J Biol Inorg Chem (2014) 19:389–398
DOI 10.1007/s00775-013-1079-0

ORIGINAL PAPER

Determination of the catalytic activity of binuclear metallohydrolases using isothermal titration calorimetry

Marcelo M. Pedroso · Fernanda Ely · Thierry Lonhienne · Lawrence R. Gahan · David L. Ollis · Luke W. Guddat · Gerhard Schenk

Received: 15 August 2013 / Accepted: 12 December 2013 / Published online: 12 January 2014

Abstract Binuclear metallohydrolases are a large and diverse family of enzymes that are involved in numerous metabolic functions. An increasing number of members find applications as drug targets or in processes such as bioremediation. It is thus essential to have an assay available that allows the rapid and reliable determination of relevant catalytic parameters (k_{cat}, K_m, and k_{cat}/K_m). Continuous spectroscopic assays are frequently only possible by using synthetic (i.e., nonbiological) substrates that possess a suitable chromophoric marker (e.g., nitrophenol). Isothermal titration calorimetry, in contrast, affords a rapid assay independent of the chromophoric properties of the substrate—the heat associated with the hydrolytic reaction can be directly related to catalytic properties. Here, we demonstrate the efficiency of the method on several selected examples of this family of enzymes and show that, in general, the catalytic parameters obtained by isothermal titration calorimetry are in good agreement with those obtained from spectroscopic assays.

Electronic supplementary material The online version of this article (doi:10.1007/s00775-013-1079-0) contains supplementary material, which is available to authorized users.

M. M. Pedroso · F. Ely · T. Lonhienne (✉) · L. R. Gahan · L. W. Guddat · G. Schenk (✉)
School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia
e-mail: t.lonhienne@uq.edu.au

G. Schenk
e-mail: schenk@uq.edu.au

D. L. Ollis
Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia

Keywords Isothermal titration calorimetry · Kinetic assays · Metalloenzymes · Organophosphate-degrading enzymes

Introduction

Binuclear metallohydrolases form a large group of enzymes that cover a wide range of metabolic functions ranging from bone resorption to signal transduction and DNA replication, to name but a few [1–3]. Several enzymes from this family are current targets for drug design and development, and others have found application in biotechnology. As an example, organophosphate-degrading phosphotriesterases have been shown to be very efficient bioremediators owing to their ability to hydrolyze and thus detoxify many commonly used pesticides and some nerve agents (including sarin and VX) [3–6]. Both drug design and the development of enzymes with tailored catalytic properties require access to kinetic assays that facilitate the measurement of catalytic parameters (i.e., k_{cat}, K_m, and k_{cat}/K_m) and inhibition constants (i.e., K_i). Such assays are frequently based on spectrophotometric measurements that record the consumption of reagents and/or the formation of products. However, often biologically relevant reactants do not have spectroscopic properties that facilitate simple and rapid assays, and hence researchers frequently use synthetic (nonbiological) substrates with specific chromophoric fingerprints to study enzymatic reactions. An example is *para*-nitrophenol phosphate. Many metallohydrolases are capable of cleaving the ester bond of this molecule, and the yellow color of the product *para*-nitrophenol makes rate measurements rapid and facile. However, for some applications such as the assessment of substrate specificity or the development of a catalyst

with increased reactivity towards a particular molecule, it is desirable to use native reactants as substrates. It is thus essential to make available an assay that facilitates rate measurements that are independent of spectroscopic properties of reactant molecules.

Here, we describe an assay that uses isothermal titration calorimetry (ITC), a method that records changes in heat associated with bond breakage (i.e., hydrolysis), and as such is applicable for measurements with any potential substrate of a particular enzyme. We have chosen three enzyme systems to illustrate the method, an organophosphate-degrading phosphotriesterase from *Agrobacterium radiobacter* (OpdA) [7–11], a glycerophospodiesterase from *Enterobacter aerogenes* (GpdQ) [12–16], and a cyclic nucleotide diesterase from *Mycobacterium tuberculosis* (Rv0805) [17–19]. Both OpdA and GpdQ are promising bioremediators; in particular, OpdA has already been shown in field trials to be competent in detoxifying pesticide-polluted environments [20]. Current research aims to evolve mutants of these enzymes with improved catalytic efficiency towards specific pesticides and nerve agents [3, 5, 6, 21, 22]. Rv0805 is involved in cell signaling pathways. It has been postulated that this enzyme, owing to its significance in maintaining cellular cyclic adenosine monophosphate (cAMP) levels, may be a target for the treatment of tuberculosis [17, 23, 24].

Materials and methods

Materials

Escherichia coli BL21(DE3) and DH5α host cells were purchased from Novagen. All chromatographic devices (fast protein liquid chromatography system and relevant resins) were from GE Healthcare, and all chemicals, buffers, and

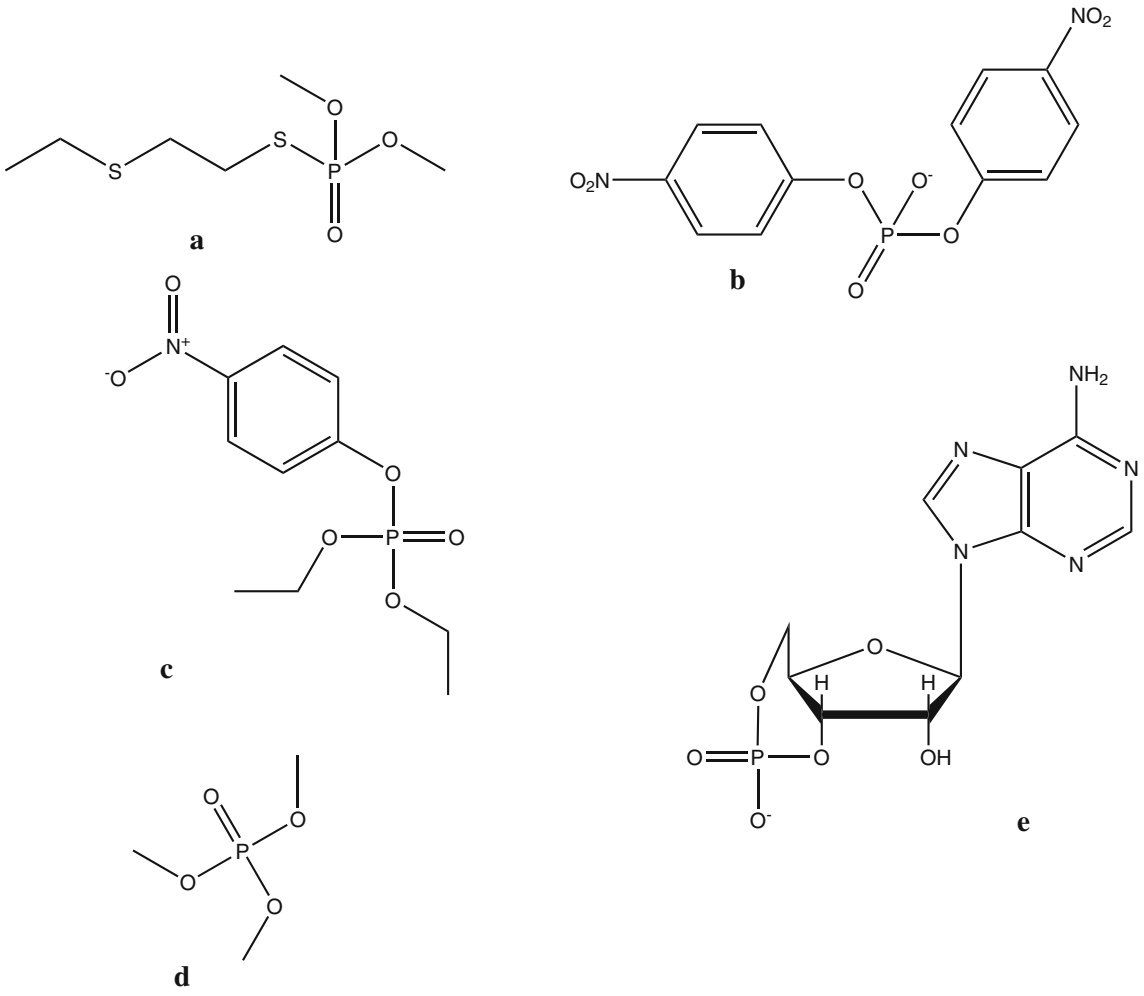

Fig. 1 Substrates used in this study: **a** demeton-S; **b** bis(*para*-nitrophenol) phosphate (bpNPP); **c** paraoxon; **d** trimethyl phosphate (TMP); **e** cyclic adenosine monophosphate (cAMP)

substrates (see Fig. 1), unless mentioned otherwise, were purchased from Sigma Chemical Company.

Protein expression and purification

The expression and purification of OpdA and GpqQ were described in detail elsewhere and will only be briefly summarized here [9, 14]. The OpdA gene was previously cloned into the recombinant plasmid pETMCSI, which was used to transform competent *E. coli* BL21 (DE3) cells. A single colony was inoculated in terrific broth supplemented with 1 mM $CoSO_4$ and 50 μM ampicillin. The culture was grown at 37 °C at 200 rpm for 48 h. Following centrifugation (approximately 30 g wet mass) the cells were disrupted using a French press at 1,000 psi. The soluble fraction was applied to a MonoS HR column, equilibrated with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer, pH 7.0, supplemented with 0.5 mM $CoSO_4$; enzymes were eluted using a gradient of 0–1 M NaCl. The fractions containing activity were concentrated and loaded onto a Sephacryl S-200 column, equilibrated with the same buffer as in the previous step, and were eluted with buffer supplemented with 0.2 M NaCl. The purified OpdA was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and its concentration was determined by measuring the absorption at 280 nm ($\varepsilon_{280} = 29{,}280\ M^{-1}\ cm^{-1}$ per monomeric unit).

The GpdQ-encoding expression vector pCY76::GpdQ was used to transform competent *E. coli* DH5α cells. The cells were grown in 4 L of terrific broth, containing 50 μg/mL ampicillin and 0.1 mM $CoCl_2$, for 36 h at 30 °C. Then the cells were harvested by centrifugation and lysed using a French press at 1,000 psi. The soluble fraction was loaded onto a HiPrep 16/10 DEAE column, equilibrated with 20 mM tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl) buffer, pH 8.0, and the proteins were eluted with a linear NaCl gradient from 0 to 1 M. Fractions containing activity against 2 mM bis(*para*-nitrophenyl) phosphate (bpNPP) were dialyzed against 20 mM HEPES buffer, pH 8.0, containing 1.5 M $(NH_4)_2SO_4$. The dialysate was then loaded onto a HiLoad 26/10 phenyl Sepharose column, equilibrated with 20 mM HEPES buffer, pH 8.0, containing 1 M $(NH_4)_2SO_4$. Fractions containing phosphodiesterase activity were concentrated to approximately 4 mL and loaded onto a HiPrep 16/10 Sephacryl S-200 gel filtration column, equilibrated with 20 mM HEPES, pH 8.0, containing 0.15 M NaCl. GpdQ concentration was measured at 280 nm using $\varepsilon_{280} = 39{,}880\ M^{-1}\ cm^{-1}$ per monomeric unit.

The Rv0805 gene, cloned into the vector pET47B, was purchased from GeneArt. The construct was transferred into competent *E. coli* BL21(DE3) cells. The transformed cell culture was incubated at 37 °C until an optical density of approximately 0.5 was reached. Subsequently, induction of recombinant protein expression was achieved by adding (isopropyl β-D-1-thiogalactopyranoside, 1 mM final concentration) and continuing incubation at 30 °C under constant shaking (200 rpm) for another 24 h. The cells were harvested by centrifugation, resuspended in lysate buffer containing 50 mM TrisHCl, pH 8.5, with 5 mM mercaptoethanol, 150 mM NaCl, and EDTA-free protease inhibitor. The cells were lysed using a French press (1,000 psi). Since the Rv0805 construct contains an N-terminal hexahistidine tag, the soluble fraction was applied to a Ni(II) affinity column (Ni(II)-IMAC resin), equilibrated with 50 mM TrisHCl, pH 8.5, with 5 mM mercaptoethanol, 150 mM NaCl, and 20 mM imidazole. The protein was eluted with an isocratic gradient using 50 mM TrisHCl, pH 8.5, with 5 mM mercaptoethanol, 150 mM NaCl, 200 mM imidazole, and 10 % glycerol. Rv0805 concentration was measured at 280 nm using $\varepsilon_{280} = 19{,}940\ M^{-1}\ cm^{-1}$ per monomeric unit.

Apoenzyme preparation and enzyme reconstitution

Most binuclear metallohydrolases display some promiscuity with respect to the metal ions they may bind [2, 3, 17, 25–27]; hence, the metal ion composition of a purified enzyme is often affected by the availability of metal ions in the purification buffers. Since differences in metal ion compositions may affect both the reactivity of a metalloenzyme and its interaction with substrates (see, e.g., [8, 11, 12, 23]), care was taken to prepare samples with well-defined metal content.

Apoenzymes were obtained by incubating approximately 3 mg of protein (i.e., OpdA, GpdQ, or Rv0805) in a 3-mL solution containing 5 mM EDTA, 5 mM 1,10-phenanthroline, 5 mM 2,6-pyridine dicarboxylic acid, 5 mM 8-hydroxyquinone-5-sulfonic acid, 5 mM 2-mercaptoethanol in 20 mM HEPES buffer, pH 7.0, at 4 °C. After 48 h for Rv0805 and 24 h for OpdA and GpdQ, the protein sample was separated from the chelating solution using an Econo-Pac 10DG gel filtration column equilibrated with 50 mM TrisHCl, pH 8.5, containing 100 mM NaCl. The absence of metal ions in the protein solutions was confirmed by atomic absorption spectroscopy.

Subsequently, enzyme activities were reconstituted by the addition of the desired metal ion and incubation for at least 24 h at 4 °C. GpdQ and OpdA activities were reconstituted with $CoSO_4$, and Rv0805 activity was reconstituted using $MnCl_2$.

ITC data collection

ITC data were collected with an iTC200 system from MicroCal. All data analysis was performed using the program Origin. All measurements were conducted at 25 °C. Catalysis was recorded by measuring the change in the thermal power triggered by the enzymatic hydrolysis of the

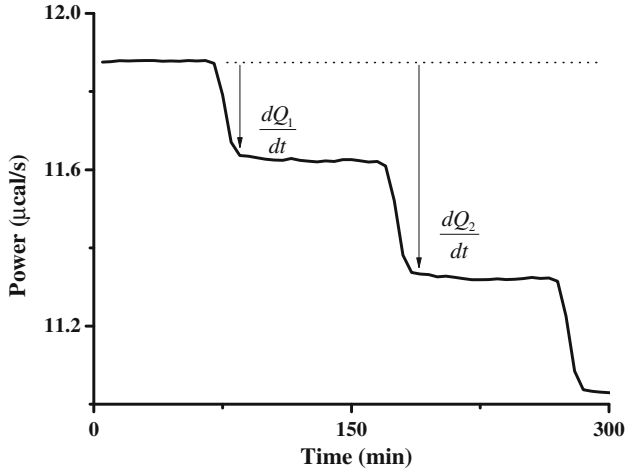

Fig. 2 Illustrative multiple-injection experiment. In subsequent injections, substrate was added to the enzyme; each injection followed after thermal equilibrium from the previous injection had been reached

substrate after injection. The samples were buffered using 50 mM TrisHCl, pH 8.5, containing 100 mM NaCl, to keep the ionic strength constant throughout the experiment.

ITC data analysis

Conversion of substrate to product by enzymes generates heat corresponding to the enthalpic component (ΔH) of the reaction. Therefore, the rate of an enzymatic reaction is directly proportional to the rate of heat generation and can be obtained as described previously [28] and summarized in the following paragraph.

The rate of heat generation of an enzymatic reaction is defined as the thermal power, expressed by Eq. 1:

$$\text{Power} = \frac{\mathrm{d}Q}{\mathrm{d}t}. \tag{1}$$

Practically, the thermal power of a reaction is given by subtracting the baseline (no reaction) from the calorimetric trace associated with substrate consumption (Fig. 2).

The amount of heat involved in the conversion of n moles of substrate to product is given by Eq. 2:

$$Q = n \cdot \Delta H_{\text{app}} = [\text{P}]_{\text{total}} \cdot V \cdot \Delta H_{\text{app}}, \tag{2}$$

where V is the volume of the reaction cell, [P] is the concentration of the product generated by the enzymatic reaction, and ΔH_{app} is the molar enthalpy for the reaction, which needs to be determined experimentally (see below).

Since the reaction rate is defined as the concentration change of the product over time $d[\text{P}]dt$, it can be obtained by the derivative of Eq. 2, resulting in Eq. 3:

$$\text{Rate} = \frac{\mathrm{d}[\text{P}]}{\mathrm{d}t} = \frac{1}{V \cdot \Delta H_{\text{app}}} \cdot \frac{\mathrm{d}Q_i}{\mathrm{d}t}. \tag{3}$$

Here, i is the ith value of $\mathrm{d}Q/\mathrm{d}t$ obtained after each injection.

The experimental determination of ΔH_{app} is achieved by allowing a known amount of substrate to react to completion in a single-injection experiment (Fig. 3; a second injection was routinely performed to verify reproducibility of the data and exclude product inhibition). ΔH_{app} arises from the integration of the power over time obtained by the total digestion of the substrate as described by Eq. 4:

$$\Delta H_{\text{app}} = \frac{1}{V \cdot [\text{S}]_{\text{total}}} \int_{t=0}^{t=\infty} \frac{\mathrm{d}Q(t)}{\mathrm{d}t}\mathrm{d}t, \tag{4}$$

where $[\text{S}]_{\text{total}}$ is the initial concentration of substrate in the reaction cell.

The reaction rates can be measured with two different methods, i.e., single-injection or multiple-injection experiments. The first method consists of a continuous rate measurement after a single injection of the substrate in the cell containing enzyme or vice versa. When the substrate has been completely digested, the thermal power returns to the preinjection baseline. ΔH_{app} is determined by the integration of the area under the peak (Eq. 4). From the analyses of the decay curve resulting from the substrate decomposition, catalytic parameters such as k_{cat} and K_{m} can be obtained on the basis of the calculation of the remaining substrate concentration at any time in the reaction course as shown in Eq. 5:

$$S_t = S_{t=0} - \frac{\int_{t=0}^{t} \frac{\mathrm{d}Q(t)}{\mathrm{d}t}\mathrm{d}t}{\Delta H_{\text{app}} \cdot V}. \tag{5}$$

The rate can then be plotted as a function of the substrate concentration, generating a continuous kinetic curve.

The second method consists of a known concentration of enzyme solution placed in the cell, and a number of consecutive injections with substrate are performed. In this method the thermal power trace does not return to the origin (like in the single-injection model), but instead reaches a constant value corresponding to the saturation of the enzyme by the substrate. Integration of the thermal power over time can be used to construct a plot of the reaction rate versus the total substrate concentration (as shown in Fig. 2) [28]. This method assumes that no appreciable substrate degradation occurs during the measurements.

Rate versus substrate concentration profiles were accordingly fitted to the Michaelis–Menten equation (Eq. 6) [29]:

$$v = \frac{V_{\text{max}} \cdot S}{K_{\text{m}} + S}, \tag{6}$$

where V_{max} represents the maximum velocity, K_{m} is the Michaelis–Menten constant, S is the substrate concentration, and v is the initial velocity.

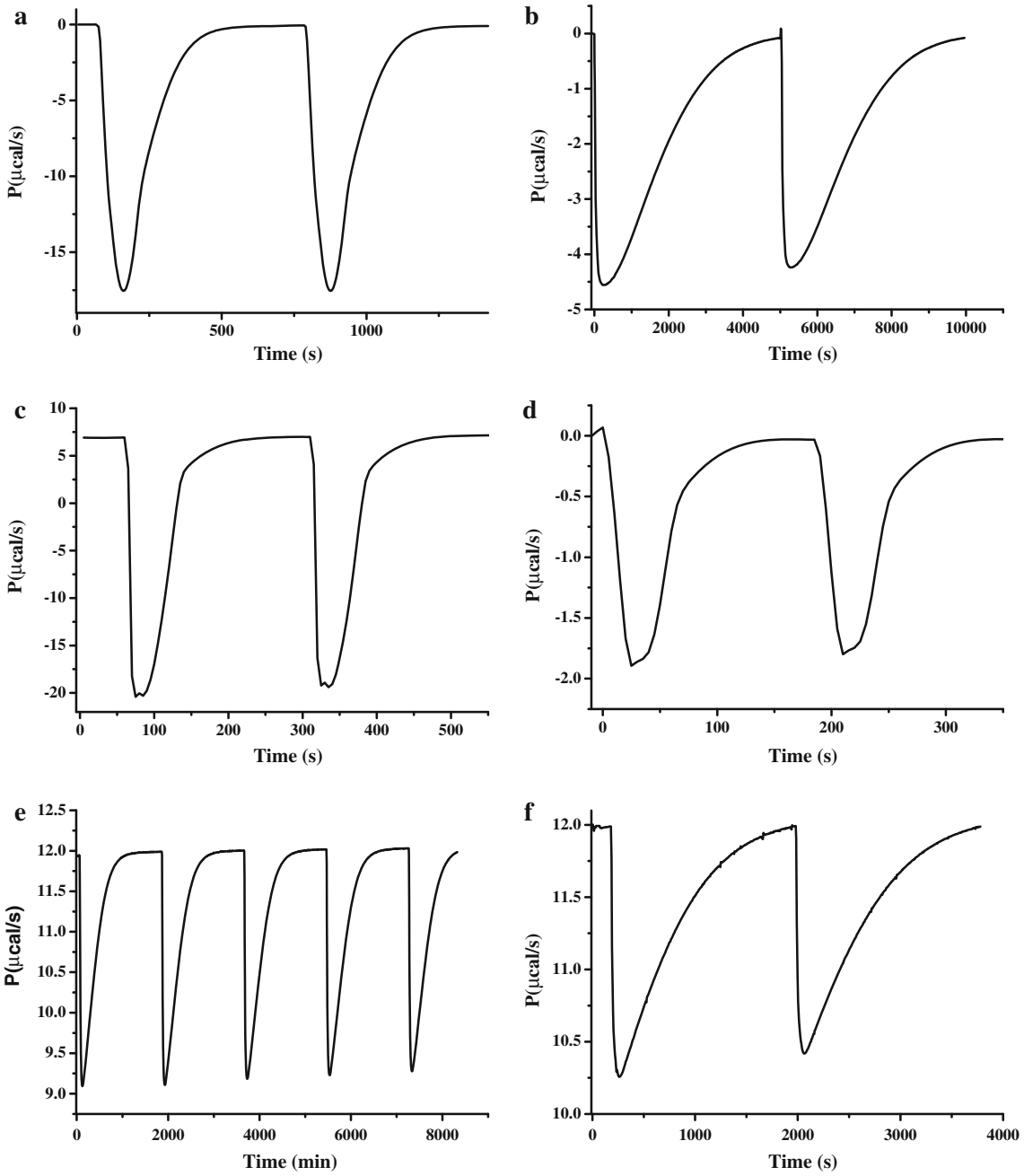

Fig. 3 Single-injection experiment for the following enzyme/substrate pairs: **a** OpdA and paraoxon, **b** OpdA and demeton-S, **c** GpdQ and bpNPP, **d** GpdQ and TMP, **e** Rv0805 and bpNPP, and **f** Rv0805 and cAMP. The reaction proceeds until all substrate has been converted to product, i.e., until the power returned to the baseline. The integration of this curve gives rise to the value of ΔH_{app} (see the text for details). A second injection of substrate was also performed, illustrating that product inhibition plays no significant role in these reactions

Results and discussion

In establishing a method for a task such as measuring catalytic parameters, representative systems need to be carefully selected. For the purpose of this study, our selection was guided by (1) the availability of comparative experimental data obtained from alternative methods (i.e., spectrophotometric assays) and (2) the necessity to have an assay available for reactants that are not amenable to simple kinetic measurements (i.e., substrates that lack chromophoric markers). For the enzyme OpdA, the substrate commonly used for functional studies is the organophosphate pesticide paraoxon (Fig. 1); on hydrolysis the product *para*-nitrophenolate is released, which has a

Table 1 Comparison of catalytic parameters obtained by isothermal titration calorimetry (*ITC*) and continuous or discontinuous spectroscopic assays

Enzyme	Substrate	ΔH_{app} (kcal mol^{-1})	ITC		Spectroscopic assays	
			k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
OpdA						
	Paraoxon	−4.0	1,541 (3)	149 (2)	1,426[a]	103
	Demeton-S	−7.9	0.7 (0.08)	227 (23)	0.8 (0.2)[b]	114 (36)
GpdQ						
	bpNPP	−16.6	1.7 (0.7)	155 (10)	1.7[c]	120
	TMP	−0.3	2.5 (0.5)	335 (21)	~0.3[d]	~1,000
Rv0805						
	bpNPP	−18.3	1.6 (0.3)	350 (1.7)	1.7 (0.07)[b]	221 (2)
	3′,5′-cAMP	−10.0	10.8 (1.1)	39 (3)	8.5 (2.1)[b]	67 (6)

bpNPP bis(*para*-nitrophenol) phosphate, *3′,5′-cAMP* 3′,5′-cyclic adenosine monophosphate, *TMP* trimethyl phosphate

[a] From Ely et al. [9]

[b] This study

[c] From Hadler et al. [14]

[d] From Gerlt and Whitman [13]

characteristic pH-independent isosbestic wavelength at 347 nm ($\varepsilon = 5{,}176$ M^{-1} cm^{-1}) [30]. However, since OpdA is a very efficient and commercially available bioremediator (http://www.orica-landguard.com), its catalytic efficiency may be geared towards the hydrolysis of specific pesticides or nerve agents such as demeton-S or VX, respectively, using in vitro evolution techniques [8, 22, 31]. These substrates lack a chromophoric marker that would facilitate a simple and continuous spectrophotometric assay.

To demonstrate the aptitude of ITC as a technique to measure catalytic parameters, paraoxon and demeton-S were used as OpdA substrates. The published catalytic parameters determined for paraoxon with the spectrophotometric assay are listed in Table 1. Because of solubility problems, paraoxon was placed into the cell with a final concentration of 3 mM and in a single injection OpdA was added. The final enzyme concentration was 1.64 nM. The reaction was monitored for approximately 40 min (2,500 s) until all substrate had been converted to product (Fig. 4). The complete conversion (Fig. 4a) is necessary in order to obtain the experimental value of ΔH_{app} (according to Eq. 2). As controls to account for nonenzymatic drifts in the baseline and thermal dilution effects, respectively, the same experiment was repeated twice, once in the absence of substrate and once without enzyme (see Figs. S1–S5, for the latter controls; no measurable dilution effects were recorded for the former control). The heat associated with these two controls was subtracted from the overall reaction heat. The resulting ΔH_{app} was thus estimated to be −4.007 kcal mol^{-1}. With use of Eq. 3, the experimental data can be converted to catalytic rates (Fig. 4b). The plot of these rates versus paraoxon concentration (calculated using Eq. 5) displays Michaelis–Menten-type saturation behavior and can be fit using Eq. 6, resulting in k_{cat} and K_m values of approximately 1,500 s^{-1} and 150 μM, respectively (Table 1). The data obtained with the spectroscopic assays are thus in good agreement with the values obtained here by ITC.

The above results indicate that ITC is a convenient technique to assess kinetic parameters for OpdA-catalyzed reactions. We thus expanded our study to include more "difficult" substrates, i.e., substrates that lack a chromophoric marker necessary for a simple spectrophotometric detection of product formation and/or substrate consumption. In the organophosphate pesticide demeton-S, as well as in the nerve agent VX, hydrolysis occurs at a P–S bond (Fig. 1). The resulting free thiol is not directly spectrophotometrically detectable, and an assay would require a coupled reaction, whereby, for instance, the thiol moiety is reacted with 5,5‘-dithiobis(2-nitrobenzoic acid) [7]. With use of ITC, the activity for demeton-S can be easily and rapidly determined in the same manner as described above for paraoxon. Since demeton-S is a rather slow substrate, the OpdA concentration in the cell was increased to a final concentration of 260 μM and the multiple-injection method was used (in an initial single-injection experiment ΔH_{app} was estimated with a 2 μM OpdA solution and $[S]_{final} = 3.8$ mM). In total, 38 consecutive injections of 1 μL of a 5 mM demeton-S solution were performed (Fig. 5) to determine the catalytic parameters. The resulting values for k_{cat} and K_m are approximately 0.7 s^{-1} and 227 μM, respectively (Table 1). For comparison, the catalytic rates obtained by ITC and the coupled assay are

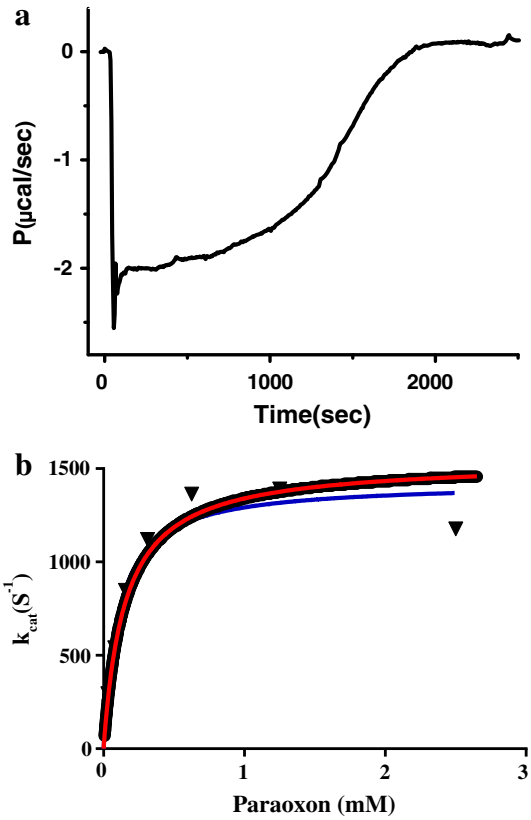

Fig. 4 Hydrolysis of paraoxon by OpdA. **a** Experimental data obtained from a single-injection experiment ($[E]_{tot} = 1.64$ nM ($V = 200$ µL), $[S]_{tot} = 3$ mM). **b** The dependences of the catalytic rates on the substrate concentration measured by isothermal titration calorimetry (ITC) (*circles*) and a continuous spectroscopic assay (*triangles*) [9] were compared. The data sets were fit using Eq. 6

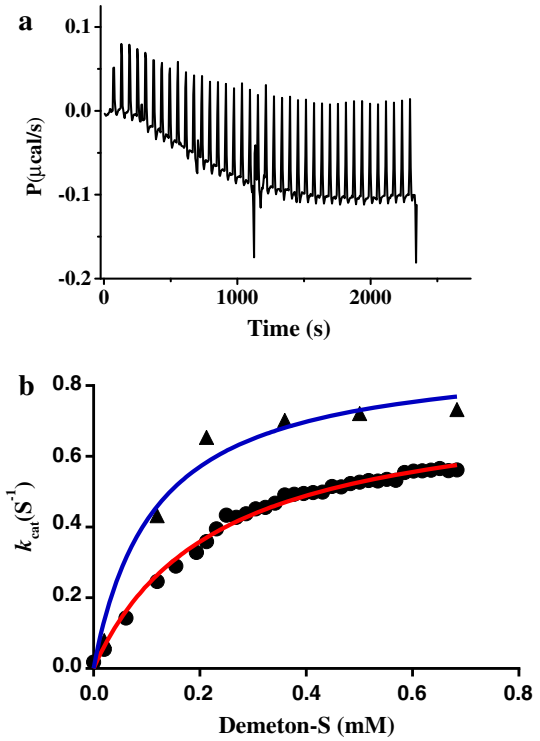

Fig. 5 Hydrolysis of demeton-S by OpdA. **a** Experimental data obtained from a multiple-injection experiment ($[E]_{final} = 260$ µM; $[S]_{stock} = 5$ mM; injection volume 1 µL; 38 injections). **b** The dependences of the catalytic rates on the substrate concentration measured by ITC (*circles*) and a discontinuous spectroscopic assay (*triangles*) were compared. The data sets were fit using Eq. 6

shown in Fig. 5b. Although the agreement between the two data sets is not as good as that observed for the reaction with paraoxon (Fig. 4b), the discrepancy is acceptable (approximately 10 % for the rates, 50 % for the K_m values; Table 1). The estimated standard deviations reported for the ITC-based assays were generally smaller than those obtained from the alternative assays. In the case of the measurements with demeton-S, the two methods thus provide parameters that are, within errors, comparable.

As mentioned above, OpdA is a member of a large family of phosphorolytic binuclear enzymes. The enzyme GpdQ is another promising bioremediator, but it is also one of the most promiscuous phosphatases known since it has been shown to act on monoester, diester, and triester substrates [7, 14, 15]. Among its many substrates, GpdQ is able to degrade (1) TMP and methylphosphonate, which are extremely stable aliphatic phosphoesters [13, 32], and (2) the molecule EA 2192, the toxic by-product of VX hydrolysis [33]. Here, we measured GpdQ activity by ITC using the substrates bpNPP and TMP. The former is the standard substrate used for functional studies; like paraoxon, its hydrolysis can be easily monitored spectrophotometrically because of the *para*-nitrophenolate leaving group. TMP is a slow substrate that is not amenable to a simple continuous spectrophotometric assay. GpdQ is not prone to product inhibition (two consecutive single injections with each of the two substrates were performed and both isotherms had virtually the same shape; Fig. 3c, d). Using a single injection, we determined ΔH_{app} as described above ($[S]_{final} = 4.3$ and 3.6 mM for bpNPP and TMP, respectively). Catalytic parameters for the hydrolysis of the two substrates were determined by the multiple-injection method (Figs. 6, 7). For the reaction with bpNPP, 30 injections of substrate (10 mM stock concentration) were added to GpdQ (2.7 µM at the beginning of the experiment; Fig. 6). The values obtained for k_{cat} and K_m, approximately 1.7 s^{-1} and approximately 120 µM, respectively (Table 1), are in excellent agreement with the values obtained by measuring the release of the *para*-nitrophenolate product [14]. A similar approach (50 injections) was used to determine the kinetic parameters for the reaction of GpdQ with TMP (Fig. 7). The values for k_{cat} and K_m are approximately 2.5 s^{-1} and approximately 335 µM, respectively. Although the agreement with previously reported values (using a discontinuous assay) is not

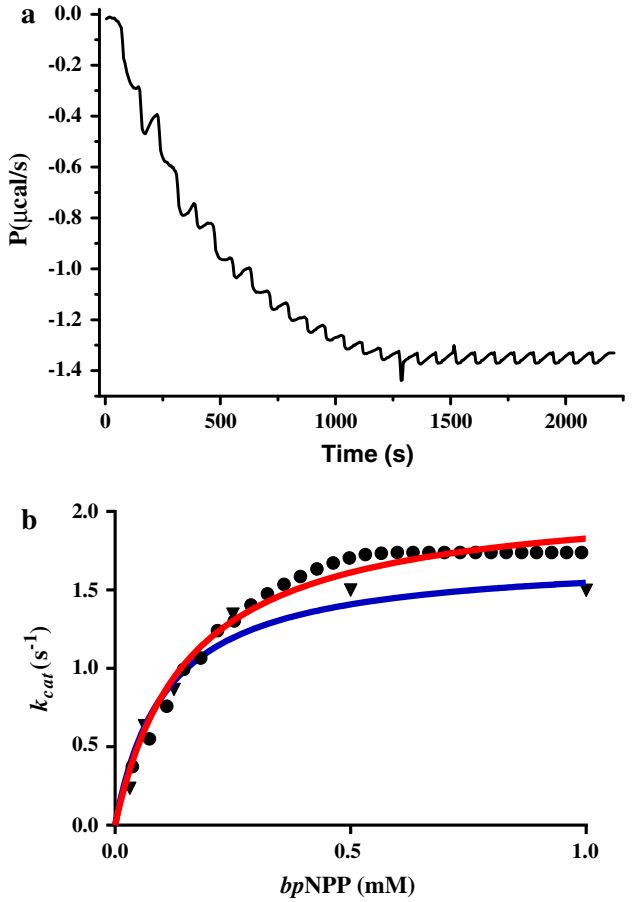

Fig. 6 Hydrolysis of bpNPP by GpdQ. **a** Experimental data obtained from a multiple-injection experiment ($[E]_{final}$ = 2.7 µM; $[S]_{stock}$ = 10 mM; 30 injections of 1 µL). **b** The dependences of the catalytic rates on the substrate concentration measured by ITC (*circles*) and a continuous spectroscopic assay (*triangles*) [14] were compared. The data sets were fit using Eq. 6

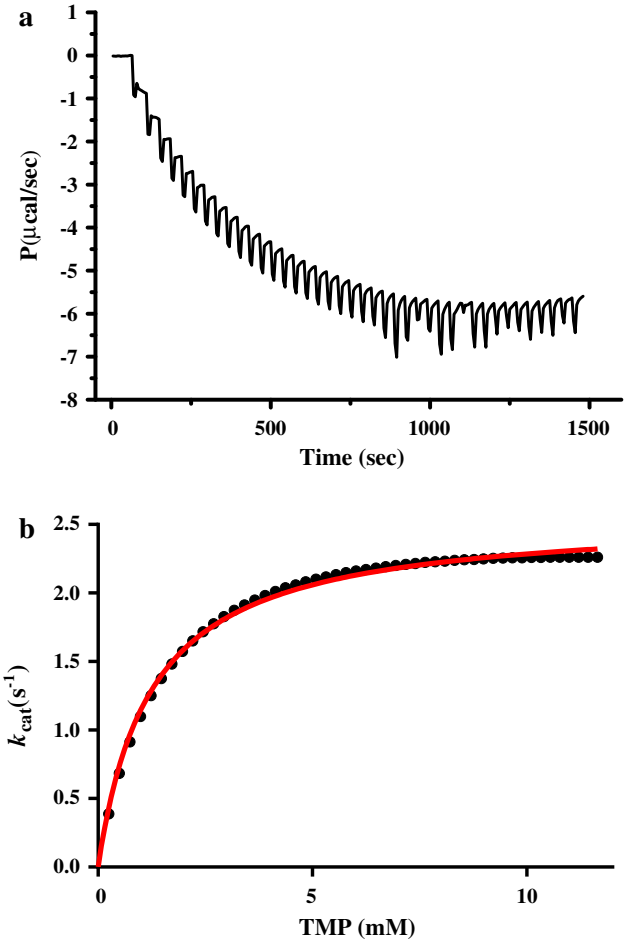

Fig. 7 Hydrolysis of TMP by GpdQ. **a** Experimental data obtained from a multiple-injection experiment ($[E]_{final}$ = 100 µM; $[S]_{stock}$ = 100 mM; 50 injections of 0.5 µL). **b** The dependence of the catalytic rates on the substrate concentration was determined by ITC and the data set was fit using Eq. (6). Only estimates of the turnover number and the Michaelis constant were previously reported [13]

as good as the that observed with the substrate bpNPP (Table 1), the slowness of the reaction and the likely heterogeneity in the metal ion composition in the previous study [13] may be major contributors to the observed discrepancy.

Rv0805 was included in this study because it is the closest known sequence analogue of GpdQ, sharing about 20 % sequence identity [34], but has a largely different substrate specificity. Rv0805 is involved in bacterial signal transduction by regulating the intracellular concentration of cyclic nucleotides [17–19]. The biologically relevant substrates for Rv0805 such as cAMP and cyclic diguanylate are again not amenable to a simple continuous spectrophotometric assay (a discontinuous assay to measure the rate of phosphate formation is used instead) [17, 19].

As for GpdQ, the ITC method was initially tested using bpNPP as a substrate (Figs. 3e, 8). Catalytic parameters were determined with the multiple-injection approach (45 injections, 4.0 µM Rv0805 at the beginning of the experiment; Fig. 8). The catalytic parameters obtained are again in excellent agreement with those measured with a continuous spectrophotometric assay (Table 1).

The same approach was used to monitor the reaction of Rv0805 with cAMP (Figs. 3f, 9; 16 injections, 1.0 µM Rv0805 at the beginning of the experiment). The estimated catalytic parameters are listed in Table 1. To compare these values with those obtained from an alternative method, we applied a discontinuous spectroscopic method as described by Carter and Karl [35]. In brief, cAMP (ranging from 10 µM to 1 mM) was mixed with enzyme (0.21 µM final concentration). The reaction (V_{tot} = 30 mL) was allowed to run for 25 min. Every 60 s, 1-mL aliquots were removed and mixed with 100 µL of 20 mM EDTA to quench the reaction. The mixture was then diluted to ensure that the concentration of the reaction product, assuming complete turnover, did not exceed 80 µM.

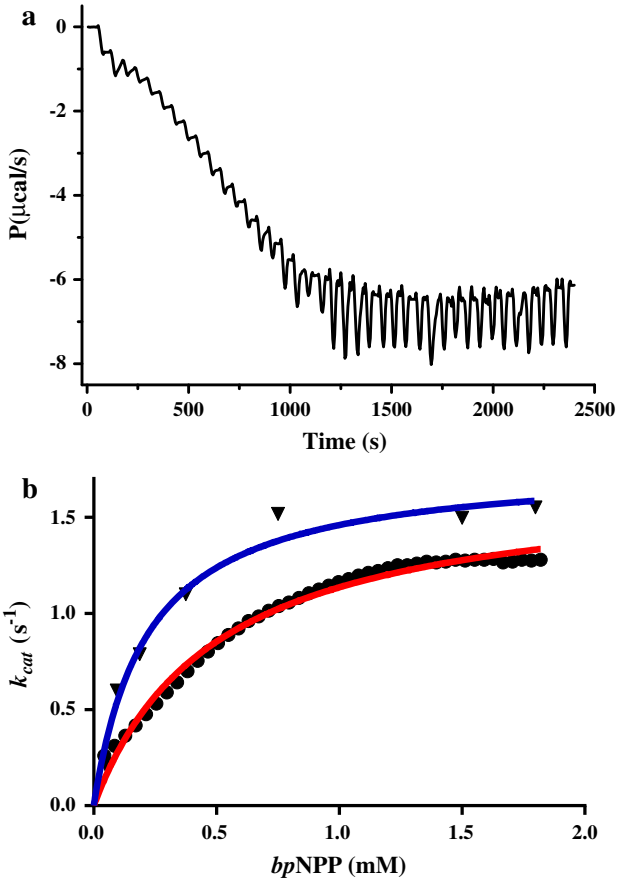

Fig. 8 Hydrolysis of bpNPP by Rv0805. **a** Experimental data obtained from a multiple-injection experiment ($[E]_{final} = 20$ μM; $[S]_{stock} = 14$ mM; 45 injections of 0.7 μL). **b** The dependences of the catalytic rates on the substrate concentration measured by ITC (*circles*) and a continuous spectroscopic assay (*triangles*) were compared. The data sets were fit using Eq. 6

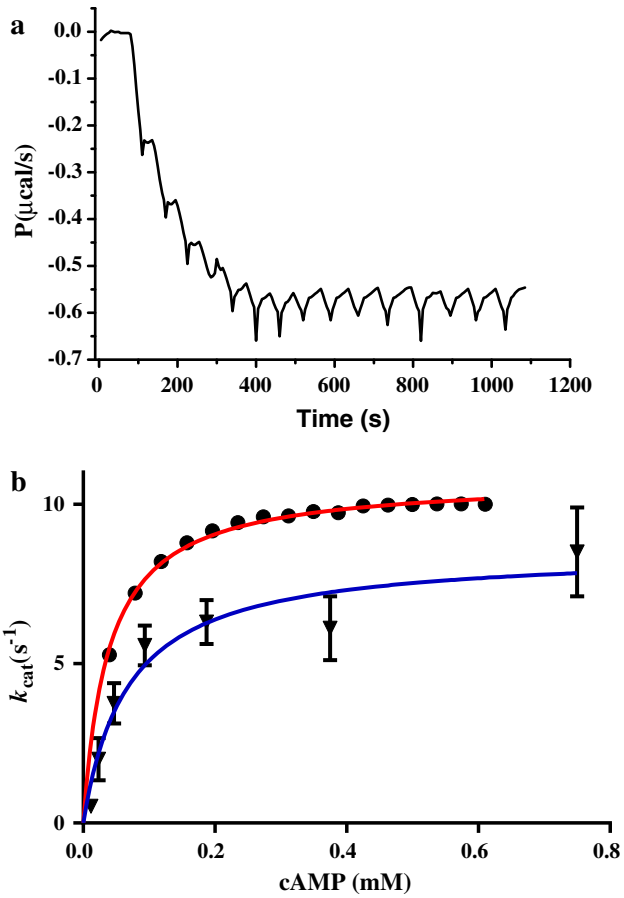

Fig. 9 Hydrolysis of cAMP by Rv0805. **a** Experimental data obtained from a multiple-injection experiment ($[E]_{final} = 1$ μM; $[S]_{stock} = 10$ mM; 16 injections of 1 μL). **b** The dependences of the catalytic rates on the substrate concentration measured by ITC (*circles*) and a discontinuous spectroscopic assay (*triangles*) were compared. The data sets were fit using Eq. 6

Subsequently, malachite green was added to the aliquot and after 15 min the absorption was measured at 620 nm. The catalytic parameters obtained (Table 1) are in good overall agreement with those from the ITC experiment, but the latter was significantly more rapid.

In summary, ITC has been shown to be a simple, efficient, and reliable method to measure enzymatic activities for a representative group of binuclear metallohydrolases. The catalytic parameters obtained are largely in good agreement with comparable values obtained from spectrophotometric assays. ITC is not envisioned to replace conventional spectrophotometric assays for functional studies that can use substrates with convenient chromophoric markers. Instead, ITC may provide a conduit to assess the interactions between enzymes and their natural substrates, in particular if the latter are not amenable to rapid, simple, and continuous spectrophotometric assays. Binuclear metallohydrolases are a large family of enzymes with a growing number of members. Their precise biological role(s) is(are) often poorly understood, obscured by a lack of knowledge about the identity of their preferred substrates. Furthermore, the range of substrates may frequently be very broad ("promiscuous" use of substrates). A uniform approach to assess/compare a variety of substrates is thus desirable to probe both the reaction mechanism of hydrolysis and the biological function(s) of these enzymes. Here, ITC was demonstrated to be very powerful, reliable, and rapid to assess the catalytic properties of several binuclear metallohydrolases towards various substrates. It is anticipated that this approach can be widely applied by bioinorganic chemists/enzymologists, thus contributing significantly to our understanding of the function and mechanism of such systems, but it may also be useful in the development of (1) variants of such enzymes with altered catalytic properties (e.g., different substrate preferences) or (2) specific inhibitors as future drug leads.

Acknowledgments This work was financially supported by the Australian Research Council, Discovery Projects Scheme (DP120104263). G.S. also acknowledges the receipt of an ARC Future Fellowship (FT120100694). M.P. is supported by an International Postgraduate Research Scholarship and University of Queensland International Living Allowance Scholarship.

References

1. Wilcox DE (1996) Chem Rev 96:2435–2458
2. Mitić N, Smith SJ, Neves A, Guddat LW, Gahan LR, Schenk G (2006) Chem Rev 106:3338–3363
3. Schenk G, Mitić N, Gahan LR, Ollis DL, McGeary RP, Guddat LW (2012) Acc Chem Res 45:1593–1603
4. Raushel FM (2002) Curr Opinion Microbiol 5:288–295
5. Ely F, Foo J-L, Jackson CJ, Gahan LR, Ollis DL, Schenk G (2007) Curr Top Biochem Res 9:63–78
6. Bigley AN, Raushel FM (2013) Biochim Biophys Acta 1834: 443–453
7. Yang H, Carr PD, McLoughlin SY, Liu JW, Horne I, Qiu X, Jeffries CMJ, Russell RJ, Oakeshott JG, Ollis DL (2003) Protein Eng 16:135–145
8. Jackson CJ, Foo J-L, Tokuriki N, Afriat L, Carr PD, Kim H-K, Schenk G, Tawfik DS, Ollis DL (2009) Proc Natl Acad Sci USA 106:21631–21636
9. Ely F, Hadler KS, Gahan LR, Guddat LW, Ollis DL, Schenk G (2010) Biochem J 432:565–573
10. Ely F, Hadler KS, Mitić N, Gahan LR, Ollis DL, Plugis NM, Russo MT, Larrabee JA, Schenk G (2011) J Biol Inorg Chem 16:777–787
11. Ely F, Pedroso MM, Gahan LR, Ollis DL, Guddat LW, Schenk G (2012) J Inorg Biochem 106:19–22
12. Daumann LJ, McCarthy BY, Hadler KS, Murray TP, Gahan LR, Larrabee JA, Ollis DL, Schenk G (2013) Biochim Biophys Acta 1834:425–432
13. Gerlt JA, Whitman GJR (1975) J Biol Chem 250:5053–5058
14. Hadler KS, Tanifum EA, Yip SH-C, Mitić N, Guddat LW, Jackson CJ, Gahan LR, Nguyen K, Carr PD, Ollis DL, Hengge AC, Larrabee JA, Schenk G (2008) J Am Chem Soc 130: 14129–14138
15. Hadler KS, Mitić N, Ely F, Hanson GR, Gahan LR, Larrabee JA, Ollis DL, Schenk G (2009) J Am Chem Soc 131:11900–11908
16. Hadler KS, Mitić N, Yip SH-C, Gahan LR, Ollis DL, Schenk G, Larrabee JA (2010) Inorg Chem 49:2727–2734
17. Shenoy AR, Capuder M, Draskovic P, Lamba D, Visweswariah SS, Podobnik M (2007) J Mol Biol 365:211–225
18. Shenoy AR, Sreenath N, Podobnik M, Kovacevic M, Visweswariah SS (2005) Biochemistry 44:15695–15704
19. Keppetipola N, Shuman S (2008) J Biol Chem 283:30942–30949
20. Sutherland TD, Horne I, Weir KM, Coppin CW, Williams MR, Selleck M, Russell RJ, Oakeshott JG (2004) Clin Exp Pharmacol Physiol 31:817–821
21. Dawson RM, Pantelidis S, Rose HR, Kotsonis SE (2008) J Hazard Mater 157:308–314
22. Yip SHC, Foo J-L, Schenk G, Gahan LR, Carr PD, Ollis DL (2011) Protein Eng Des Sel 24:861–872
23. Shenoy AR, Srinivas A, Mahalingam M, Visweswariah SS (2005) Biochimie 87:557–563
24. Podobnik M, Tyagi R, Matange N, Dermol U, Gupta AK, Mattoo R, Seshadri K, Visweswariah SS (2009) J Biol Chem 284:32846–32857
25. Jackson C, Carr PD, Kim HK, Liu J-W, Herrald P, Mitic N, Schenk G, Smith CA, Ollis DL (2006) Biochem J 397:501–508
26. Mitić N, Noble CJ, Gahan LR, Hanson GR, Schenk G (2009) J Am Chem Soc 131:8173–8179
27. Mitić N, Hadler KS, Gahan LR, Hengge AC, Schenk G (2010) J Am Chem Soc 132:7049–7054
28. Todd MJ, Gomez J (2001) Anal Biochem 296:179–187
29. Segel IH (1993) Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley, New York
30. Aubert SD, Li Y, Raushel FM (2004) Biochemistry 43: 5707–5715
31. Liu J-W, Hadler KS, Schenk G, Ollis D (2007) FEBS J 274:4742–4751
32. Gerlt JA, Westheimer FH (1973) J Am Chem Soc 95:8166–8168
33. Ghanem E, Li Y, Xu C, Raushel FM (2007) Biochemistry 46:9032–9040
34. Hadler KS, Huber T, Cassady AI, Weber J, Robinson J, Burrows A, Kelly G, Guddat LW, Hume DA, Schenk G, Flanagan JU (2008) BMC Res Notes 1:78
35. Carter SG, Karl DW (1982) J Biochem Biophys Methods 7:7–13