J Biol Inorg Chem (2009) 14:111–121
DOI 10.1007/s00775-008-0428-x

ORIGINAL PAPER

Effect of metal binding and posttranslational lysine carboxylation on the activity of recombinant hydantoinase

Cheng-Yang Huang · Ching-Chen Hsu · Mei-Chun Chen · Yuh-Shyong Yang

Received: 15 March 2008 / Accepted: 27 August 2008 / Published online: 10 September 2008

Abstract Bacterial hydantoinase possesses a binuclear metal center in which two metal ions are bridged by a posttranslationally carboxylated lysine. How the carboxylated lysine and metal binding affect the activity of hydantoinase was investigated. A significant amount of iron was always found in *Agrobacterium radiobacter* hydantoinase purified from unsupplemented cobalt-, manganese-, or zinc-amended *Escherichia coli* cell cultures. A titration curve for the reactivation of apohydantoinase with cobalt indicates that the first metal was preferentially bound but did not give any enzyme activity until the second metal was also attached to the hydantoinase. The pH profiles of the metal-reconstituted hydantoinase were dependent on the specific metal ion bound to the active site, indicating a direct involvement of metal in catalysis. Mutation of the metal binding site residues, H57A, H59A, K148A, H181A, H237A, and D313A, completely abolished hydantoinase activity but preserved about half of the metal content, except for K148A, which lost both metals in its active site. However, the activity of K148A could be chemically rescued by short-chain carboxylic acids in the presence of cobalt, indicating that the carboxylated lysine was needed to coordinate the binuclear ion within the active site of hydantoinase. The mutant D313E enzyme was also active but resulted in a pH profile different from that of wild-type hydantoinase. A mechanism for hydantoinase involving metal, carboxylated K148, and D313 was proposed.

Keywords Hydantoinase · Metalloenzyme · Carboxylated lysine · Site-directed mutagenesis · Structure–function relationship

Electronic supplementary material The online version of this article (doi:10.1007/s00775-008-0428-x) contains supplementary material, which is available to authorized users.

C.-Y. Huang · C.-C. Hsu · M.-C. Chen · Y.-S. Yang (✉)
Department of Biological Science and Technology,
National Chiao Tung University,
Hsinchu, Taiwan
e-mail: ysyang@faculty.nctu.edu.tw; ysyang@mail.nctu.edu.tw

Y.-S. Yang
Instrument Technology Research Center
and National Nano Device Laboratories,
National Applied Research Laboratories,
Hsinchu, Taiwan

Introduction

Hydantoinase (EC 3.5.2.2) is a metalloenzyme widely distributed in living organisms and was first described in the 1940s as occurring in plants and animals [1, 2]. In the 1950s, the enzyme isolated from calf liver was shown to catalyze the hydrolysis of dihydrouracil and dihydrothymine and was known as dihydropyrimidinase [3]. Owing to their broad substrate specificities, hydantoinase and dihydropyrimidinase were also classified as imidases [4]. Imidases from bacteria and mammals are generally known as hydantoinase and dihydropyrimidinase, respectively [5]. Hydantoinase catalyzes the reversible hydrolytic ring opening of six-membered or five-membered cyclic imides such as dihydropyrimidines and 5′-monosubstituted hydantoins to the corresponding 3-ureido acids and carbamoyl amino acids, respectively [6]. Hydantoinase also plays a role in industry in the preparation of optically active compounds [7–9]. Hydantoinase is used in combination with carbamoylase as biocatalysts for the enantiospecific enzymatic production of nonproteinogenic amino acids from racemic hydantoins [7, 8]. The products are valuable

precursors for the production of β-lactam antibiotics or synthetic peptides.

On the basis of an analysis of the amino acid sequences, hydantoinase was suggested to belong to the group of metal-dependent amidohydrolases [10]. Members of this family contain either a mononuclear metal center or a binuclear metal center [10]. For some bimetal-containing amidohydrolases, such as dihydroorotase [11], urease [12], and phosphotriesterase [13], their metal ions were found to be bridged by a carboxylated lysine ligand. The crystal structures of hydantoinase from *Burkholderia pickettii* [14], *Thermus* sp. [15], and *Bacillus stearothermophilus* [16] were resolved, and a carboxylated lysine located within the active site was also observed to bridge the two metals (Fig. 1). Recently, the crystal structure of hydantoinase from *Bacillus* sp. AR9 was determined; however, the usual posttranslational carboxylation of the active-site lysine residue was not found [17]. These observations raise an interesting question as to why the lysine needs to be carboxylated for hydantoinase and its effect on metal binding and enzyme activity. In the work reported here, we examined the metal contents and characterized the effects of metal binding on the activity of recombinant hydantoinase from *Agrobacterium radiobacter* by site-directed mutagenesis on specific amino acids involved in metal binding. The importance of the posttranslational modification of the carboxylated lysine was studied by using short-chain carboxylic acids for the chemical rescue of the mutant K148A.

Materials and methods

Materials

The water used had a resistance of more than 18 MΩ, and was purified by reverse osmosis followed by passage through a Millipore reagent water system (Millipore). Candidate substrates for hydantoinase, such as barbituric acid, allantoin, dihydroorotate, uracil, pyromellitic diimide, phthalic anhydride, 5-bromouracil, 2,3-naphthalene dicarboximide, 1,8-naphthalimide, 3,4,5,6-tetrachlorophthalimide, 4-amino-1,8-naphthalimide, 2*H*-1,3-benzoxazine-2,4(3*H*) dione, biuret, bemegride, 5,5-dimethylhydantoin, uridine, 5-ethylmethylhydantoin, parabanic acid, rhodanine, 2,4-thiazolidinedione, *N*-methylmaleimide, *N*-cyclohexylmaleimide, and oxindole, were obtained from Aldrich (USA). Diethylaminoethyl Sepharose, chelating Sephacel (fast flow), and HiTrap™ desalting columns were purchased from GE Healthcare Bio-Sciences (USA). 1,3-Bis[tris

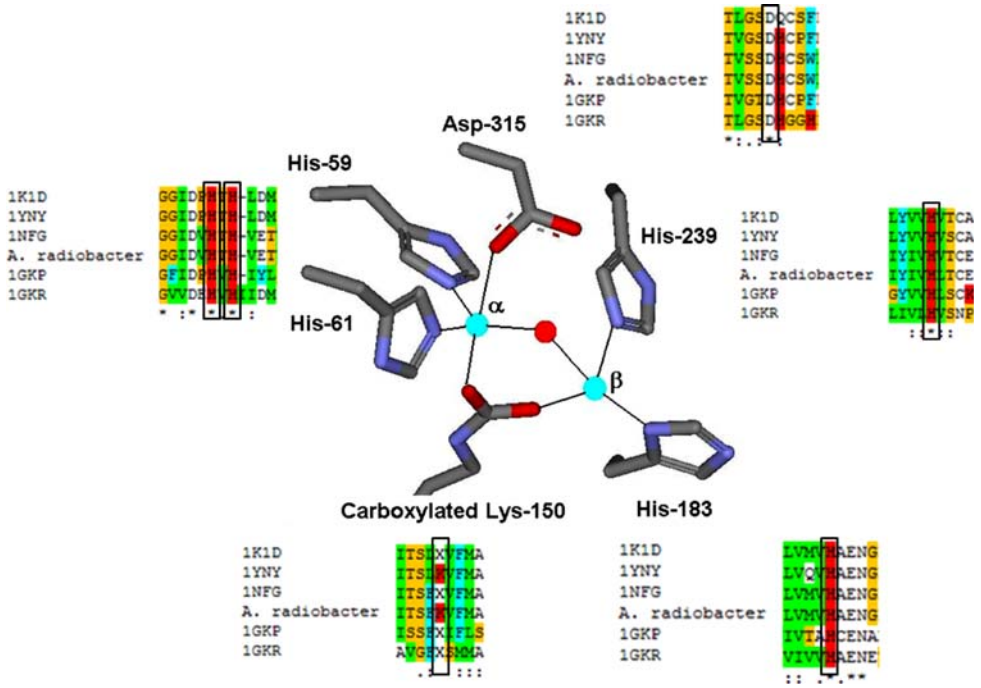


Fig. 1 Binuclear metal center within the active site of hydantoinase. The coordinates were obtained from 1GKP of the Protein Data Bank based on *Thermus* sp. at 1.3-Å resolution [15]. The amino acid sequence numbers correspond to His-57, His-59, Lys-148, His-181, His-237, and Asp-313 of those of *Agrobacterium radiobacter* hydantoinase studied in the work reported here. The aligned sequence are *Thermus* sp. (1GKP), *Arthrobacter aurescens* (1GKR), *Burkholderia pickettii* (1NFG), *Bacillus* sp. AR9 (1YNY), *Bacillus stearothermophilus* (1K1D), and *Agrobacterium radiobacter* hydantoinase by using MUSIC (http://genome.life.nctu.edu.tw/MUSIC/). The six conserved ligands were highlighted by *rectangles*

(hydroxymethyl)methylamino]propane, sodium acetate, phenylmethanesulfonyl fluoride, 8-hydroxy-5-quinolinesulfonic acid, and EDTA were purchased from Sigma (USA). Sodium chloride, sodium hydroxide, potassium phosphate, glycine, sodium dodecyl sulfate (SDS), and zinc acetate were obtained from J.T. Baker (USA). All other reagents were of the highest grades commercially available.

Enzyme assay

A rapid spectrophotometric assay [4] was used as the standard assay. Briefly, the decrease in absorbency at 298 nm was measured upon hydrolysis of phthalimide as the substrate at 25 °C. To start the reaction, the purified hydantoinase (5–100 μg) was added into a 1-mL solution, containing 1 mM phthalimide and 100 mM tris(hydroxymethyl)aminomethane (Tris)–HCl at pH 7.9. Under these conditions, a change in A_{298} of 2.26 represents the hydrolysis of 1 μmol of the substrate. The hydrolysis of the substrate was monitored with a UV/vis spectrophotometer (Hitachi U 3300). The hydrolysis of a number of candidate substrates was assayed spectrophotometrically in an entirely similar manner except for the wavelength used; necessary modifications of the assay were needed for specific compounds. The extinction coefficient of each substrate was determined experimentally by direct measurement with a spectrophotometer.

Protein concentration

The protein concentrations of enzyme solution were determined by bicinchoninic acid protein assay (Pierce, USA) using bovine serum albumin as a standard.

Expression and purification of recombinant hydantoinase

The procedure for the purification of recombinant *Agrobacterium radiobacter* hydantoinase and its mutant proteins was modified according to a previous report [18]. Total hydantoinase activities in cell cultures grown in zinc, cobalt, or manganese ions (range from 0 to 5 mM) were determined and the maximal activities were obtained at 1 mM metal ion concentration. Cell growth was inhibited by these metal ions at a concentration above 2 mM. Thus, metal ions at 1 mM concentration were amended for cell culture and for routine purification of hydantoinase.

Escherichia coli BL21 (DE3) cells were transformed with plasmid pHDT200 and grown in Luria–Bertani medium supplemented with 50 μg ampicillin per milliliter plus metal ion (1 mM zinc chloride, cobalt chloride, manganese chloride, nickel chloride, or cadmium chloride) at 37 °C with rapid shaking. When A_{600} of the cell cultures reached 0.6, isopropyl β-D-thiogalactopyranoside was added to 50 μM, the temperature of flasks was changed to 25 °C, and growth continued for an additional 25 h. Cells were chilled on ice, harvested by centrifugation, and frozen at −80 °C. After thawing, cells were resuspended in 20 mM Tris–HCl buffer (pH 7.9) and disrupted by sonication (the power was set at 3.5; each pulse lasted 2 s, with 2 s between pulses, and this continued for 5 min; the complete pulse sequence was repeated three times) with ice cooling between pulses. The disrupted cell suspensions were centrifuged at 50,000*g* for 30 min. The supernatant solutions were chromatographed with a diethylaminoethyl Sepharose HR 10/16 column (GE Healthcare Bio-Sciences). After being washed with 20 mM Tris–HCl (pH 7.9), hydantoinase was eluted with a 0–0.3 M NaCl gradient in 20 mM Tris–HCl (pH 7.9) in a total volume of 500 mL. Fractions were examined for the presence of hydantoinase protein by SDS polyacrlyamide gel electrophoresis (PAGE) analysis, and those containing enzyme of sufficient purity were pooled and directly applied to a column (1.6-cm diameter × 10 cm; GE Healthcare Bio-Sciences) of chelating Sephacel (fast flow) that was treated with 5 times gel volume containing 0.2 M zinc acetate and then equilibrated with buffer A (20 mM Tris and 0.5 M NaCl, pH 7.0). The loaded column was washed with 100 mL buffer A. The enzyme was then eluted with a linear glycine gradient from 0 to 1 M with buffer A and buffer A plus 1 M glycine in 500 mL. The fractions were examined by SDS-PAGE analysis, and those containing more than 95% pure hydantoinase were pooled, dialyzed against the dialysis buffer (20 mM Tris–HCl, pH 8.0) with two buffer changes, and concentrated to approximately 10 mg/mL. The mutant proteins could be purified to homogeneity in a manner similar to native hydantoinase. In general, about 10 mg purified hydantoinase (or mutant protein) was obtained per 0.5-L cell culture.

Preparation of apohydantoinase

The preparation of the apo form of hydantoinase was according to the published procedure [19]. Briefly, purified enzyme (10 mg/mL; 10–15 mL) was dialyzed against a chelating buffer (pH 6.5) that contained 2-morpholinoethanesulfonic acid (50 mM) and 8-hydroxy-5-quinolinesulfonic acid (15 mM) at room temperature for 2 days. The enzyme solution was dialyzed against *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer (pH 7.0, 10 mM) at 4 °C for 4 h, and then passed through a HiTrap™ desalting column and eluted with HEPES buffer (pH 7.0, 10 mM) at 4 °C. The activity of the resultant enzymes (apohydantoinase) detected by the standard assay was less than 0.4% of that of co-reconstituted enzyme and the metal content was about 0.01–0.09 mol of

zinc, manganese, cobalt and iron per mole of the enzyme monomer according to inductively coupled plasma mass spectrometry (ICP-MS) (Elan 5000, PerkinElmer, USA). The apohydantoinase was stored at 4 °C and was stable for a minimum of 1 month.

Preparation of metal-reconstituted hydantoinase

The reconstitution of apoenzymes with metal ion was according to the published procedure [19]. The apoenzyme (10 mg/mL in 4 mL) was dialyzed at 4 °C for 2 days in HEPES buffer (4 L, 10 mM at pH 7) plus one of the following metal ions (1 mM): Co^{2+}, Mn^{2+}, or Zn^{2+}. The approximate time for recovery of the maximum enzyme activity under the conditions was about 40 ± 5, 30 ± 5, and 10 ± 2 min for Co^{2+}, Mn^{2+}, and Zn^{2+}, respectively. ICP-MS was used to measure the concentration of each cation in the protein samples. Prior to the ICP-MS analysis, the protein solution was passed through a HiTrap™ desalting column and elution with HEPES buffer (pH 7.0, 10 mM) at 4 °C was used to remove excess metal ions in the protein solution. Measurements for each sample were repeated five times and the standard deviation was calculated. Normally, two or more samples were used for each determination by ICP-MS. Less than 1 mL enzyme sample (0.05–0.2 mg/mL) was used for each determination.

The pH profiles

K_m and k_{cat} of the enzymatic reaction were determined over the pH range of 5.5–9.5. The reaction was buffered with 0.1 M sodium acetate ($pK_a = 4.8$) and 0.1 M 1,3-bis [tris(hydroxymethyl)methylamino]propane ($pK_a = 6.8$ and 9.0) at the desired pH, and appropriate amounts of the enzyme (about 5–400 μg) were added to start the reaction. The pH of the buffers was determined at 25 °C. K_m and k_{cat} were obtained by nonlinear regression (Enzyme Kinetics module of SigmaPlot) using ten to 15 measurements determined at different substrate concentrations. Reaction rates obtained at each pH value were fitted to the following equation using Sigma-Plot, $(V_{max})_H = V_{max}K_a/K_a + [H^+]$, then the pK_a value was given, where $(V_{max})_H$ is the maximal degradative reaction rate at a particular pH, V_{max} is the maximal rate when all the enzyme sites are in the appropriate ionic form, and is used to represent k_{cat}, and K_a is the acid dissociation constant for a catalytic residue at the active site [20]. A plot of k_{cat}/K_m or K_m as a function of pH was also fitted to this equation in a similar manner.

Site-directed mutagenesis

Each hydantoinase mutant was generated according to the Stratagene QuickChange mutagenesis protocol (Stratagene, La Jolla, CA) using the plasmid pHDT2000 as the template [18]. Each mutant was confirmed by nucleotide sequencing. Expression and purification of the mutant proteins were the same as described for wild-type hydantoinase protein. The oligonucleotide primers for the preparation of mutants were GGCATCGACGTTGCTACGCATGTCG AG and CTCGACATGCGTAGCAACGTCGATGCC for H57A; ATCGACGGTCATACGGCTGTCGAGACGGTC and GACCGTCTCGACAGCCGTATGAACGTCGAT for H59A; AGCCATGAAGACCGCGAAGGAGGTGATGCC and GGCATCACCTCCTTCGCGGTCTTCATGGCTTA for K148A; GCGATAAGCCATGAAGACACAGAAGGA GGTG and GGCATCACCTCCTTCTGTGTCTTCATGG CTTA for K148C; GCGATAAGCCATGAAGACATCG AAGGAGGTG and GGCATCACCTCCTTCGATGTC TTCATGGCTTA for K148D; GCGATAAGCCATGAA-GACCGAGAAGGAGGTG and GGCATCACCTCCTT CTCGGTCTTCATGGCTTA for K148S; CTCGTCATG GTGGCCGCGGAGAACGGC and GCCGTTCTCCGC GGCCACCATGACGAG for H181A; GCCCCGATCTA CATCGTGGCTCTGACCTGCG and TTCTTCGCAGGT CAGAGCCACGATGTAGAT for H237A; CTCGAAACG GTCTCCTCGGCCCATTGTTCCT and GAGCCAGGAA CAATGGGCCGAGGAGACCGT for D313A; and CTCG AAACGGTCTCCTCGGAGCATTGTTCCT and GAGCC AGGAACAATGCTCCGAGGAGACCGT for D313E. The underlined sequences denote the mutated amino acids.

Results

Metal contents and activities of recombinant hydantoinase

The addition of divalent metal ions to the standard growth medium was found to substantially increase the hydantoinase activity. Metal contents and activities of hydantoinase purified from unsupplemented zinc-, cobalt-, and manganese-amended cultures are shown in Table 1. Cadmium and nickel ions were also tested as supplements but the resulting hydantoinase activity was similar to that from unsupplemented medium.

Mixtures of metals were found in purified hydantoinase as shown in Table 1. Significant amounts of iron, zinc, and manganese were observed in recombinant hydantoinase obtained from the cell culture without supplementing additional metal ions. It was also determined that the metal contents for zinc, iron, manganese, cobalt, and nickel were 12.7 ± 0.06, 6.66 ± 0.19, 0.34 ± 0.004, 0.19 ± 0.004, and 0.02 ± 0.003 μM, respectively, in unsupplemented cell culture. The total metal contents (for zinc, cobalt, manganese, and iron) in recombinant hydantoinase were near to 2 and 1.6 per enzyme subunit for purified hydantoinase

Table 1 Metal contents and specific activities of recombinant hydantoinase purified from zinc-, cobalt-, and manganese-amended and unsupplemented cultures

Metal ion amended (1 mM) in cell culture	Metal-amended[a] hydantoinase (μmol/min/mg)	Metal-reconstituted hydantoinase (μmol/min/mg)	Metal per enzyme subunit[b]			
			Zn	Co	Mn	Fe
Zn	0.15 ± 0.04	0.22 ± 0.03	1.4 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.6 ± 0.3
Co	0.85 ± 0.10	1.1 ± 0.1	0.3 ± 0.1	1.2 ± 0.3	0.1 ± 0.1	0.5 ± 0.2
Mn	0.34 ± 0.05	0.45 ± 0.08	0.2 ± 0.1	0.0 ± 0.0	1.5 ± 0.2	0.3 ± 0.2
Unsupplemented	$<10^{-4}$	–	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	1.1 ± 0.3

Specific activity was determined using phthalimide as a substrate

[a] Metal-amended hydantoinase was enzyme purified from metal-amended cell culture but that had not gone through the procedure for metal reconstitution as described in "Materials and methods"

[b] The metal contents in hydantoinase were determined by inductively coupled plasma mass spectrometry

obtained from metal-amended and unsupplemented media (Table 1), respectively. Interestingly, zinc, presumably the native metal ion in hydantoinase, was neither the major metal found in recombinant hydantoinase from unsupplemented medium nor the metal that gave the highest hydantoinase activity (Table 1). The results concerning metal contents in recombinant hydantoinase shown in Table 1 indicate that preparation of apohydantoinase and reconstitution of the apohydantoinase with metals were needed for proper understanding of the effect of metal on the catalytic properties of recombinant hydantoinase.

Reactivation of apohydantoinase

A titration curve for the reactivation of apohydantoinase with cobalt is shown in Fig. 2a. This plot shows a slow increase in hydantoinase activity at low metal ion content (less than one cobalt per enzyme subunit) followed by a sharp increase in hydantoinase activity that reached maximal activity when the ratio of metal to protein was 2. A kinetic model (Fig. 2b) for metal activation of apohydantoinase was proposed for the nonlinear increase in catalytic activity with an increasing ratio of cobalt to protein as shown in Fig. 2a. It was suggested that in this model only protein with a fully assembled binuclear metal center (M_1M_2-hydantoinase) was catalytically active and the first metal (M_1) was preferentially bound to the binuclear center. It is unlikely that metal binding of apohydantoinase occurred in a cooperative manner. In such a case, the metal would have been preferentially bound in pairs and a plot with a linear increase in catalytic activity would have been observed. Thus, the binding of the first metal ion to apohydantoinase was proposed to be tighter than that of the second metal ion.

The reconstitution of apohydantoinase with different divalent metal ions resulted in a differential recovery of catalytic activity as shown in Table 1. The metal-reconstituted hydantoinase gave significantly higher specific activities than those of enzyme purified directly from metal-amended cultures. Incubation of apohydantoinase with Cd^{2+} and Ni^{2+} did not significantly enhance hydantoinase activity. The metal content per subunit enzyme was

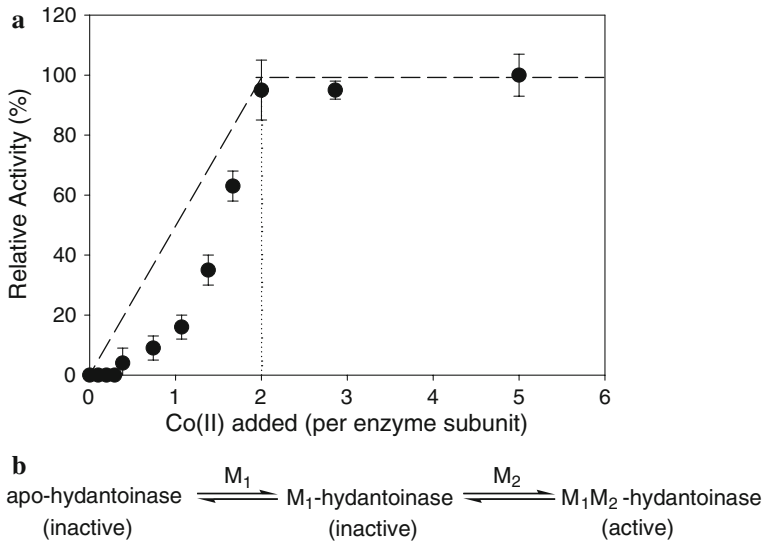


Fig. 2 Reactivation of apohydantoinase with cobalt ions. **a** Titration curve of hydantoinase activity with cobalt ion. The apohydantoinase (50 μM) was incubated with the indicated amounts of cobalt chloride in 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid at pH 7.0 (100-μL final volume) for 4 days at 4 °C. The activity of hydantoinase was then determined by standard assay. **b** Proposed kinetic model for the metal-activated hydantoinase

2.6 ± 0.1, 2.5 ± 0.1, and 2.8 ± 0.1 for cobalt-, zinc-, and manganese-reconstituted hydantoinase, respectively. These results indicate that the hydantoinase might have more than two metal binding sites; two of them are needed for maximal activity (Fig. 2a).

Effect of metals on the substrate specificity of hydantoinase

The kinetic constants for the metal-reconstituted hydantoinase for several representative substrates are shown in Table 2. Significant changes of K_m, k_{cat}, and k_{cat}/K_m were found for each substrate with different metals in hydantoinase, which indicates that the substrate specificity of hydantoinase was metal-dependent. However, the variations were all within 1 order of magnitude.

Effect of metals on the pH profiles of hydantoinase

The pH dependence of enzyme activity and catalytic efficiency (k_{cat} and k_{cat}/K_m) of the metal-reconstituted hydantoinase was determined (the pH profiles are given in the electronic supplementary material). Metal ions appeared to play a significant role in enzyme function. Both k_{cat} and k_{cat}/K_m were notably influenced by the variation in metal ions in the optimal pH range. According to the pH profiles, a general base-catalyzed reaction of hydantoinase was expected, and a group within the active site of hydantoinase must be ionized for catalytic activity. The kinetic pK_a values of the cobalt-, zinc-, and manganese-reconstituted hydantoinase derived from pH profiles were 6.3 ± 0.1, 6.0 ± 0.1, and 6.6 ± 0.1, respectively, for k_{cat} and 7.0 ± 0.1, 6.7 ± 0.1, and 6.8 ± 0.1, respectively, for k_{cat}/K_m. These results reflect the ionization of the active site functional group being dependent on the specific metal ion bound to the binuclear metal center.

Mutational analysis for the metal binding site

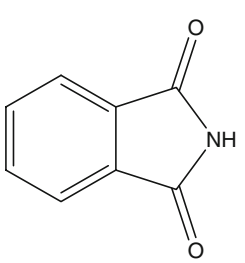
The role of conserved residues (as shown in Fig. 1) for metal binding of hydantoinase was probed by site-directed mutagenesis, and the metal content and specific activity of

Table 2 Substrate specificity of metal-reconstituted hydantoinase

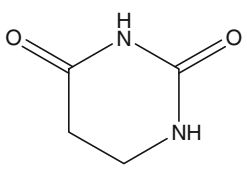
Metal ion	Substrate[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Co	Dihydrouracil	50 ± 20	14.0 ± 6.0	290 ± 130
	Phthalimide	6.0 ± 2.0	7.7 ± 2.0	1,320 ± 430
	5-Leucinylhydantoin	4.3 ± 0.2	64 ± 1	15,000 ± 700
Zn	Dihydrouracil	20 ± 3	3.7 ± 1.5	190 ± 70
	Phthalimide	5.3 ± 1.6	2.2 ± 0.4	420 ± 130
	5-Leucinylhydantoin	5.3 ± 0.5	10 ± 1	1,900 ± 190
Mn	Dihydrouracil	80 ± 20	4.2 ± 2.0	50 ± 30
	Phthalimide	8.0 ± 1.0	5.9 ± 1.4	740 ± 180
	5-Leucinylhydantoin	5.6 ± 0.4	50 ± 4	8,900 ± 700

Enzyme activity was determined according to the procedure for the standard assay except that different substrates were used. The large standard errors reflect the difficulty of obtaining enzyme activity at high substrate concentrations owing to the low solubility of the substrates. The following compounds were judged as inactive for all the metal-reconstituted hydantoinases examined: barbituric acid, dihydroorotate, uracil, pyromellitic diimide, phthalic anhydride, 5-bromouracil, 2,3-naphthalene dicarboximide, bemegride, *N*-methylphthalimide, rhodanine, 1,8-naphthalimide, *N*-methylmaleimide, 4-amino-1,8-naphthalimide, 2*H*-1,3-benzoxazine-2,4(3*H*)dione, 3,4,5,6-tetrachlorophthalimide, biuret, 5,5-dimethyl hydantoin, uridine, parabanic acid, 5-ethylmethylhydantoin, oxindole, 2,4-thiazolidinedione, *N*-cyclohexylmaleimide, and *N*-hydroxyphthalimide

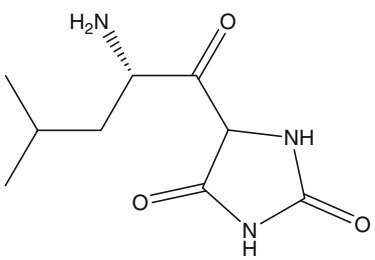
[a] Structures of the three representative substrates:



Phthalimide



Dihydrouracil



5-leucinyl-hydantoin

Table 3 The specific activity and metal content of the cobalt-reconstituted hydantoinase and mutants

Enzyme	Cobalt content per enzyme subunit	Specific activity[a] (μmol/min/mg)
Wild type	2.5 ± 0.1	1.1 ± 0.1
H57A	1.2 ± 0.1	$<10^{-4}$
H59A	1.2 ± 0.1	$<10^{-4}$
K148A	0.5 ± 0.1	$<10^{-4}$
K148C	0.8 ± 0.2	$<10^{-4}$
K148D	0.8 ± 0.2	$<10^{-4}$
K148S	0.9 ± 0.0	$<10^{-4}$
H181A	1.2 ± 0.1	$<10^{-4}$
H237A	1.2 ± 0.1	$<10^{-4}$
D313A	1.4 ± 0.1	$<10^{-4}$
D313E	2.2 ± 0.1	0.05 ± 0.01

The mutant enzymes were purified from cobalt-amended cultures; apohydantoinase was prepared and then reconstituted with cobalt. Prior to inductively coupled plasma mass spectrometry analysis, the protein solution was passed through a HiTrap™ desalting column and elution with *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid buffer (pH 7.0, 10 mM) at 4 °C was used to remove excess metal ions

[a] Assayed with phthalimide as the substrate

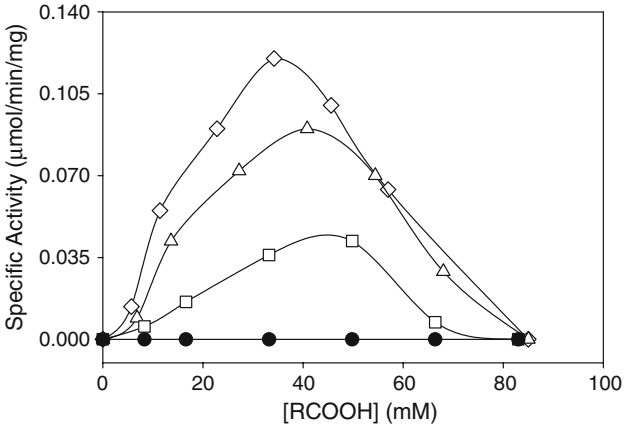


Fig. 3 Chemical rescue of K148A with short-chain carboxylic acids. Specific activity was determined on the basis of a standard assay. The reaction mixture included K148A, cobalt chloride (5 mM) and various concentrations of acetic acid (*squares*), propionic acid (*triangles*), or butyric acid (*diamonds*) and reagents needed for standard assay. The control experiment (*circles*) included various concentrations of butyric acid in the absence of metal ion. Carboxylic acids were dissolved in the assay buffer with the pH adjusted. High concentration of carboxylic acids (more than 50 mM) caused irreversible precipitation of K148A

the hydantoinase mutants are listed in Table 3. As expected, the catalytic activities for these mutant proteins were severely impaired. Only D313E was found to be active, and its specific activity was about 20-fold less than that of wild-type hydantoinase. D313E also contained about two metals per enzyme subunit (Table 3). K148 mutants (K148A, K148C, K148D, K148S), unable to possess a carboxylated side chain, were found to have 0.5–0.9 metal per enzyme subunit, less than those of other mutants. Other mutant proteins (H57, H59, H181, and H237 shown in Table 3) were found to possess 1.2–1.4 metals per subunit. The results shown in Table 3 indicate that the binuclear metal center was essential for the catalytic activity of hydantoinase. These data were in agreement with the titration curve and the proposed model presented in Fig. 2 suggesting that only the M_1M_2-hydantoinase form was active.

Chemical rescue of K148A by short-chain carboxylic acids

In the presence of a high concentrations of cobalt (5 mM), the K148 mutants, K148A, K148C, K148S, and K148D, were activated to values of 1.4×10^{-3}, 4.9×10^{-3}, 1.6×10^{-3}, and 0.7×10^{-3} μmol/min per milligram, respectively. Addition of a short-chain carboxylic acid, such as acetic acid, propionic acid, or butyric acid, to the reaction mixtures further improved the activity of K148A by nearly 2 orders of magnitude, as shown in Fig. 3. High concentrations of short-chain carboxylic acids, 45, 40, and 35 mM for acetic acid, propionic acid, and butyric acid, respectively, were needed to give the maximal activity of K148A. Taken together, the studies of mutational analysis and chemical rescue for K148 indicate that the posttranslational modification of carboxylated Lys-148 was essential for binuclear metal coordination and assembly of the active site of hydantoinase.

Dual roles of D313 in metal binding and catalysis

In contrast to D313A, D313E maintained two metals per subunit and exhibited enzyme activity (Table 3). The pH dependence of D313E (Fig. 4) was found to differ from those of wild-type hydantoinase. The kinetic pK_a values of D313E derived from Fig. 4 were 6.7 ± 0.1 and 7.4 ± 0.1 for k_{cat} and k_{cat}/K_m, respectively, which were significantly higher (0.4 and 0.7, respectively) than those of wild-type hydantoinase. These observations from site-directed mutagenesis and associated kinetic data indicated that D313 had dual roles in both metal binding and in catalysis.

Discussion

It is interesting to observe that iron appeared to be the most favorable metal ion of recombinant *Agrobacterium radiobacter* hydantoinase expressed in *Escherichia coli*. Significant amounts of iron were found in the recombinant hydantoinase even when a large excess of zinc, cobalt, or manganese ion was added in the cell culture (Table 1). However, unlike other iron enzymes, such as cytosine

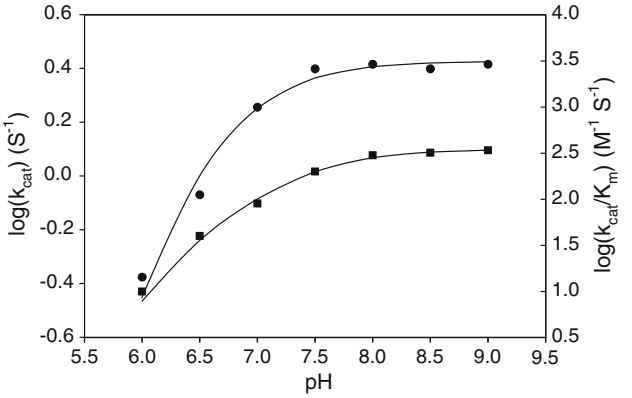


Fig. 4 The pH profiles of D313E using 5-leucinylhydantoin as the substrate. Enzyme activity was determined using 100 μg to 5 mg D313E. *Circles* denote k_{cat} and *squares* denote k_{cat}/K_m

deaminase [21], methionine aminopeptidase [22], and deformylase [23], iron did not activate apohydantoinase activity. It has been observed that recombinant hydantoinase exhibits no enzyme activity without supplementation with a large excess of active metal ions, and metal-reconstituted hydantoinase exhibits higher enzyme activity [19]. At that time, the occupation of the metal binding site by iron was not suspected. The results shown in Table 1 clearly explain why metal supplementation in the cell culture was needed to obtain active recombinant hydantoinase and why metal-reconstituted hydantoinase exhibited higher enzymatic activity. Both zinc and iron are the most abundant and important metal nutrients for growth of bacteria [24]. It is unclear why iron is the preferred metal incorporated into recombinant hydantoinase and why the highest activity of hydantoinase is observed when the cell cultures are supplemented with cobalt. It is unlikely that cobalt has a biological role in vivo because of its low bioavailability in the cell [24].

Both the metal titration (Fig. 2) and the metal contents (Tables 1, 3) for bacterial hydantoinase suggest that there are two metal ions within the active site needed for hydantoinase activity. However, significantly higher metal content (about 2.5 metals per enzyme subunit) was determined in highly active metal-reconstituted hydantoinase (Table 3). It was not surprising that additional metal binding sites on the protein surface were available because hydantoinase could efficiently bind to a chelating column (described in "Materials and methods" for protein purification). Dihydroorotase has been found to have nearly three metals per monomer [25]. The recent crystal structure of *Bacillus* sp. hydantoinase reveals that there is a third metal binding site near the binuclear active site of the enzyme [17]. The significant amounts of metals found in K148 mutants (their carboxylated sites were eliminated) and more than two metals per subunit found in wild-type hydantoinase (Table 3) indicate the presence of extra metal binding sites in addition to the binuclear active site. The metal center in the amidohydrolase superfamily is vital for enzyme activity [26] and a mononuclear or binuclear metal center is the structural landmark for hydrolytic enzymes with the TIM-barrel structural fold (named after triosephosphateisomerase, consisting of eight α-helices and eight parallel β-strands that alternate along the peptide backbone) [27]. Mutation of each of the amino acids of the binuclear metal center (Fig. 1) partially eliminated a metal binding site, as presented in Table 3. The above observations regarding metal contents might also reconcile the seemingly contradictory data reported previously [28] for the possibility of metal contamination in recombinant hydantoinase. Metal quantitation indicates that only a mutation at lysine could eliminate the binding of nearly two metals (K148A) as compared with wild-type hydantoinase. This finding is consistent with the crystal structure shown in Fig. 1 and indicates that posttranslational lysine caboxylation is necessary for binuclear metal binding.

Hydantoinase of bacterial origin and its mammalian counterpart, dihydropyrimidinase, exhibit a broad substrate spectrum [4]. As shown in Table 2, the K_m valuess of the three representative substrates are high in the millimolar ranges, especially for those of dihydrouracil. Variation of the active-site metal center kept the K_m valuess within the same order of magnitude, but the K_m values of mammalian dihydropyrimidinase are 1 order of magnitude lower than those of bacterial enzymes using dihydropyrimidine and 6-methyldihydropyrimidine as substrates [4], which suggests that the mammalian dihydropyrimidinase has its important biological role in the reductive degradation of pyrimidine degradation. The specific biological function of hydantoinase in bacteria is unclear [5].

The sizes of the metal have been proposed as the factors that affect the activity of hydantoinase [19]. In Table 2, the effect of metal on the substrate specificity of hydantoinase is examined for three representative substrates, a six-membered ring (dihydropyrimidine), a bicyclic compound (phthalimide), and a five-membered ring (5-leucinylhydantoin). The results indicate that the metal ion not only affected the activity of hydantoinase but also significantly affected its substrate selectivity. The differences in catalytic efficiency (k_{cat}/K_m) between the best and worst substrates tested were 10-, 52- and 178-fold for zinc, cobalt, and manganese, respectively, in the metal-reconstituted hydantoinases. This indicates that zinc hydantoinase gave less discrimination between dihydrouracil and 5-leucinylhydantoin, while manganese hydantoinase saw dihydrouracil and 5-leucinylhydantoin as two very distinct substrates.

Activity of K148A could be chemically rescued by small organic acids in the presence of a high concentration of metal ions (Fig. 3), and smaller carboxylic acids lead to

better hydantoinase activity (acetate < propionate < butyrate) (Fig. 3). The variation may be due to the accessibility of the carboxylic acids to the active site. The maximal activity of K148A was still tenfold less than that of wild-type hydantoinase (Table 3, Fig. 3). One of the reasons for the difference in hydantoinase activity might be because carbamate and carboxylate have different chemical properties, as shown in Fig. 5a and b. A resonance structure of carbamate gives both oxygen atoms of a carbamate formal negative charges (Fig. 5a), perhaps promoting the bimetal binding [29, 30]. A partially positive charge, which is not available for carboxylate (Fig. 5b), is also formed in carbamate resulting from lysine carboxylation. This structural feature for the active sites of phosphotriesterase [13], urease [12], dihydroorotase [11], and isoaspartyl dipeptidase [31] is very similar to that of hydantoinase. A combination of site-directed mutagenesis and chemical rescue studied for phosphotriesterase [32] gives results similar to those of hydantoinase obtained in this study. In contrast, chemical rescue for another carbamate-dependent enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), not a member of the amidohydrolase superfamily, was not successful [33], suggesting that the role of carbamate in RUBISCO may be not as the same as that of phosphotriesterase and hydantoinase. Other zinc amidohydrolases, such as dihydroorotase and allantoinase, contain carbamated lysine and catalyze similar reactions [26, 34, 35]. However, the titration curve for addition of the metals into the active site of phosphotriesterase indicates a cooperative manner for assembly of the binuclear metal center [36], different from our study on hydantoinase (Fig. 2). The titration curve for the reactivation of apohydantoinase indicates that the first metal was preferentially bound but did not give any enzyme activity until the second metal was also bound to the hydantoinase. The relative binding affinity of the two metal binding sites for cobalt was estimated to be roughly 5:1 according to the slopes of the metal titration curve shown in Fig. 2a. It is, however, difficult to determine which is the preferred binding site (α or β as shown in Fig. 1). A mutagenesis study (Table 3) showed that either metal binding site can be occupied by cobalt with the removal of the metal ligand by site-directed mutagenesis in the other metal binding site. It is interesting to observe that D313, which coordinates to the α metal (Fig. 1), can be replaced with glutamate and retain full metal binding capacity (Table 3). It appears that the α metal may be the preferred one when both metal binding sites are available.

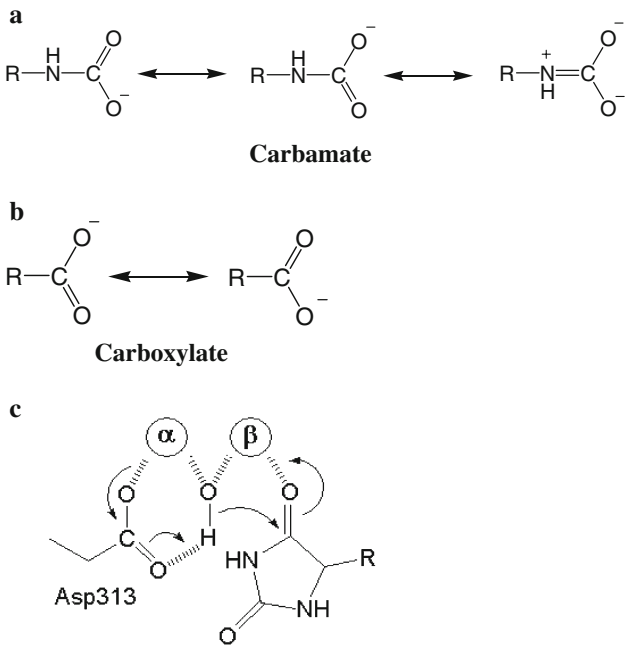


Fig. 5 Mechanism of hydantoinase-catalyzed reaction. **a** Resonance forms for carbamate of carboxylated lysine. **b** Resonance forms for carboxylate. **c** Proposed mechanism for the action of hydantoinase. For the hydrolysis of 5′-monosubstituted hydantoin, a nucleophilic attack by the bridging hydroxide was facilitated by the transfer of the proton from the hydroxide to the carboxylate of Asp-313

Variations in pK_a values with different metal ions in hydantoinase indicate that ionization of the active-site functional groups is dependent on the specific metal binding to the binuclear metal center. As shown in Fig. 5c, metal ions are proposed to directly promote the activation of the hydroxyl group for the nucleophilic attack on hydantoin. Asp-313 was proposed to be involved in the coordination of metal and activation of the hydroxyl group. The pK_a values of k_{cat}, 6.3 ± 0.1, 6.0 ± 0.1, and 6.6 ± 0.1 for cobalt-, zinc-, and manganese-reconstituted hydantoinase, respectively, indicate that the pK_a of the hydroxyl group activated by metal ion (Fig. 5c) may be significantly affected by the metal ion in the active site. The pK_a values of k_{cat}/K_m, 7.0 ± 0.1, 6.7 ± 0.1, and 6.8 ± 0.1 for cobalt-, zinc-, and manganese-reconstituted hydantoinase, respectively, indicate that the overall catalytic efficiency, which includes substrate binding, may be affected in a different manner. In Fig. 5c, Asp-313 is shown to coordinate one metal ion (α) and to function as a base to stabilize the hydroxyl group activated by the other metal ion (β) and to stabilize the intermediate, the dihydroxyl hydantoin (not shown) following the nucleophilic attack by the hydroxyl group. The pK_a values obtained with the D313E mutant (Fig. 4) were significantly higher (0.4 and 0.7 for k_{cat} and k_{cat}/K_m, respectively) than those of wild-type hydantoinase, which supports the mechanism shown in Fig. 5c suggesting that D313 is directly involved in the metal assisted imide hydrolysis and stabilization of the intermediate or transition state of hydantoin hydrolysis. The significant perturbation in the active-site structure would be expected with the mutation of aspartic acid to glutamate. The

increase in pK_a values may indicate that glutamate is less effective in functioning as a metal coordinator or in stabilizing the transition state of the reactant in the hydantoinase active site.

The chemical mechanism of the binuclear metal center containing amidohydrolase [26] likely has three steps: (1) the hydrolytic water molecule must be activated for nucleophilic attack, (2) the amide bond of the substrate must be made more electrophilic by polarization of the carbonyl-oxygen bond, and (3) the leaving-group nitrogen must be protonated as the carbon–nitrogen bond is cleaved. Thus, a single group must be unprotonated for the catalytic activity [37]. The respective kinetic pK_a obtained from the pH profiles of the metal-reconstituted hydantoinase varies, suggesting the deprotonation of the enzyme is associated with the specific metal bound to the active site. Moreover, on the basis of the crystal structure of dihydroorotase [11], the bimetal ion is proposed to function as a Lewis acid for polarizing the carbonyl group of the substrate. The carbonyl oxygen of the substrate directly interacting with the metal center will diminish the electron density and facilitate nucleophilic attack by the bridging hydroxide. In the final step, the amide nitrogen of the substrate must be protonated. The crystal structure of hydantoinase shows that the D313 is responsible for the proton transfer. This residue has been proposed as the group that shuttles the proton from the bridging hydroxide to and from the substrate and product during catalysis [34]. This is consistent with our finding that the kinetic pK_a values of D313E derived from the pH profile were significantly shifted compared with those of the wild-type enzyme. The aspartate located in the active site of related amidohydrolases, phosphotriesterase [38] and dihydroorotase [39], has also been demonstrated to be involved in proton transfer reactions with the bridging hydroxide.

It has been speculated that the functionally related amidohydrolases, such as hydantoinase (dihydropyrimidinase), dihydroorotase, and allantoinase, use a similar catalytic mechanism for the hydrolysis of the cyclic amide ring [28, 34, 40]. The chemical mechanism of hydantoinase presented here was similar to that of *Escherichia coli* dihydroorotase [39], and thus it further raises an important question as to whether their mammalian counterparts use the same mechanism. Three lines of evidence may suggest that the difference in enzyme properties between mammalian dihydropyrimidinase and bacterial hydantoinase requires further attention. The most interesting finding in the amidohydrolase superfamily family is their metal content. Hydantoinases from bacteria and yeast are found to contain two metal ions; however, the mammalian counterparts have one metal ion [19]. Second, bacterial hydantoinase is generally EDTA-labile, while its mammalian counterparts are EDTA-resistant (even above 100 mM), suggesting that bacterial hydantoinase and dihydropyrimidinase have different metal binding affinities, or the metal in dihydropyrimidinase is inaccessible to EDTA. Third, the activities of animal dihydropyrimidinases are significantly higher than those from yeast and bacteria [34, 41]. Similarly, these observations are also found for dihydroorotase. In higher organisms, dihydroorotase is found within a large polyfunctional protein CAD, which contains one metal ion [42]; in bacteria such as *Escherichia coli*, however, dihydroorotase is found to have two metal ions [11]. The one metal/two metal phenomena were observed in other enzyme families with interesting association to the amidohydrase superfamily described above. The aminopeptidase from *Aeromonas proteolytica* contains two zinc ions in the active site and is bridged by an aspartic acid site chain [43]. This structure may highly resemble that of K148A mutant rescued by carboxylic acids (Fig. 3). Glyoxalase II of the metallo-β-lactamase family from *Arabidopsis thaliana* is shown to bind a mixture of zinc, iron, or manganese, but the binding of iron and zinc to this enzyme occurs exclusively as bimetal centers [44]. However, metallo-β-lactamase from *Bacillus cereus* contains a mononuclear zinc in the active site [45]. Another metallo-β-lactamase, L1 from *Stenotrophomonas maltophilia*, was found to contain 1 and 2 equiv of zinc and 2 equiv of zinc plus hydrolyzed nitrocefin (an antibiotic) [46]. At present, no structural data are available for mammalian dihydroorotase and dihydropyrimidinase that contain one metal per enzyme subunit.

Acknowledgments We thank Yi-Rong Chen for purification of D313E mutant protein. This research was supported by grants from the National Science Council, Taiwan (96-2627-B-009-004).

References

1. Bernheim F, Bernheim MLC (1946) J Biol Chem 163:683–685
2. Eadie G, Bernheim F, Bernheim MLC (1949) J Biol Chem 181:449–458
3. Wallach DP, Grisolia S (1957) J Biol Chem 226:277–288
4. Yang YS, Ramaswamy S, Jakoby WB (1993) J Biol Chem 268:10870–10875
5. Syldatk C, May O, Altenbuchner J, Mattes R, Siemann M (1999) Appl Microbiol Biotechnol 51:293–309
6. Vogels GD, Van der Drift C (1976) Bacteriol Rev 40:403–468
7. Schoemaker HE, Mink D, Wubbolts MG (2003) Science 299:1694–1697
8. Altenbuchner J, Siemann-Herzberg M, Syldatk C (2001) Curr Opin Biotechnol 12:559–563
9. May O, Nguyen PT, Arnold FH (2000) Nat Biotechnol 18: 317–320
10. Holm L, Sander C (1997) Proteins 28:72–82
11. Thoden JB, Phillips GN Jr, Neal TM, Raushel FM, Holden HM (2001) Biochemistry 40:6989–6997

12. Jabri E, Carr MB, Hausinger RP, Karplus PA (1995) Science 268:998–1004
13. Benning MM, Kuo JM, Raushel FM, Holden HM (1995) Biochemistry 34:7973–7978
14. Xu Z, Liu Y, Yang Y, Jiang W, Arnold E, Ding J (2003) J Bacteriol 185:4038–4049
15. Abendroth J, Niefind K, Schomburg D (2002) J Mol Biol 320:143–156
16. Cheon YH, Kim HS, Han KH, Abendroth J, Niefind K, Schomburg D, Wang J, Kim Y (2002) Biochemistry 41:9410–9417
17. Radha Kishan KV, Vohra RM, Ganesan K, Agrawal V, Sharma VM, Sharma R (2005) J Mol Biol 347:95–105
18. Huang CY, Chao YP, Yang YS (2003) Protein Expr Purif 30:134–139
19. Huang CY, Yang YS (2002) Biochem Biophys Res Commun 297:1027–1032
20. Cleland WW (1979) Methods Enzymol 63:103–138
21. Ireton GC, McDermott G, Black ME, Stoddard BL (2002) J Mol Biol 315:687–697
22. D'Souza VM, Holz RC (1999) Biochemistry 38:11079–11085
23. Becker A, Schlichting I, Kabsch W, Groche D, Schultz S, Wagner AF (1998) Nat Struct Biol 5:1053–1058
24. Faraldo-Gomez JD, Sansom MS (2003) Nat Rev Mol Cell Biol 4:105–116
25. Washabaugh MW, Collins KD (1984) J Biol Chem 259:3293–3298
26. Seibert CM, Raushel FM (2005) Biochemistry 44:6383–6391
27. Gerlt JA, Raushel FM (2003) Curr Opin Chem Biol 7:252–264
28. Kim GJ, Kim HS (1998) Biochem J 330:295–302
29. Hausinger RP (2003) Nat Struct Biol 10:234–236
30. Kuchar J, Hausinger RP (2004) Chem Rev 104:509–525
31. Jozic D, Kaiser JT, Huber R, Bode W, Maskos K (2003) J Mol Biol 332:243–256
32. Kuo JM, Chae MY, Raushel FM (1997) Biochemistry 36:1982–1988
33. Smith HB, Hartman FC (1991) Biochemistry 30:5172–5177
34. Gojkovic Z, Rislund L, Andersen B, Sandrini MP, Cook PF, Schnackerz KD, Piskur J (2003) Nucleic Acids Res 31:1683–1692
35. Gerlt JA, Babbitt PC (2001) Annu Rev Biochem 70:209–246
36. Shim H, Raushel FM (2000) Biochemistry 39:7357–7364
37. Christianson DW, Cox JD (1999) Annu Rev Biochem 68:33–57
38. Aubert SD, Li Y, Raushel FM (2004) Biochemistry 43:5707–5715
39. Porter TN, Li Y, Raushel FM (2004) Biochemistry 43:16285–16292
40. May O, Habenicht A, Mattes R, Syldatk C, Siemann M (1998) Biol Chem 379:743–747
41. Huang CY, Yang YS (2003) Biochem Biophys Res Commun 312:467–472
42. Huang DT, Thomas MA, Christopherson RI (1999) Biochemistry 38:9964–9970
43. Desmarais W, Bienvenue DL, Bzymek KP, Petsko GA, Ringe D, Holz RC (2006) J Biol Inorg Chem 11:398–408
44. Wenzel NF, Carenbauer AL, Pfiester MP, Schilling O, Meyer-Klaucke W, Makaroff CA, Crowder MW (2004) J Biol Inorg Chem 9:429–438
45. Peraro MD, Vila AJ, Carloni P (2002) J Biol Inorg Chem 7:704–712
46. Costello A, Periyannan G, Yang KW, Crowder MW, Tierney DL (2006) J Biol Inorg Chem 11:351–358