A Phosphate-binding Histidine of Binuclear Metallophosphodiesterase Enzymes Is a Determinant of 2',3'-Cyclic Nucleotide Phosphodiesterase Activity*

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Binuclear metallophosphoesterases are an enzyme superfam-

ily defined by a shared fold and a conserved active site. Although

many family members have been characterized biochemically or

structurally, the physiological substrates are rarely known, and

the features that determine monoesterase versus diesterase

activity are obscure. In the case of the dual phosphononoester-

ase/diesterase enzyme CthPnk, a phosphate-binding histidine

was implicated as a determinant of 2',3'-cyclic nucleotide

phosphodiesterase activity. Here we tested this model by comparing

catalytic repertoires of Mycobacterium tuberculosis Rv0805,

which has this histidine in its active site (His98), and Escherichia

coli YfcE, which has a cysteine at the equivalent position (Cys74).

We find that Rv0805 has a previously unappreciated 2',3'-cyclic

nucleotide phosphodiesterase function. Indeed, Rv0805 was

150-fold more active in hydrolyzing 2',3'-cAMP than 3',5'-cAMP.

Changing His98 to alanine or asparagine suppressed

the 2',3'-cAMP phosphodiesterase activity of Rv0805 without

adversely affecting hydrolysis of bis-p-nitrophenyl

phosphate. Further evidence for a defining role of the

histidine derives from our ability to convert the inactive YfcE protein to a

vigorous and specific 2',3'-cNMP phosphodiesterase by intro-
ducing histidine in lieu of Cys74. YfcE-C74H cleaved the P–O2'

bond of 2',3'-cAMP to yield 3'-AMP as the sole product.

Rv0805, on the other hand, hydrolyzed other P–O2' or P–O3'
to yield a mixture of 3'-AMP and 2'-AMP products, with a bias

toward 3'-AMP. These reaction outcomes contrast with that of

CthPnk, which cleaves the P–O3' bond of 2',3'-cAMP to gen-

erate 2'-AMP exclusively. It appears that enzymic features other

than the phosphate-binding histidine can influence the orienta-
tion of the cyclic nucleotide and thereby dictate the choice of the

leaving group.

The binuclear metallophosphoesterases comprise a vast

enzyme superfamly distributed widely among taxa. A proto-
typal member is bacteriophage λ phosphatase (λ-Pase),2 which

has been characterized structurally and biochemically (1–7).

λ-Pase uses Mn2+ to catalyze phosphoester hydrolysis with a

variety of substrates, including phosphopeptides, phosphopro-

teins, nucleoside 2',3'-cyclic phosphates, and “generic” organic

phosphomonoesters and diesters such as p-nitrophenyl phos-

phate and bis-p-nitrophenyl phosphate. Although the physio-

logical substrate(s) and biological function of A-Pase remain

obscure, other well studied members of the binuclear metallo-

phosphoesterase superfamily play key physiological roles in cel-

lular pathways of signal transduction (e.g. the phosphoprotein

phosphatase calcineurin), DNA repair (e.g. the DNA nuclease

Mre11), or RNA processing (e.g. the RNA debranching enzyme

Dbr1) (8–10).

The signature feature of the metallophosphoesterase super-

family is an active site composed of two metal ions (typically

manganese, iron or zinc) coordinated with octahedral geo-

metry by a cage of histidine, aspartate, and asparagine side chains

(Fig. 1). The metals directly coordinate the scissile phosphate

anion, as does the metal-binding asparagine. Plausible catalytic

mechanisms have been proposed based on crystal structures of

superfamily members with phosphate or sulfate in the active

site (5, 9, 11–13) and mutational studies of a few exemplary

enzymes. We construe the active site configuration of the

Mycobacterium tuberculosis Rv0805 protein bound to a phos-

phate anion (14) to mimic the substrate complex of the phos-

phoesterase reaction (Fig. 1, top panel). In this structure, a met-

al-bridging water is situated 3 Å from the phosphorus atom.

The almost perfectly apical orientation of this water to the

putative “leaving” oxygen atom implicates the metal-bridged

water as the nucleophile in the hydrolysis reaction. A puta-

tive mimetic of the product complex is exemplified by the

active site of the Escherichia coli YfcE protein (15) (Fig. 1, middle panel). Here, the tetrahedral sulfate anion has under-

gone stereochemical inversion relative to the phosphate in

Rv0805, and the former metal-bridged water is incorporated

into the anion product.

The rapid pace of identification and structural/biochemical

characterization of new members of the binuclear metallophos-

phoesterase superfamily via genome mining has not been

matched by progress in understanding the biological functions

and relevant substrates of most of these enzymes. The empirical

approach is to produce recombinant protein and survey for

activity with a broad a range of phosphoester substrates and

then surmise a role based on the results. In this way, it was

inferred that the M. tuberculosis Rv0805 enzyme depicted in

Fig. 1 functions as a 3',5'-cyclic nucleotide phosphodiesterase

(16). By contrast, the E. coli YfcE protein catalyzed manganese-

dependent hydrolysis of bis-p-nitrophenyl phosphate (15) but

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2 The abbreviations used are: λ-Pase, λ phosphatase; CIP, calf intestine

phosphatase.
Metallophosphodiesterase Substrate Specificity Determinants

had no activity with any natural phosphodiesters tested (nucleic acid, cyclic nucleotides, and phosphatidyl choline) or any of 57 natural phosphomonoester substrates (nucleotides, sugars, and amino acids).

Our studies have focused on Clostridium thermocellum polymethylketone kinase-phosphatase (CthPnkp) as a model to probe the binuclear metallophosphodiesterase mechanism and the determinants of substrate specificity (7, 17–20). CthPnkp catalyzes 5’ and 3’ RNA end-healing reactions that prepare broken RNA termini for sealing by RNA ligase (17, 20). The central 3’ end-healing domain of CthPnkp belongs to the binuclear metallophosphodiesterase superfamily; extensive mutational analysis underscores the strong similarity of the active site of CthPnkp to that of λ-Pase with respect to the metal and phosphate ligands (7, 18, 19). Biochemically, CthPnkp is a Ni²⁺- dependent phosphodiesterase/monoesterase, active on nucleotides (2’,3’-cAMP, 3’-AMP and 2’-AMP) and generic substrates (bis-p-nitrophenyl phosphate, p-nitrophenyl phosphate, and p-nitrophenyl phenylphosphonate). The phosphodiesterase and monoesterase reactions rely on overlapping but different ensembles of active site functional groups. The enzyme is remarkably plastic, insofar as CthPnkp can be transformed toward narrower metal and substrate specificities via mutations of the active site. For example, certain changes (e.g. replacing the metal-binding His¹⁸⁹ residue with aspartate) transform CthPnkp into a Mn²⁺-dependent phosphodiesterase devoid of monoesterase activity (19).

We have analyzed in depth the 2’,3’-cyclic phosphodiesterase activity of CthPnkp, in light of the fact that 2’,3’-cyclic phosphate termini are the predominant products of several known RNA damage pathways (7). We found that alanine, glutamine, or asparagine mutations at the phosphate-binding residue His²⁶⁴ of CthPnkp (corresponding to His⁷⁶ in λ-Pase or His⁹⁸ in Rv0805; Fig. 1) crippled the 2’,3’-cyclic phosphodiesterase activity, whereas the same changes enhanced the generic phosphodiesterase activity of CthPnkp with bis-p-nitrophenyl phosphate.

Our results prompted speculation that binuclear metallophosphoesterases might evolve distinct biochemical specificities via subtle changes at the active site (7). In particular, we predicted a correlation between 2’,3’-cyclic nucleotide phosphodiesterase activity and the presence of a phosphate-binding histidine analogous to His⁷⁶ in λ-Pase or His⁹⁸ in Rv0805.

The 2’,3’-cyclic phosphodiesterase activity of CthPnkp is Ni²⁺-dependent (18), as is the 2’,3’-cyclic nucleotide phosphodiesterase activity of CthPnkp and CthPnkp (corresponding to His⁷⁶ in λ-Pase or His⁹⁸ in Rv0805). Therefore, we hypothesized that CthPnkp has the potential to convert a substrate (e.g. cGMP) into a product (e.g. bis-p-nitrophenyl phosphate, p-nitrophenyl phosphate, and p-nitrophenyl phenylphosphonate) that might be analogous to that of CthPnkp or CthPnkp.

FIGURE 1. Active sites of binuclear metallophosphodiesterases Rv0805 and YfcE. The top and middle panels show stereo views of the active sites of M. tuberculosis Rv0805 (top panel; Protein Data Bank code 2HY1) and E. coli YfcE (middle panel; Protein Data Bank code 1SU1, protomer D). The amino acid side chains coordinating the binuclear metal cluster and either the phosphate ion in Rv0805 or sulfate ion in YfcE are shown. The metal ions are colored magenta. Water is colored red. The phosphate-binding histidine in Rv0805 (His²⁶⁴) is replaced by a cysteine in YfcE (Cys⁷⁴). The middle panel shows models of two potential orientations of 2’,3’-cGMP in the active site of Rv0805. The 2’,3’-cGMP molecule was imported from Protein Data Bank code 1GSP. The cyclic phosphoester was superimposed on the phosphate anion in the Rv0805 structure. When the ribose O₂⁻ is apical to the metal-bridged water nucleophile (left), the reaction yields a 3’-PO₄ nucleotide product. When the ribose O₃⁻ atom is apical to the water nucleophile (right), the product is a 2’-PO₄ nucleotide. The His²⁶⁴ side chain is poised to donate a hydrogen bond to the leaving ribose oxygen atom in the modeled 2’,3’-cGMP substrate in either orientation.

EXPERIMENTAL PROCEDURES

Materials

p-Nitrophenyl phosphate, bis-p-nitrophenyl phosphate, p-nitrophenol, cAMP, cGMP, and cUMP were purchased from Sigma. Malachite green reagent was purchased from BIOMOL Research Laboratories.

Purification of Rv0805

The M. tuberculosis gene Rv0805 was amplified by two-stage overlap extension PCR (21) from genomic DNA with Pfu DNA polymerase using primers designed to eliminate an internal BamHI site while introducing an Ndel restriction site at the start codon and a BamHI site 3’ of the stop codon. The PCR product was digested with Ndel and BamHI and inserted into pET16b to generate an expression plasmid encoding the 318-amino acid Rv0805 polypeptide fused to an N-terminal His₁₀ tag. Missense mutations H98N and H98A were introduced into the Rv0805 open reading frame by PCR using the two-stage overlap extension method (21). The inserts were sequenced to
verify that there were no unwanted coding changes. Wild type and mutant pET-Rv0805 plasmids were transformed into E. coli strain BL21(DE3). Cultures (200 ml) of E. coli BL21(DE3)/pET-Rv0805 were grown at 37 °C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until the A600 reached ~0.6. The cultures were chilled on ice for 30 min, adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside and 2% (v/v) ethanol, and then incubated at 17 °C for 16 h with continuous shaking. The cells were harvested by centrifugation, and the pellet was stored at -80 °C. All of the subsequent procedures were performed at 4 °C. Thawed bacteria were resuspended in 20 ml of buffer A (50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 10% sucrose). Lysozyme, phenylmethylsulfonyl fluoride, and Triton X-100 were added to final concentrations of 1 mg/ml, 1 mM, and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 1-ml columns of nickel-nitritolactoside-acid-agarose (Qiagen) that had been equilibrated with buffer A. The columns were washed with 8 ml of the same buffer and then eluted stepwise with 4-ml aliquots of 25, 50, 200, and 500 mM imidazole in buffer B (50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 10% glycerol). The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The His10-Rv0805 proteins adsorbed to the column and were recovered predominantly in the 200 mM imidazole eluates. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The Rv0805 preparations were stored at −80 °C.

**Purification of YfcE**

The open reading frame encoding the 184-amino acid YfcE polypeptide was amplified from E. coli genomic DNA with primers that introduced an NdeI site at the start codon and a BamHI site 3’ of the stop codon. The PCR product was digested with NdeI and BamHI and inserted into pET16b to generate an expression plasmid encoding His10-tagged YfcE. Missense mutations C74A, C74N, and C74H were introduced by PCR using the two-stage overlap extension method (21). The pET-YfcE plasmids were transformed into E. coli BL21(DE3). The His10-YfcE proteins were purified from soluble extracts of 200-ml cultures of isopropyl-β-D-thiogalactopyranoside-induced bacteria as described above for His10-Rv0805.

**Hydrolysis of p-Nitrophenyl Phosphate and Bis-p-nitrophenyl Phosphate**

Reaction mixtures (25 µl) containing 50 mM Tris-HCl (pH 8.5), 0.5 mM MnCl2, 10 mM p-nitrophenyl phosphate or bis-p-nitrophenyl phosphate, and Rv0805 or YfcE as specified were incubated at 37 °C. The reactions were quenched by adding 20 mM EDTA (YfcE reactions) or 5% SDS (Rv0805 reactions), followed by 0.9 ml of 1 M Na2CO3. Release of p-nitrophenol was determined by measuring A410 and interpolating the value to a p-nitrophenol standard curve.

**Hydrolysis of Cyclic Nucleotides**

Reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 8.5), 0.5 mM MnCl2, 10 mM cyclic nucleotide as specified, and either Rv0805, YfcE, or calf intestine phosphatase (CIP) as specified were incubated for 10 min at 37 °C. (CIP was present in excess and did not limit the extent of phosphate release.) The reactions were quenched by adding 20 mM EDTA, followed by 1 ml of malachite green reagent. Release of phosphate was determined by measuring A620 and interpolating the value to a phosphate standard curve.

**Kinetic Parameters**

**Hydrolysis of Bis-p-nitrophenyl Phosphate**—Reaction mixtures (25 µl) containing 50 mM Tris-HCl (pH 8.5), 0.5 mM MnCl2, increasing concentrations (0.313, 0.625, 1.25, 2.5, or 5.0 mM) of bis-p-nitrophenyl phosphate, and a fixed amount of the specified enzyme (2 pmol Rv0805 or YfcE-C74H corresponding to 0.08 µM enzyme; 5 pmol YfcE, 0.2 µM enzyme) were incubated at 37 °C for either 5 min (Rv0805 and YfcE-C74H) or 6 min (YfcE).

**Hydrolysis of p-Nitrophenyl Phosphate**—Reaction mixtures (25 µl) containing 50 mM Tris-HCl (pH 8.5), 0.5 mM MnCl2, increasing concentrations (0.625, 1.25, 2.5, 5.0, or 10 mM) of p-nitrophenyl phosphate, and a fixed amount of the specified enzyme (100 pmol Rv0805 or YfcE, corresponding to 4 µM enzyme; 50 pmol YfcE-C74H, 2 µM enzyme) were incubated for 5 min at 37 °C.

**Hydrolysis of 2’,3’-cAMP**—Reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 8.5), 0.5 mM MnCl2, 1 unit CIP, increasing concentrations (0.625, 1.25, 2.5, 5.0, or 10 mM) of 2’,3’-cAMP, and either 7.5 pmol Rv0805 (0.75 µM enzyme) or 25 pmol YfcE-C74H (2.5 µM enzyme) were incubated at 37 °C for 15 min (YfcE-C74H) or 5 min (Rv0805). The enzyme concentrations and incubation times were chosen to ensure that ≥36% of the substrate was converted to product at the lowest substrate concentrations tested (the ranges were from 9 to 36% conversion). The extents of p-nitrophenol or Pi production were first plotted as a function of substrate concentration. Km and kcat were then calculated from Eadie-Hofstee plots of the data. The Km and kcat values reported in Table 1 are averages from two independent substrate titration experiments ± mean absolute error.

**RESULTS**

2’,3’-Cyclic Phosphodiesterase Activity of M. tuberculosis Rv0805—Shenoy et al. (16) found that Rv0805 catalyzes Mn2+-dependent cleavage of bis-p-nitrophenyl phosphate and hydrolysis of 3’,5’-cAMP to 5’-AMP. The ability of Rv0805 to hydrolyze 2’,3’-cyclic nucleotides was not reported. Here, we produced Rv0805 in E. coli as a His10 fusion and purified the enzyme from a soluble bacterial extract by nickel-agarose chromatography (Fig. 2). The recombinant protein hydrolyzed 10 mM bis-p-nitrophenyl phosphate in the presence of 0.5 mM MnCl2, to yield p-nitrophenol; the extent of product formation was proportional to input enzyme (Fig. 3A). From the slope of the titration curve, we calculated a turnover number of 12.6 s⁻¹. Formation of p-nitrophenol by Rv0805 displayed a hyperbolic dependence on the concentration of bis-p-nitrophenyl phosphate (not shown). From an Eadie-Hofstee plot, we calculated a Km of 0.9 mM and kcat of 12.4 s⁻¹ (Table 1). (The kinetic parameters reported previously by Shenoy et al. (16) were: Km = 1.3 mM bis-p-nitrophenyl phosphate and kcat = 4.2 s⁻¹.)
displayed much weaker activity as a phosphomonoesterase (Fig. 3B). It hydrolyzed 10 mM p-nitrophenyl phosphate to p-nitrophenol with a specific activity of 0.5 s⁻¹. From the results of a substrate titration experiment, we calculated a $K_m$ of 1.7 mm p-nitrophenyl phosphate and $k_{cat}$ of 0.55 s⁻¹ (Table 1). Thus, the catalytic efficiency ($k_{cat}/K_m$) of Rv0805 was 43-fold greater for the phosphodiesterase substrate.

2',3'-Cyclic nucleotide phosphodiesterase activity was tested by reacting Rv0805 with 10 mM 2',3'-cAMP in the presence of 0.5 mM MnCl₂. CIP was included in the reaction to liberate inorganic phosphate from any nucleoside phosphomonoesters formed by Rv0805. In the experiment shown in Fig. 4, Rv0805 converted 16% of the input 10 mM 2',3'-cAMP to a CIP-sensitive phosphomonoester. By contrast, a control reaction with CIP alone released <1% of the Pi from 2',3'-cAMP. Also, no free phosphate was released from 2',3'-cAMP by Rv0805 in the absence of CIP.

To query whether Rv0805 displays specificity toward a particular nucleotide and whether the enzyme discriminates between a 2',3'-cyclic phosphate and a 3',5'-cyclic phosphate, we reacted the enzyme with 10 mM 2',3'-cGMP, 3',5'-cAMP, 3',5'-cGMP, or 3',5'-CUMP substrates. Rv0805 converted 16% of the input 2',3'-cGMP to a CIP-sensitive phosphomonoester; thus, the enzyme did not have a preference for 2',3'-cAMP versus 2',3'-cGMP. The salient finding was that Rv0805 displayed much weaker activity as a 3',5'-cyclic phosphodiesterase, converting only 0.2, 1, and 1.6% of the input 3',5'-cAMP, 3',5'-cGMP, and 3',5'-cUMP substrates to CIP-sensitive phosphomonoesters, respectively (Fig. 4A). An enzyme titration experiment (Fig. 3D) showed that the specific activity of Rv0805 as a 2',3'-cyclic AMP phosphodiesterase was 150-fold higher than as a 3',5'-cyclic AMP phosphodiesterase (estimated turnover numbers of 150 and 1 min⁻¹, respectively). The kinetic data reported by Shenoy et al. (16) for hydrolysis of 3',5'-cAMP indicated a $k_{cat}$ value of ~1.7 min⁻¹, which agrees with the value we observe. Thus, the conclusion by Shenoy et al., that Rv0805 is a 3',5'-cyclic nucleotide phosphodiesterase, is open to question in light of our determination that it has 2 orders of magnitude higher activity with 2',3'-cyclic nucleotide substrates. Additional experiments revealed that Rv0805 had essentially no detectable ability to hydrolyze phosphomonoester substrates 5'-AMP, 3'-AMP, or 2'-AMP (10 mM) in Tris-HCl buffer (pH 8.5) the presence of 0.5 mM MnCl₂. Specifically, a 10-µl reaction mixture so constituted with 21 µM Rv0805 released ~0.1 nmol of inorganic phosphate (the lower limit of detection of the assay) as product from 100 nmol of input 5'-AMP, 3'-AMP, or 2'-AMP substrate during a 10-min incubation at 37 °C, which corresponds to a turnover number of ~0.05 min⁻¹, a value 3,000-fold lower than the activity of Rv0805 with 2',3'-cAMP.

Formation of a CIP-sensitive adenylate by Rv0805 displayed a hyperbolic dependence on the concentration of 2',3'-cAMP. From an Eadie-Hofstee plot, we calculated a $K_m$ of 1.6 mm and $k_{cat}$ of 2.8 s⁻¹ (Table 1). To determine the chemical identity of the products of the reaction with 2',3'-cAMP, we performed cellulose TLC analysis of the reaction mixture as a function of enzyme in the presence of Rv0805 only (no CIP). The TLC plate was developed with buffer containing saturated ammonium nitrate essentially no detectable ability to hydrolyze phosphomonoester substrates 5'-AMP, 3'-AMP, or 2'-AMP (10 mM) in Tris-HCl buffer (pH 8.5) the presence of 0.5 mM MnCl₂. Specifically, a 10-µl reaction mixture so constituted with 21 µM Rv0805 released ~0.1 nmol of inorganic phosphate (the lower limit of detection of the assay) as product from 100 nmol of input 5'-AMP, 3'-AMP, or 2'-AMP substrate during a 10-min incubation at 37 °C, which corresponds to a turnover number of ~0.05 min⁻¹, a value 3,000-fold lower than the activity of Rv0805 with 2',3'-cAMP.

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that Rv0805 preferentially cleaves the P–O2 bond of 2',3'-cAMP to a mixture of more quickly migrating products. Their order of migration away from the origin ([9]) reveals that YfcE has a cysteine (Cys74) at the position corresponding to Rv0805 His98 and Pnkp His264 (Fig. 1). We seized on YfcE as a promising scaffold to test the key prediction of our substrate-specificity model, by engineering a gain-of-function in a metallophosphodiesterase enzyme that ordinarily lacks the ability to hydrolyze 2',3'-cyclic nucleotides.

We produced wild type YfcE as a His6 tag and purified it from a soluble bacterial extract by nickel-agarose chromatography (Fig. 2). YfcE hydrolyzed 10 mM bis-p-nitrophenyl phosphate in the presence of 0.5 mM MnCl2 to yield p-nitrophenol; from the slope of the titration curve (Fig. 5A), we estimated a turnover number of 22 s⁻¹. From the results of a substrate titration experiment, we calculated a Km of 4.8 mM bis-p-nitrophenyl phosphate and a kcat of 18 s⁻¹ (Table 1). (The kinetic parameters reported previously by Miller et al. (15) were: Km = 9.7 mM bis-p-nitrophenyl phosphate and kcat of 20 s⁻¹.) Unlike Miller et al., we were able to detect a generic YfcE phosphomonoesterase activity. YfcE hydrolyzed 10 mM p-nitrophenyl phosphate to p-nitrophenol in an enzyme concentration-dependent manner (Fig. 5B). From the slope of the titration curve, we estimated a turnover number of 0.4 s⁻¹. Kinetic parameters for the YfcE phosphomonoesterase reaction determined from a substrate titration experiment were: Km = 10.6 mM p-nitrophenyl phosphate and kcat = 0.9 s⁻¹ (Table 1). The catalytic efficiency (kcat/Km) of YfcE is thereby 44-fold greater for the generic phosphodiesterase substrate. YfcE had feeble activity in hydrolysis of 2',3'-cAMP to a CIP-sensitive nucleoside monoester (Fig. 5C). From the slope of the titration curve, we estimated a turnover number of 0.03 s⁻¹.
Transformation of YfcE into a 2',3'-Cyclic Nucleotide Phosphodiesterase—YfcE mutants with alanine, asparagine, or histidine in lieu of Cys\textsuperscript{74} were purified (Fig. 2) and surveyed for phosphodiesterase and monooxygenase activities (Fig. 5). The specific activities in hydrolysis of bis-p-nitrophenyl phosphate (Fig. 5A) varied within a 5-fold range according to the amino acid at position 74, as follows: His (56 s\textsuperscript{-1}) > Asn (40 s\textsuperscript{-1}) > Cys (22 s\textsuperscript{-1}) > Ala (10 s\textsuperscript{-1}). Greater salutary effects of the histidine and asparagine changes were observed for phosphomonoesterase activity with p-nitrophenyl phosphate (Fig. 5B), with the following hierarchy of specific activities: Asn (6 s\textsuperscript{-1}) > His (2.5 s\textsuperscript{-1}) > Cys (0.4 s\textsuperscript{-1}) ≈ Ala (0.3 s\textsuperscript{-1}). The increase of generic Mn\textsuperscript{2+} dependent monooxygenase activity in YfcE-C74H versus C74A reciprocated the loss of generic monooxygenase function observed when the Rv0805 and CthPnkp histidines were mutated to alanine. The YfcE-C74N and C74H titration curves deviated downward from linearity at the higher levels of input enzyme at which ≥ 30% of the 10 mM p-nitrophenyl phosphate substrate was converted to p-nitrophenol and P\textsubscript{i} (Fig. 5B). This reflected product inhibition by P\textsubscript{i}. From a separate experiment entailing prior addition of increasing amounts of phosphate to a reaction mixture containing 10 mM p-nitrophenyl phosphate and 0.2 μM YfcE-C74H, we determined an IC\textsubscript{50} value of 2.5 mM P\textsubscript{i} (data not shown).

The instructive finding was that the C74H mutation uniquely conferred a gain of function in hydrolysis of 10 mM 2',3'-cAMP (Fig. 5C). From the slope of the titration curve, we estimated a turnover number of 1.3 s\textsuperscript{-1}. To delineate the specificity of this novel catalyst, we tested C74H cyclic phosphodiesterase activity in parallel with 10 mM 2',3'-cAMP, 2',3'-cGMP, 3',5'-cAMP, 3',5'-cGMP, and 3',5'-cUMP substrates (Fig. 6A). This experiment highlighted YfcE-C74H as strictly specific for 2',3'-cAMP; there was scant activity with the 3',5'-cNMP substrates. Control assays verified that YfcE-C74H catalyzed no detectable phosphate release from 2',3'-cAMP in the absence of CIP (Fig. 6A). Moreover, YfcE-C74H had no detectable ability to hydrolyze phosphomonoester substrates 5'-AMP, 3'-AMP, or 2'-AMP (10 mM) in the presence of 0.5 mM MnCl\textsubscript{2} to yield inorganic phosphate as product. Specifically, a 10-μl reaction mixture so constituted with 140 μM YfcE-C74H released ≤ 0.1 nmol of inorganic phosphate (the lower limit of detection of the assay) as product from 100 nmol of input 5'-AMP, 3'-AMP, or 2'-AMP substrate during a 15-min incubation at 37 °C, which corresponds to a turnover number of ≅ 0.005 min\textsuperscript{-1}, a value 15,000-fold lower than the activity of YfcE-C74H with 2',3'-cAMP.

To determine the chemical identities of the products of the reaction with 2',3'-cAMP, we performed cellulose TLC analysis of the reaction mixture as a function of reaction time in the presence of YfcE-C74H only (no CIP). YfcE-C74H quantitatively converted the 2',3'-cAMP substrate to a single product that comigrated with the 3'-AMP standard (Fig. 6B). We conclude that YfcE-C74H cleaves exclusively the P–O\textsubscript{2} bond of 2',3'-cAMP. Steady-state kinetic parameters for the YfcE-C74H cyclic phosphodiesterase derived from 2',3'-cAMP titra-
tion experiments were as follows: $K_m = 35 \text{ mM}$ 2',3'-cAMP and $k_{\text{cat}} = 2.95 \text{ s}^{-1}$ (Table 1). It is notable that the $k_{\text{cat}}$ of the “designed” YfcE-C74H 2',3'-cAMP phosphodiesterase was similar to that of the “natural” Rv0805 enzyme, although the affinity of the YfcE-C74H protein for the substrate was 22-fold less than Rv0805.

These results attest to the transformative power of the active site histidine as a determinant of cyclic nucleotide phosphodiesterase activity. As discussed below, we surmise that enzymic groups other than the phosphate-binding histidine might contribute to cyclic nucleotide binding and orientation of the leaving group.

**DISCUSSION**

The present study supports a model, suggested by our studies of CthPnkp (7), that the presence of a phosphate-binding histidine in the active site of phosphodiesterase members of the binuclear metallophosphodiesterase superfamily is a determinant of 2',3'-cyclic nucleotide phosphodiesterase activity. Here we focused on two structurally characterized metallophosphodiesterases that differ in having a histidine (Rv0805) or a cysteine (YfcE) at this active site position. Although Rv0805 had been dubbed a 3',5'-cyclic nucleotide phosphodiesterase by other investigators (16), our characterization of this enzyme shows it to be 150-fold more active on 2',3'-cAMP than 3',5'-cAMP. Thus, the presence of an active site histidine in Rv0805 correctly predicted its heretofore unexamined capacity for 2',3'-cNMP hydrolysis. Changing the histidine to alanine or asparagine suppressed 2',3'-cAMP phosphodiesterase activity of Rv0805 without affecting the hydrolysis of a generic non-nucleotide phosphodiester substrate. Even more compelling evidence for the defining role of the histidine derives from our ability to convert the otherwise inactive YfcE protein into an active and highly specific 2',3'-cNMP phosphodiesterase by introducing a histidine in lieu of Cys34.

The correlation of an active site histidine and 2',3'-cNMP phosphodiesterase activity applies to other metallophosphodiesterase superfamily members, including λ-Pase (7) and the recently characterized Deinococcus radiodurans enzyme DR1281 (23). DR1281 resembles Rv0805 in its 35-fold higher $k_{\text{cat}}$ for Mn$^{2+}$-dependent hydrolysis of bis-p-nitrophenyl phosphate versus p-nitrophenyl phosphate and its selective hydrolysis of 2',3'-cNMPs versus 3',5'-cNMPs (23). The correlation between a nonhistidine residue and the absence of cyclic phosphodiesterase activity seen here with YfcE is reminiscent of the properties of the structurally characterized Methanococcus jan-naschii enzyme MJ0936 (24). MJ0936 has vigorous activity in hydrolyzing bis-p-nitrophenyl phosphate but is unable to cleave p-nitrophenyl phosphate. Although possessed of a generic phosphodiesterase activity, MJ0936 reportedly had no detectable cyclic phosphodiesterase activity with the 2',3'- or 3',5'-forms of cAMP or cGMP (24). The crystal structure of manganese-bound MJ0936 (Protein Data Bank code 1S3N) (24) reveals the similarity of its active site to that of Rv0805, except for the presence of an asparagine in lieu of the phosphate-coordinating histidine.

Although the histidine is clearly a major determinant of 2',3'-cNMP phosphodiesterase activity, the outcomes of the hydrolysis step can differ significantly from one enzyme to another. CthPnkp-H189D (a diesterase-only mutant in which a metal-binding histidine is changed to aspartate) catalyzes hydrolysis of the P–O3' bond of 2',3'-cAMP or -cGMP to yield exclusively 2'-AMP or 2'-GMP products (7, 20). By contrast, YfcE-C74H (Fig. 6) and DR1281 (23) both catalyze hydrolysis of the P–O2' bond of 2',3'-cAMP to yield 3'-AMP and 2'-AMP products, with a clear bias toward generation of 3'-AMP (Fig. 3). λ-Pase also hydrolyzes either P–O2' or P–O3' to yield both 3'-AMP and 2'-AMP products, albeit with a preference for 2'-AMP formation (7). We surmise that the histidine facilitates an aspect of phosphohydrolase chemistry common to both pathways (conceivably entailing protein donation to the ribose O2' or O3' leaving atom), but constituents other than the histidine dictate the reaction outcome by influencing substrate orientation and affinity.

The critical issue of substrate orientation in determining which products are formed is illustrated in Fig. 1 (bottom panel), in which we have modeled a 2',3'-cGMP molecule (imported from a crystal structure of RNase T1; Protein Data Bank code 1GSP) (33) into the active site of Rv0805 by superimposing the cyclic phosphate moiety of cGMP on the phosphate anion in the Rv0805 structure. This results in two potential configurations for opening the cyclic phosphate. When the ribose O2' is apical to the metal-bridged water nucleophile, the reaction yields a 3'-PO$_4$ nucleotide product. When the ribose O3' atom is apical to the water nucleophile, the product is a 2'-PO$_4$ nucleotide. The His$^{\text{98}}$ side chain is poised to donate a hydrogen bond to the leaving ribose oxygen atom in the modeled substrate complex with 2',3'-cGMP in either orientation (Fig. 1, bottom panel). The asymmetry of the binding modes is apparent in the locations of the bulky purine base relative to the (pseudo-mirror-symmetrical) cyclic phosphate-ribose ring system. It is likely that steric constraints on the adoption of one orientation versus the other are responsible for stringent and opposite choice of leaving groups during the 2',3'-cNMP phosphodiesterase reactions of CthPnkp-H189D versus YfcE-C74H and DR1281. We speculate that Rv0805 and λ-Pase are less constrained with respect to the binding orientations of the cyclic nucleotide, thereby allowing formation of either 2'-NMP or 3'-NMP products. Further evaluation of this hypothesis and elucidation of the structural elements that dictate substrate orientation, will hinge on determining crystal structures of metallophosphodiesterases enzymes with a 2',3'-cyclic nucleotide bound in the active site.

Are there useful biological inferences to be drawn from the fact that a metallophosphodiesterase family member has a vigorous and relatively specific 2',3'-cyclic nucleotide phosphodiesterase activity in vitro? In the case of CthPnkp, we have suggested that this activity is relevant to the repair of RNA 2',3'-cyclic ends (7, 20), which are natural intermediates in RNA processing (25) and RNA catabolism (26). RNA 2',3'-cyclic ends are also the end products of RNA-cleaving toxins (27–30). An initial clue to a nucleic acid repair role for CthPnkp was the covariant linkage of its metallophosphoesterase domain to a polynucleotide kinase module (17) that is known to catalyze 5'...
end healing reactions in RNA repair pathways (25, 27). In the case of Rv0805 and λ-Pase, there are no physiologically instructive flanking domains, and there is, to our knowledge, no genetic evidence implicating either protein in a particular biological process in its native context. The prospect that M. tuberculosis Rv0805 acts on broken RNAs is worthy of consideration, given that M. tuberculosis encodes at least seven MazF-like endoribonuclease toxins that generate site-specific breaks with 2′,3′-cyclic ends (30–32).

Finally, the correlation between a nonhistidine residue (Cys74) in the YfcE active site and the absence of cyclic phosphodiesterase activity hints at a novel specificity within the superfamily. We think it is unlikely that the histidine substitution by cysteine in YfcE is a sporadic event resulting in a “crippled” metallophosphodiesterase diesterase enzyme. Indeed, Miller et al. (15) described a novel clade of bacterial YfcE-like binuclear metallophosphodiesterases, embracing predicted polypeptides from diverse bacterial genera, each of which has a cysteine in lieu of the phosphate-binding histidine. A current data base search identifies members of this cysteine-containing bacterial subfamily in Shigella, Citrobacter, Enterobacter, Klebsiella, Yersinia, Serratia, Erwinia, Vibrio, Shewanella, Photobacterium, Aeromonas, Clostridium, Bacteroides, Ruminococcus, Thermotoga, Moorella, Treponema, and others. The fact that YfcE can hydrolyze 2′,3′-cNMPs when mutated to His74 hints that this subfamily evolved toward a narrow substrate specificity. The “real” substrate for YfcE-like phosphodiesterases (and thus the specificity determining role of the active site cysteine) remains to be discovered.

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