3 (3.5) |
| $R_{merge}$    | 0.084 (0.55)      | 0.064 (0.81) | 0.095 (0.501) |
| Structural Refinement |                   |           |                       |
| $R$-factor/$R_{free}$ | 0.172/0.184 | 0.169/0.180 |                       |
| no. of reflections | 91848/4844 (5%) | 93309/4910 (5%) |                       |
| resolution (Å) | 15–1.31           | 15–1.31   |                       |
| rmsd           |                   |           |                       |
| bond lengths (Å) | 0.008             | 0.008     |                       |
| bond angles (deg) | 1.2               | 1.3       |                       |
| average $B$ factor (Å$^2$) (no. of atoms) | 18.4 (2914) | 18.7 (2914) |                       |
| protein        | 15.2 (8)          | 19.7 (8)  |                       |
| Zn             | 13.6 (2)          | 13.2 (2)  |                       |
| SO$_4$         | 17.8 (5)          | 35.9 (23) |                       |
| GMP            |                   |           |                       |
| water          | 25.3 (245)        | 24.7 (215) |                       |

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Tris base (pH 7.5), 50 mM NaCl, 1 mM β-ME, and 1 mM EDTA and eluted with the same buffer supplemented with 200 mM NaCl. The yPDE1 protein was finally purified with a Superdex 200 column using a buffer of 20 mM Tris base (pH 7.5), 1 mM β-ME, 1 mM EDTA, and 50 mM NaCl. The combined yPDE1 fractions were concentrated to 6–10 mg/mL and stored in −80 °C for use.

**Enzymatic Assay.** The enzymatic assay was assayed using [3H]cAMP or [3H]cGMP as a substrate, as previously reported for human PDE. The yPDE1 activity was tested in a buffer of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MnCl₂, 0.01 mM ZnCl₂, 1 mM EDTA, or 1 mM DTT. In addition, the yPDE1 activity was assayed after dialysis against a buffer of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA, three times (1 h, 2 h, and overnight).

**Catalysis, Structure Determination, and Comparison.** The crystal of native yPDE1 (6 mg/mL) was grown by the hanging drop method against a buffer of 20 mM HEPES (pH 7.5), 5% PEG3350, 40 mM lithium sulfate, and 12% 2-methyl-2,4-pentanediol (MPD) at room temperature, or a similar buffer but with 15% glycerol and 20% MPD instead of 12% MPD. The native yPDE1 crystals diffracted to 1.31 Å resolution and are in space group C222₁, with the following cell dimensions: a = 74 Å, b = 85 Å, and c = 130 Å (Table 1). The selenomethionyl yPDE1 (11 mg/mL) was crystallized at room temperature by the hanging drop method against a well buffer of 6% PEG3350, 30 mM sodium citrate (pH 5.6), 60 mM ammonium acetate, 75–100 mM ammonium sulfate, and 3% glycerol. The Se-yPDE1 crystals are in space group P2₁ with the following cell dimensions: a = 68 Å, b = 57 Å, c = 102 Å, and β = 106.7°. The yPDE1–GMP complex was prepared by soaking the native yPDE1 crystal in the crystallization buffer with 50 mM cGMP at room temperature for 2 days.

The single-wavelength anomalous diffraction (SAD) data for selenomethionyl yPDE1 were collected at the k-edge of Se (λ = 0.97920), and the data of native yPDE1 and its complex with cGMP were collected at λ = 1.075 on beamline X29 of the Brookhaven National Laboratory (Table 1). All the data were processed with HKL. Three Se sites were found for the SAD data with SHELX and used to phase the structure with PHENIX. Improvement of the SAD phases yielded a figure of merit of 0.66 for the data at 2.3 Å resolution, and the Fₚ map clearly revealed the trace of the yPDE1 structure. The atomic model was built with O and refined with REFMAC.

The yPDE1 structure was compared with structures in the Protein Data Bank by the online program Dalil (http://www. ebi.ac.uk/Tools/structure/dalilite). The results were ranked by Dalil’s Z score and rmsd. A structure with a Z score of >2.0 is assumed thought to be partially similar with that of yPDE1, and a higher Z score indicates better similarity.

### RESULTS

**Yeast PDE1 Hydrolyzes both cAMP and cGMP.** The native bakers’ yeast yPDE1 was shown to contain two zinc ions per molecule by an atomic absorption spectrometer. In a plain assay buffer without divalent metals, our recombinant yPDE1 shows similar enzymatic efficacy for hydrolysis of cAMP and cGMP: Kₘ of 110 μM and kₐ of 16.9 s⁻¹ for cAMP and Kₘ of 105 μM and kₐ of 11.8 s⁻¹ for cGMP (Table 2). Thus, the enzymatic efficacy kₐ/Kₘ of yPDE1 is 0.153 and 0.113 for cAMP and cGMP, respectively. The specificity constant (kₐ/Kₘ) for cAMP is 1.4 suggests a dual activity of yPDE1 on hydrolysis of both cAMP and cGMP, with a slightly better efficacy on cAMP than cGMP. Our Kₘ of yPDE1 for cAMP is comparable with the early report of Kₘ values of 100–120 μM, but our yPDE1 enzyme is much more active, as shown by a Vₘₐₓ at least 20-fold higher than those of the previously reported proteins.

Because human PDEs require magnesium or manganese for maximization of their activities, a few types of divalent metal ions were added to the assay buffer to test if they promote the activity of yPDE1. In summary, addition of 10 μM ZnCl₂, 2 mM MnCl₂, or 10 mM MgCl₂ to the assay buffer did not significantly promote or inhibit the catalytic activities of our recombinant yPDE1 upon hydrolysis of cAMP and cGMP (Table 2). These results are consistent with the early observations that yPDE1 from S. cerevisiae or C. albicans was not sensitive to the divalent metals. In addition, the dialysis of native yPDE1 against 1 mM EDTA overnight did not change the catalytic efficiency (kₐ/Kₘ) for cAMP but slightly decreased kₐ/Kₘ for cGMP (Table 2), implying that the dialysis was not efficient for the extraction of the zinc ions that coordinate with four yPDE1 residues. On the other hand, DTT significantly inhibits our yPDE1 activity, in contrast to the fact that DTT promotes the activity of human PDEs.

**Architecture of the yPDE1 Structure.** The 369 amino acids of yPDE1 are folded into 16 β-strands, 10 α-helices, and two 3₁₀-helices (Figure 1A). The 16 β-strands can be divided into two sheets that form a sandwich and a hydrophobic core of the molecule. The center of the molecular core looks like a distorted β-barrel and is flanked by the helices. Two molecules of yPDE1 are tightly associated into a dimer (Figure 1B,C). For the massive interactions between two monomers, we assume that the dimeric form of yPDE1 is a biologic unit for its function. This argument is supported by the gel filtration experiment in which the recombinant yPDE1 protein appears to be a dimer in the solution (data not shown) and also by the structural assembly in which two monomers jointly form the active site of yPDE1 (Figure 1).

The active site pocket of yPDE1 can be divided into three portions. The bottom part of the pocket is composed of residues His128, His130, Asp132, His133, His213, Asp244, and His326, which are entirely contributed by molecule A of the dimer. Residues Glu289, Tvr292, His294, and Lys328 of molecule A constitute one wall of the catalytic pocket, while Pro142, Tyr145, Trp157, Pro176, and Leu178 of molecule B form another wall of the pocket (Figure 2A). Two divalent metal ions sit in the bottom of the pocket, each of which forms four coordinations with His128, His130, His213, and Asp244, or with Asp132, His133, Asp244, and His326 of the same molecule of the dimer (Figure 2B). In addition, a sulfate ion was found to coordinate with the two zinc ions in the structure of unliganded yPDE1. This sulfate ion must come from the excess...
Table 2. Kinetics of Yeast PDE1 (1–369)

| Enzyme          | Ion in Assay Buffer | \(K_M\) (μM) | \(V_{max}\) (μmol mg^{-1} min^{-1}) | \(k_{cat}/K_M\) (s^{-1}/μM) | \(k_{cat}/V_{max}\) (s^{-1}/μM) | \(k_{cat}/K_M\) (s^{-1}/μM) |
|-----------------|---------------------|--------------|-------------------------------------|-----------------------------|--------------------------------|-----------------------------|
| yPDE1 10 mM MgCl\(_2\) | 98.8 ± 8.5 | 30.1 ± 0.7 | 210 ± 10 | 0.213 | 0.070 | 0.014 |
| yPDE1 001 mM ZnCl\(_2\) | 115.7 ± 7.4 | 24.8 ± 0.4 | 174 ± 1.1 | 0.150 | 0.060 | 0.011 |
| yPDE1 2 mM MnCl\(_2\) | 117.5 ± 16.8 | 16.8 ± 0.6 | 118 ± 0.9 | 0.100 | 0.040 | 0.009 |
| yPDE1 no ion | 110.1 ± 142 | 24.1 ± 0.8 | 169 ± 1.3 | 0.153 | 0.060 | 0.011 |
| yPDE1* no ion | 81.2 ± 7.4 | 20.5 ± 0.5 | 143 ± 0.4 | 0.176 | 0.070 | 0.014 |
| yPDE1* no ion with 1 mM EDTA | 77.3 ± 12.0 | 15.8 ± 0.6 | 113 ± 1.5 | 0.144 | 0.050 | 0.009 |

\(^{a}\)yPDE1 protein was dialyzed three times against a buffer of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA (1 h, 2 h, and overnight).
yPDE1, while both of them have the common phosphate interactions with the divalent metals (Figure 3). Considering the fact that both MBL-like protein and yPDE1 require a dimer for catalysis and have identical metal binding residues, yPDE1 might have an evolutionary relationship with the MBL-like protein. In short, the structural comparison described above may suggest that the zinc ions in yPDE1 may stabilize and facilitate the proper folding of the yPDE1 structure, in addition to their main role as the catalytic ions.

**DISCUSSION**

**A Putative Mechanism for the Catalysis of yPDE1.** It has been well established that hydrolysis of a phosphodiester bond by class I PDEs is accomplished via a nucleophilic attack of the bridging water or hydroxide ion. However, it is unknown whether yPDE1 hydrolyzes cAMP and cGMP with the same catalytic mechanism as human PDEs. Historically, an inversion of configuration at phosphorus was proposed for the stereochemistry of the cAMP hydrolysis by PDE in 1979. Later, the crystal structures of PDE4 and PDE9-cGMP revealed a hydroxide ion (or a water molecule) that bridges the divalent metal ions may serve as the nucleophile to attack the phosphorus. This hydroxide ion may be activated by an aspartic or glutamic acid (Asp318 or Glu230 in PDE4D2). After formation of an intermediate covalent bond between the hydroxide ion and the phosphorus, a histidine (His160 in PDE4D2) may donate a proton to complete the hydrolysis of the phosphodiester bond. This model is consistent with the mechanism of many other zinc enzymes that use an aspartic acid as a general base to activate a water or hydroxide ion.

Yeast PDE1 contains two zinc ions as the catalytic metals, instead of zinc and magnesium or manganese in human PDEs. The crystal structure reveals a hydroxide ion or water molecule that bridges two zinc ions of yPDE1, in a pattern similar to that seen in the case of human PDEs. This hydroxide ion forms a hydrogen bond with Asp230 and may thus be activated by Asp132 to initiate the nucleophilic attack (Figures 2B and 4). After the reaction intermediate forms, His294 that forms a hydrogen bond with the phosphate group of GMP in the crystal structure of yPDE1-GMP may serve as the proton donor to complete the conversion of cGMP to GMP. Therefore, class II yPDE1 may share a common catalytic mechanism with class I PDEs.
PDEs for the hydrolysis of cAMP and cGMP, although they possess different structures and divalent metals.

Implication of the Physiological Roles of yPDE1. Although class II PDEs are found in a diverse number of species, including *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces pombe*, *Cryptococcus neoformans*, *Dictyostelium discoideum*, *Leishmania mexicana*, *Trypanosoma brucei*, *Vibrio fischeri*, and *Trypanosoma cruzi*, their physiological roles remain unclear. The low affinity of cAMP for yPDE1 renders it nonfunctional at the usual physiological concentration of cAMP. However, several studies have shown critical roles of yPDE1 under extreme environments or stresses. Deletion of yPDE1, but not yPDE2, resulted in a much higher level of accumulation of cAMP upon addition of glucose or intracellular acidification, suggesting a specific role of yPDE1 on agonist-induced cAMP signaling. Also, deletion of PDE1 in *C. albicans* changed the level of expression of genes, stress responses, cell wall and membrane biogenesis, adherence, and virulence, suggesting irreplaceable roles of class II PDEs in stress, adhesion, and virulence. Furthermore, the genetic interactions of PDE1 with GPA2, a *Gα* protein controlling yeast differentiation, caused synthetic defects in growth, morphogenesis, and responses to some stresses, suggesting that yPDE1 and Gpa2 modulate agonist-induced cAMP signaling.

While the cAMP signaling pathway of yPDE1 appears to play a secondary role in yeast physiological processes, the function of the cGMP signaling pathway in yeast has not been well characterized. Most studies focused on the cAMP signaling in which yPDE1 and yPDE2 are involved, but only a few papers about the function of the cGMP signaling in yeast have been published. Because only the cAMP activity of the four yPDE2 genes was reported, it is unknown if they also have cGMP hydrolysis activities. Our study reveals for the first time that yPDE1 is capable of hydrolyzing both cAMP and cGMP with similar efficacies. However, it remains to be elucidated whether the cGMP activity of yPDE1 plays an independent role in yeast physiological processes or an auxiliary function to the cAMP signaling pathway.

ASSOCIATED CONTENT

Accession Codes

The coordinates and structural factors of unliganded yPDE1 and its GMP complex have been deposited as Protein Data Bank entries 4OJV and 4OJX.

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Notes

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ABBREVIATIONS

PDEs, phosphodiesterases; cGMP, cyclic guanosine 3′,5′-monophosphate; cAMP, cyclic adenosine 3′,5′-monophos-
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