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Probing the active site of rat porphobilinogen synthase using newly developed inhibitors

Nan Li^{a,b}, Xiusheng Chu^a, Xiaojun Liu^a, Ding Li^{a,*}

^a Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, PR China ^b Department of Chemical and Environmental Engineering, Wuyi University, Jiangmen, Guangdong Province 529020, PR China

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ABSTRACT

The structurally related tetrapyrrolic pigments are a group of natural products that participate in many of the fundamental biosynthetic and catabolic processes of living organisms. Porphobilinogen synthase catalyzes a rate-limiting step for the biosyntheses of tetrapyrrolic natural products. In the present study, a variety of new substrate analogs and reaction intermediate analogs were synthesized, which were used as probes for studying the active site of rat porphobilinogen synthase. The compounds **1**, **3**, **6**, **9**, **14**, **16**, and **28** were found to be competitive inhibitors of rat porphobilinogen synthase with inhibition constants ranging from 0.96 to 73.04 mM. Compounds **7**, **10**, **12**, **13**, **15**, **17**, **18**, and **26** were found to be irreversible enzyme inhibitors. For irreversible inhibitors, loose-binding inhibitors were found to be irreversible indig to the analogs were found to be important for their binding to the enzyme. This study increased our understanding of the active site of porphobilinogen synthase.

1. Introduction

The structurally related tetrapyrrolic pigments are a group of natural products that include the haems, the chlorophylls, the corrinoids (e.g. coenzyme B_{12}), the cyclic tetrapyrroles factor F_{430} and the linear tetrapyrroles (bilins) [1,2]. Regulation of tetrapyrrole biosynthesis has been found to be crucial to plant and bacteria metabolism and gene expression [3–8]. These compounds participate in many of the fundamental biosynthetic and catabolic processes of living organisms. They are all intensely colored and almost every living organism has an absolute requirement for one or more of them. It is for this reason that they are called the "pigments of life."

Porphobilinogen synthase (PBG synthase; PBGS; EC 4.2.1.24) is also named 5-aminolaevulinic acid dehydratase (ALA¹ dehydratase), which catalyses an asymmetric condensation of two molecules of 5-aminolaevulinic acid (ALA) to give the monopyrrole porphobilinogen (PBG) as shown in Fig. 1. The substrate that becomes the acetyl-containing half of PBG is called A-side ALA; the

E-mail address: bhdingli@cityu.edu.hk (D. Li).

propionyl-containing half of PBG derives from P-side ALA. This has also led to the terminology of the "A" and "P" binding sites in the enzyme which bind the A-side and P-side ALAs, respectively.

PBG synthase is highly conserved throughout the archaea, eubacteria, and eukarya. This enzyme has been purified from a variety of sources including human erythrocytes [9], bacteria such as *Escherichia coli* [10], and plants such as spinach [11]. There are some differences among these PBG synthases in terms of their metal requirements, kinetic parameters, pH optima, inactivation by inhibitors and susceptibility to oxidation. It is often claimed that almost all PBG synthases require a divalent cation for activity with animal enzymes using zinc and plant enzymes using magnesium. Representatives of both zinc and magnesium classes exist in some bacteria [12]. The human PBG synthase can adopt different nonad-ditive quaternary assemblies (morpheein forms), which are a high activity octamer, a low activity hexamer, and two structurally distinct dimer conformations [13,14].

The X-ray structures of PBG synthases from several species have been determined [15–21]. In these structures, the enzyme is a homo-octamer, and the active site of each subunit is located in a pronounced cavity formed by loops at the C-terminal ends of the β -strands. All eight active sites are oriented towards the outer surface of the octamer and appear to be independent. The catalysis has been shown to proceed by formation of a Schiff-base link at the P-site at first between the 4-keto group of substrate ALA and an invariant lysine residue, equivalent to Lys263 in yeast PBG synthase. The structures of several PBG synthases complexed with the inhibitors 4,7-dioxosebacic acid, 5-fluorolaevulinic, and several

^{*} Corresponding author. Fax: +86 852 2788 7406.

¹ Abbreviations used: ALA, 5-aminolaevulinic acid; DHP, 1,2-dihydropyrane; DMF, dimethyl formamide; DMSO, dimethyl sulfuroxide; DTT, dithiothreitol; IPTG, isopro-pyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBG, porphobilinogen; PBGS, porphobilinogen synthase; PCC, pyridinium chlorochromate; PCR, polymerase chain reaction; PPTS, pyridinium p-toluenesulfonate; PTSA, p-toluenesulfonic acid; SDS, sodium dodecylsulfate; THF, tetrahydrofuran; THP, tetra-hydropyran; UV/vis, ultraviolet-visible spectroscopy.



Fig. 1. Reactions catalyzed by PBG synthase.

bisubstrate analogs have been solved at high resolution [22–24]. These inhibitors form two Schiff bases at the active site involving Lys210 as well as Lys263 in yeast PBG synthase. The structural evidence that both invariant lysines form Schiff bases with some inhibitors suggests that catalysis involves a double Schiff-base mechanism.

Two possible mechanisms have been proposed for the formation of PBG from ALA. They differ mainly in the order of C-C (aldol condensation) and C-N bond formation (Schiff base) (Fig. 1). Nandi and Shemin have proposed a mechanism that is analogous to the reaction catalyzed by the class I aldolases suggesting C-C bond formation happens before C-N bond formation [25]. Several years later, Jordan and Seehra proposed another mechanism suggesting a C-N bond formation takes place at first [26]. After either C-C bond or C-N bond forms at first in the enzymatic reaction, a long chain dicarboxylate intermediate is generated. In order to distinguish between the two major mechanistic proposals, diacids containing seven carbon atoms (suggesting that C-C bond formation occurs first) or diacids containing ten carbon atoms (suggesting C-N bond formation occurs first) were tested as inhibitors for E. coli PBG synthase [27]. The diacids analogs containing ten carbon atoms have been found to be either competitive or irreversible inhibitors for E. coli PBG synthase, which supports Jordan mechanism. However, the later studies on these compounds indicate that some PBG synthases from human and other sources are not inhibited by these compounds [22,28]. Therefore, the order of bond-making and bond-breaking reactions in the PBGS-catalyzed reaction remains uncertain and may not be conserved for all PBG synthases from different species.

It has been reported that PBG synthase activity is correlated with some diseases such as diabetes, hypothyroidism [29,30]. In humans, hereditary deficiencies in PBG synthase give rise to the rare disease porphyria [31-33], and the clinical comment of this acute porphyria is similar to lead poisoning. Human PBG synthase is a metalloenzyme that requires Zn^{II} for maximal catalytic activity and it is an important molecular target for the widespread environmental toxic metal Pb²⁺ [34,35]. Al³⁺, Ga³⁺, and In³⁺ inhibit bovine liver PBG Synthase by competing with Zn^{II}, whereas Tl³⁺ and In³⁺ inhibit bovine PBG synthase by directly oxidizing essential sulfhydryl group [36]. PBG synthase also require thiol groups for catalytic activity, and the purified enzyme is extremely sensitive to oxygen and requires thiol-reducing agents to display maximal activity [35]. In fact, mammalian PBG Synthases have several reactive cysteinyl residues that react with a variety of sulfhydryl reagents [37-39], and it has been proposed that one role for Zn^{II} is to prevent disulfide formation between essential sulfhydryl groups in PBG Synthase [40].

A number of compounds have been synthesized as substrate or product analogs in attempts to unravel PBG synthase mechanism or to inhibit the enzyme in a specific manner [25,41–43]. Some substrate analogs have been found to be competitive enzyme inhibitors. It has been found that PBG synthase is not inhibited by its direct product PBG. It has also been revealed that different enzymes exhibit different susceptibilities to various inhibitors [44,45]. In the present research, we synthesized a variety of new substrate analogs of PBG synthase, which were incubated with the enzyme as probes. The study further increased our understanding of this important enzyme.

2. Methods and materials

2.1. Materials

A Hi-Trap chelating metal-affinity column was purchased from Amersham Pharmacia Biotech. *Taq* DNA polymerase, HB101 competent cells, *E. coli* strain BL21(DE3) competent cells, agarose, Plasmid Mini kit, and synthesized oligonucleotides came from Invitrogen Life Technologies. Restriction enzymes came from MBI Fermentas. All other reagents were of research grade or better and were obtained from commercial sources.

2.2. Cloning of the functional rat PBG synthase

A rat liver Quick-Clone cDNA library was purchased from Clontech (Palo Alto, CA). The gene of rat PBG synthase was amplified by PCR using primers that were designed to add six continuous histidine codons to the 5' primer. The sequence of the forward primer was 5'-cg cgc gga tcc aggagga atttaaa atg aga gga tcg cat cac cat cac cac cag tcc gtt ctg cac ag 3', containing a BamHI site (gga tcc), a ribosome binding site (aggagga), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 4-8 of rat liver PBG synthase. The sequence of the reverse primer was 5'-ctg cag gtc gac tta ctc ttc ctt cag cca ctt caa cag-3', containing a Sall site (gtc gac), a stop anticodon (tta), and anticodons for the last eight amino acids of rat liver PBG synthase. The PCR product was gel purified, double digested, and ligated into a pLM1 [46] expression vector resulting in the pLM1::PBGS plasmid. pLM1 vector has a T7 promoter-driven system with ampicillin resistance and can amplify in E. coli HB101. The constructed pLM1::PBGS plasmid was transformed into HB101 competent cells according to an electroporation transformation procedure (Bio-Rad) for screening purposes. The identified positive colony was grown in LB medium containing ampicillin (50 mg/L), and the plasmid pLM1::PBGS was isolated and transformed to E. coli strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned rat liver PBG synthase gene was performed, and the inserted gene sequence was identified to be the same as that previously deposited in NCBI without any mutation.

PBG synthase gene was also cloned into another vector pET28a+ using a similar method. The sequence of the forward primer was 5'-cg cgc gct agc cac cac cag tcc gtt ctg cac ag-3', containing a NheI site (gct agc), and codons for amino acids 2–8 of rat liver PBG synthase. The sequence of the reverse primer was 5'-ctg cag <u>aag ctt tta</u> ctc ttc ctt cag cca ctt caa cag-3', containing a HindIII site (aag ctt), a stop anticodon (tta), and anticodons for the last eight amino acids of rat liver PBG synthase. The constructed pET28a+::PBGS plasmid was sequenced, and the inserted gene sequence was identified to be the same as that previously deposited in NCBI without any mutation.

2.3. Expression and purification of soluble rat PBG synthase

Established methods [47] were used to prepare the enzymes to apparent homogeneity as analyzed by SDS–PAGE. The proteins were stored at -80 °C in 50 mM Tris buffer, pH 7.5, 5% glycerol, and 5 mM β -mercaptoethanol. The stability of the purified enzymes was tested by its activity and the His-tagged proteins were proved to be highly stable. The enzymes can be stored at 4 °C for

1 week without significant change of activity. The proteins were normally stored in a -80 °C freezer and were stable for at least one year tested on the basis of their activities.

Rat PBG synthase from recombinant plasmid pET28a+::PBGS was incubated with thrombin to remove the His-tag. The enzyme (150 mg) and the thrombin (5 mg) were incubated in Tris–Cl buffer (50 mM, pH 8.0) at 25 °C for different time period, and the result showed that 1-h incubation is enough for an obvious cleavage of the protein.

2.4. Activity assay

The activity of PBG synthase was determined by a calorimetric assay based on the reaction between PBG and modified Ehrlich's reagent [48]. Enzyme $(6-8 \mu g)$ was preincubated for 10 min at 37 °C in Tris-Cl buffer (100 mM, pH 8.5, containing 10 µM ZnCl₂ and 10 mM 2-mercaptoethanol). The reaction was started by the addition of ALA (10 mM final concentration) to give a final volume of 500 µl and preceded for 5 min at 37 °C. The incubation was terminated by the addition of 500 µl mercuric chloride (0.1 M) in 20% trichloroacetic acid at 0 °C to precipitate protein and thiol. The solution was stayed on ice for 10 min and then centrifuged at 10,000 rpm for 5 min at room temperature. The aliquot (500 μ l) of supernatant was transferred and reacted with an equal volume of modified Ehrlich's reagent. After 10 min, PBG was detected spectrophotometrically by the absorbance of pyrrole at 555 nm with the use of a molar absorption coefficient for PBG of $60{,}200\,M^{-1}\,cm^{-1}{.}$ One unit of enzyme activity is defined as the amount of enzyme that produce 1 µmol of PBG in 1 h at 37 °C. Determination of the $K_{\rm M}$ and the $V_{\rm max}$ was performed using the same assay buffer with varying substrate concentrations ranging from 0.1 to 5 mM.

2.5. Enzyme inactivation study

PBG synthase (40–50 µg, 15 µl) was incubated with an appropriate amount of inhibitor, and 10 µM $ZnCl_2$ in 45 µl Tris buffer (100 mM, pH 8.5) at 37 °C. At various time intervals, 10 µl aliquot of the solution was taken out individually to assay the enzyme activity in a buffer containing 2-mercaptoethanol in order to react with the remaining inhibitor. A control experiment was performed under identical conditions without inhibitor. After 60 min incubation at room temperature to ensure complete inactivation of the enzyme, the inactivated enzyme was dialyzed against 100 mM Tris buffer (pH 8.5) over 2 days at 4 °C with seven buffer changes. The residual enzyme activity was determined before and after dialysis to test for the reversibility of the inactivation. A control experiment was performed under identical conditions without inhibitor.

2.6. Determination of kinetic constant K_i of competitive inhibitors

The type of inhibition of the reversible inhibitors was determined by a double-reciprocal plot of 1/V versus 1/[S]. The K_i values of the competitive inhibitors of PBG synthase were determined by Dixon plot [49]. The reaction cocktail contained the buffer, the enzyme, and an inhibitor, which were incubated at 37 °C for 10 min. Then the reaction was started by adding the substrate. If the inhibition type of the inhibitor was not competitive, the IC₅₀ values were determined instead of the K_i values to compare the potency of the inhibitors.

2.7. Determination of IC₅₀ for inhibitors

In determination of the IC_{50} values for the inhibitors, the concentration of the substrate was about two fold of the $K_{\rm M}$ value for the enzyme. The reaction cocktail contained the buffer, the en-

zyme, and an inhibitor, which were incubated at 37 °C for 10 min. Then the reaction was started by adding the substrate. A control experiment was performed under identical conditions without the inhibitor. The IC_{50} value was determined by Dixon plot [49].

3. Results and discussion

3.1. Gene cloning, expression in E. coli, and purification of rat PBG synthase

Rat PBG synthase gene was cloned with two different vectors. which gave two recombinant plasmid pLM1::PBGS and pE-T28a+::PBGS. The expression in E. coli BL21 (DE3) was carried out at different temperatures and IPTG concentrations. It was found that both proteins expressed better at an IPTG concentration of 0.5 mM and that it resulted in a more soluble protein at room temperature than 37 °C. Nickle metal-affinity resin columns were used for single-step purifications of the two His-tagged rat PBG synthases. The protein fractions were dialyzed against a 20 mM potassium phosphate buffer, pH 7.5, 5% glycerol, and 5 mM βmercaptoethanol, as soon as possible after the purification. The addition of 5% glycerol and 5 mM β-mercaptoethanol was essential to maintain the long-term stability of the protein. The enzymes can be stored at 4 °C for 1 week without significant change of activity. The proteins were normally stored in a -80 °C freezer and were stable for at least one year tested on the basis of its activity. The purity of the enzymes was examined by SDS-PAGE and single bands corresponding to about 37 kDa proteins were observed with >95% purity (Fig. 2). The molecular mass is in agreement with the data calculated from the amino acid sequences. The overall yields of the two proteins in the purification procedure both were above 90%. For the plasmid pLM1::PBGS, 12 mg purified protein was obtained from 0.5 L culture. For the plasmid pET28a+::PBGS, 2 mg purified protein was obtained from 0.5 L culture. In order to test the effect of the six histidines added in the N-terminus of the protein, thrombin was used to remove the His-tag from rat PBG synthase from the recombinant plasmid pET28a+::PBGS. The purity of the resulting enzymes was examined using SDS-PAGE and single bands corresponding to about 37 kDa proteins were observed with >90% purity (Fig. 3).



Fig. 2. SDS–PAGE of purified rat PBG synthase from different plasmids. Lane 1, protein marker: 116.0, β -galactosidase from *E. coli*; 66.2, bovine serum albumin from bovine plasma; 45.0, ovalbumin from chicken egg white; 35.0, lactate dehydrogenase from porcine muscle; 25.0, REase Bsp98I from *E. coli*; 18.4, β -lactoglobulin from bovine milk; 14.4, lysozyme from chicken egg white; lane 2, purified rat PBG synthase from recombinant plasmid pLM1::PBGS; lane 3, purified rat PBG synthase from recombinant plasmid pET28a+::PBGS.



Fig. 3. SDS-PAGE of rat PBG synthase from recombinant plasmid pET28a+::PBGS cleaved by thrombin. Lane 1, protein marker; lane 2, PBG synthase from recombinant plasmid pET28a+::PBGS; lane 3, PBG synthase cleaved by thrombin at 25 °C for 1 h; lane 4, PBG synthase cleaved by thrombin at 25 °C for 2 h; lane 5, PBG synthase cleaved by thrombin at 25 °C for 3 h.

3.2. Characterization of rat PBG synthase

Rat PBG synthases from pLM1::PBGS, pET28a+::PBGS, and rat PBG synthase without the His-tag were assayed in 100 mM Tris-

Table 1

Kinetic constants of rat PBG synthase.

	$K_{\rm M}$ (mM)	V _{max} (µmol/h/mg)
PBGS from pLM1:: PBGS	3.22 ± 0.67	5.5 ± 0.6
PBGS from pET28a+::PBGS	0.65 ± 0.06	16.7 ± 0.5
PBGS from pET28a+::PBGS, No His-tag	2.10 ± 0.49	17.4 ± 1.2

Cl buffer (containing 10 μ M ZnCl₂) in the range of pH 7.0–9.5. All enzymes showed higher activity under basic conditions. The optimum pH value for PBG synthase from pLM1::PBGS is 8.5. For PBG synthase from pET28a+::PBGS, the enzymes with and without Histag showed same pH-activity profile with highest activity in the range of pH 8.5–9.5.

The activities of three rat PBG synthases were tested at different ZnCl_2 concentrations in Tris buffer (pH 8.5). For two enzymes from pET28a+::PBGS, the optimal concentration of Zn^{2+} ion was found to be 10 μ M. For the enzyme from pLM1::PBGS, the optimal concentration of Zn^{2+} ion was found to be 30 μ M. All three enzymes were found to have considerable activities when no Zn^{2+} ion was used, since there was a little Zn^{2+} ion in the LB culture for protein expression. All three enzymes were completely inactivated when 1 mM EDTA was added to the assay buffer, indicating that the Zn^{2+} ion was essential to the activity of rat PBG synthase.

For the kinetic analyses of rat PBG synthases, rates were measured at five or six substrate concentrations. The results of kinetic studies obtained through nonlinear curve fitting using SigmaPlot 8.0 program are summarized in Table 1. The results indicate the removal of His-tag does not have significant effect on the enzymatic activity.

Rn Hs Sc Ec	1 1 1	MHHQSVLHSGYFHPLLRAWQTTPSTVSATNLIYPIFVTDVPDDVQPIAS MQPQSVLHSGYFHPLLRAWQTATTTLNASNLIYPIFVTDVPDDIQPITS MHTAEFLETEPTEISSVLAGGYNHPLLRQWQSE-RQLTKNMLIFPLFISDNPDDFTEIDS MTDLIQRPRRLRKSPALRAMFEE-TTLSLNDLVLPIFVEEEIDDYKAVEA * . * . * . * . * . * . * . * . * . * . * . . * .	49 49 59 49
Rn	50	LPGVARYGVNQLEEMLRPLVEAGLRCVLIFGVPSRVPKDEQGSAADSEDSPTIEAVRL	107
Hs	50	LPGVARYGVKRLEEMLRPLVEEGLRCVLIFGVPSRVPKDERGSAADSEESPAIEAIHL	107
Sc	60	LPNINRIGVNRLKDYLKPLVAKGLRSVILFGVP-LIPGTKDPVGTAADDPAGPVIQGIKF	118
Ec	50	MPGVMRIPEKHLAREIERIANAGIRSVMTFGISHHTDETGSDAWREDGLVARMSRI	105
		:*.: * ::* :. :. *:*.*: .* *: * ::	
Rn	108	LRKTFPTLLVACDV C L C PYTSHG HC GLLSENGAFLAEESRQRLAEVALAYAKAGCQVVAP	167
Hs	108	LRKTFPNLLVACDV C L C PYTSHG H C GLLSENGAFRAEESRQRLAEVALAYAKAGCQVVAP	167
Sc	119	IREYFPELYIICDV C L C EYTSHG HC GVLYDDGTINRERSVSRLAAVAVNYAKAGAHCVAP	178
Еc	106	$\texttt{CKQTVPEMIVMSDT}{\mathbf{C}}{\mathbf{F}}{\mathbf{C}}{\mathbf{EYTSHG}}{\mathbf{H}}{\mathbf{C}}{\mathbf{GVLCEH}}{\mathbf{GVDN}}{\mathbf{-}}{\mathbf{DATLENL}}{\mathbf{GKQAVVAAAA}}{\mathbf{GADFIAP}}$	164
		:: .* : .*. * :* ********* :.* : :*. *: * ** :**	
Rn	168	$\texttt{SDMMDGRVEAIKAALLKHGLGNRVSVMSYSA}{\mathbf{K}} \texttt{FASCFYGPFRDAAQSSPAFGDRR}{\mathbf{C}} \texttt{YQLP}$	227
Hs	168	SDMMDGRVEAIKEALMAHGLGNRVSVMSYSA K FASCFYGPFRDAAKSSPAFGDRR C YQLP	227
Sc	179	SDMIDGRIRDIKRGLINANLAHKTFVLSYAA K FSGNLYGPFRDAACSAPSNGDRK C YQLP	238
Еc	165	$\texttt{SAAMDGQVQAIRQALDAAGFKD-TAIMSYST}{\mathbf{K}}\texttt{FASSFYGPFREAAGSALK-GDRKSYQMN}$	222
		* :**:. *: .* .: . ::**::**:. :*********	
Rn	228	PGARGLALRAVARDIQEGADILMV K PGLPYLDMVQEVKDKHPELPLAVYQVSGEFAMLWH	287
Hs	228	$\texttt{PGARGLALRAVDRDVREGADMLMV}{\mathbf{K}}\texttt{PGMPYLDIVREVKDKHPDLPLAVYHVSGEFAMLWH}$	287
Sc	239	PAGRGLARRALERDMSEGADGIIV K PSTFYLDIMRDASEICKDLPICAYHVSGEYAMLHA	298
Еc	223	PMNRREAIRESLLDEAQGADCLMVK PAGAYLDIVRELRER-TELPIGAYQVSGEYAMIKF	281
		* * * * * :*** ::* * *. ***:::: : :**:.*:*:**:	
Rn	288	GAKAGAFDLRTAVLESMTAFRRAGADIIITYFAPQLLKWLKEE- 330	
Hs	288	GAQAGAFDLKAAVLEAMTAFRRAGADIIITYYTPQLLQWLKEE- 330	
Sc	299	AAEKGVVDLKTIAFESHQGFLRAGARLIITYLAPEFLDWLDEEN 342	
Ec	282	AALAGAIDEEKVVLESLGSIKRAGADLIFSYFALDLAEKKILR- 324	
		* * * • • * • * * • * • • • • • • • • •	

Fig. 4. The sequence of PBG synthase from various sources. Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Ec, *Escherichia coli*. Residues conserved in all sequences are marked with *. Residues not conserved in all sequences but conserved in some sequences are marked with : or . based on the degree of conservation. The sequence alignment was made by using ClustalX [53,54]. The important residues for catalysis and binding are highlighted.



Fig. 5. Syntheses of PBG synthase substrate analogs.

3.3. Sequence alignment of PBG synthases from various sources

The sequence alignment of PBG synthases from various sources were carried out as shown in Fig. 4. It was found that PBG synthases from mammalian sources, yeast, and bacteria have good sequence homology. Rat PBG synthase shares 88% sequence homology with human PBG synthase. The important residues for catalysis and binding are highly conserved. This indicates rat PBG synthase is a good model enzyme for subsequent studies of synthetic substrate analogs.

3.4. Synthesis of substrate analogs and reaction intermediate analogs

Some new substrate analogs of PBG synthase were synthesized as molecular probes for the study of rat PBG synthase as shown in Fig. 5. Compounds **1** and **3** were synthesized from corresponding ester through hydrolysis reactions. 5-Bromolaevulinic acid (**2**) was synthesized from benzyl laevulinate through bromination and hydrolysis reaction. Ethyl 4-acetylbutyrate was brominated in the methanol, and the resulting α -bromoketo ester was substituted by sodium azide. Then, the azido group was hydrogenated using palladium on charcoal catalyst, which was then deprotected through hydrolysis reaction to give compound **6**.

4-Hydroxy-2-butanone was used as the starting material for syntheses of a variety of substrate analogs as shown in Fig. 5. It was brominated in methanol at 0 °C to give compound **7**, and the hydroxyl group was stable in the reaction at low temperature. The 4-hydroxyl group was protected by dihydropyran (DHP), and the resulting compound **8** was reacted with sodium azide. After deprotection, the azido group was hydrogenated to give compound **9**. The hydroxyl group of 4-hydroxy-2-butanone was substituted by bromine to give compound **12**, and then the bromine was substituted by cyano group to afford compound **14**. Compounds **10**, **11**, **13**, **15**, and **16** were synthesized using same chemical reactions as mentioned above. 5-Hydroxy-2-pentanone was used as starting material for syntheses of several other substrate analogs, including compounds **17**, **18**, and **21**, using same chemical reactions as mentioned above.



Fig. 6. Syntheses of diacids intermediate analogs for PBG synthase catalyzed reaction.

Two new intermediate analogs (dicarboxylic acids) for PBG synthase catalyzed reaction were also synthesized as shown in Fig. 6. The key intermediate compound **25** was synthesized through Wittig reaction of aldehyde (**24**) and triphenyl phosphonium ylide (**23**). Compound **25** is identified as an E- α , β -unsaturated compound, which was hydrolyzed in the solution of phosphoric acid to give compound **26**. The compound **25** was also hydrogenated using palladium on charcoal catalyst to give dimethyl 4-oxo-5decenedioate (**27**), which was then hydrolyzed with NaOH and acidified with HCl to give product **28**.

3.5. Inhibition study of rat PBG synthase with newly synthesized reversible inhibitors

All above synthetic substrate analogs were tested for their interactions with rat PBG synthase as inhibitors. Some substrate analogs were found to be reversible enzyme inhibitors, and their inhibition constants (K_i or IC₅₀) are summarized in Table 2. If the inhibition type of an inhibitor was not competitive, the IC₅₀ value was determined instead of the K_i value to compare the potency of the inhibitors.

The compounds **1**, **3**, **6**, **9**, **14**, **16**, and **28** were found to be competitive inhibitors of rat PBG synthase with inhibition constants ranging from 0.96 to 73.04 mM, indicating that their binding affinity with this enzyme was quite different. The compounds **1** and **6** are very similar to the substrate and their K_i values are close to the K_M , indicating that the two compounds are well adopted into the active site. Compound **1** has been found to be a good competitive inhibitor of PBG synthases from Pea, *E. coli.*, and yeast [44]. The K_i value of compound **1** is a little lower than that of compound **6**, indicating the chain length of the molecule is more important than the amino group in the terminal for the binding.

Compound **28** has two carboxyl groups and was expected to bind to both the A-site and P-site of the enzyme whereas the compound **1** was found to bind to the P-site of *E. coli*. enzyme [16]. The K_i value of compound **28** is three times more than that of compound **1**, indicating that the additional carboxyl group and longer carbon chain can not fit well into the A-site. The compound **28** has been found to be a good irreversible inhibitor to *E. coli* enzyme, but was a weak inhibitor to human enzyme [28].

Compound **3** has one more methylene group than compound **1** and was assumed to bind to the P-site like compound **1**, but the larger K_i value indicates the longer carbon chain is unfavorable for the binding. The Comparison of compounds **3** and **6** indicates that the amino group is important for binding. This result is also

supported by the comparison of the structures and kinetic constants of compounds **1** and **6**. Comparing with compound **16**, compound **14** lacks the amino group and has a much higher K_i value. This further indicates that the amino group plays an important role for the binding.

Compounds **9** and **16** were found to be weak competitive inhibitors of rat PBG synthase with similar inhibition constants. Compounds **11** and **21** were not competitive inhibitors but their IC₅₀ values are at the same level as those of compounds **9** and **16**. These four compounds all have the amino groups and carbonyl groups, but no carboxyl groups. All of them were not substrates, but weak inhibitors of the enzyme, which indicates the carboxyl group is important for the binding. The X-ray structure of yeast PBG synthase complexed with substrate showed the bound ALA was linked to Lys263 in the P-site and the carboxyl group was hydrogen bonded to two highly conserved residues, Ser290 and Tyr329 [18]. Therefore, if the carboxyl group is replaced by other functional groups, the analogs are not able to be fixed in the appropriate position through hydrogen bonding, and can not take part in subsequent catalytic reaction.

3.6. Inhibition study of rat PBG synthase with newly synthesized irreversible inhibitors

In the absence of thiols, PBG synthases are susceptible to aerial oxidation. However, many known inhibitors of PBG synthase were found to react with thiols such as dithiothreitol (DTT) and β -mercaptoethanol. Therefore, the inactivation experiments were carried out in well degassed thiol-free buffers, and little loss of activity was observed under these conditions. Some new substrate analogs were synthesized in the present study and were found to be irreversible enzyme inhibitors.

Table 2Inhibition constants of the reversible inhibitors.

Compounds	K_{i} (mM)	IC ₅₀ (mM)
Laevulinic acid (1)	0.96	_
6-Amino-5-oxohexanoic acid (6)	1.07	_
4-Oxo-sebacic acid (28)	2.93	_
5-Oxo-hexanoic acid (3)	3.05	_
5-Amino-4-oxopentanenitrile (16)	18.48	-
1-Amino-4-hydroxy-2-butanone (9)	26.52	-
4-Oxo-pentanenitrile (14)	73.04	-
1-Amino-4-methoxy-2-butanone (11)	-	30.00
1-Amino-5-hydroxy-2-pentanone (21)	-	32.50



Fig. 7. (a) Inactivation of rat PBG synthase by 1 mM (■) and 5 mM (▲) 5-bromo-laevulinic acid (2); (b) Inactivation of rat PBG synthase by 0.1 mM of compounds 10 (♦), 7 (■), and 15 (▲); (c) Inactivation of rat PBG synthase by 0.1 mM (♦) and 0.5 mM (▲) 4-oxo-decenedioic acid (26).

The inactivation experiments were carried out by incubating a relatively concentrated solution of thiol-free enzyme with the analogs. At various time intervals, aliquots were taken and added to the assay mixture containing ALA at a final concentration of 10 mM. The time-dependent loss of activity was noted during the incubations (Fig. 7). It should be noted that some compounds have been found to be irreversible inhibitors of PBG synthases from various sources, but none of their apparent dissociation constants (K_i) and the inactivation rate constants (k_{inact}) have been determined [50], because the reaction involves two ALAs and the inhibitions do not fit into Michael–Menton equation. Our attempt to determine the K_i and k_{inact} values of our synthetic analogs was not successful because of the same reason.

Almost all our irreversible inhibitors contain bromide except compound **26**, which is α , β -unsaturated dicarboxylic acid. 5-Bromolaevulinic acid (**2**) was found to be a weak irreversible enzyme inhibitor, as shown in Fig. 7a. This is consistent with the inactivation properties of 5-chlorolaevulinic acid for bovine and *E. coli*. PBG synthases [43,51,52]. Comparing with 5-chlorolaevulinic acid, compound **2** has a bromine atom, a more sensitive leaving group at C-5 position. Therefore, compound **2** is assumed to inactivate the enzyme in the same mechanism as that of 5-chlorolaevulinic acid. For bovine PBG synthase, 5-chlorolaevulinic acid modified the protein at Cys223, to which the ZnA was bound in the active site. It is possible that compound **2** is attacked by the same nucleophilic cysteine.

Compounds **7**, **10**, and **15** showed considerable inactivation of PBG synthase at low concentration (0.1 mM) (Fig. 7b). Comparing with compound **2**, these compounds lack the carboxyl group that is very important to bind the compound to the active site. Interestingly, the tight binding analog **2** led to weak inactivation of the enzyme, while loose-binding analog **7**, **10**, and **15** gave strong inactivation.

Compounds **12**, **13**, **17**, and **18** were all found to be irreversible inhibitors of rat PBG synthase as shown in Fig. 7c, which are simple substrate analogs with bromine atom. Compounds **13** and **18** have two bromine atoms to react with more nucleophilic residues, therefore both of them are stronger enzyme inhibitors than compounds **12** and **17**. This may indicate that more than one nucleophilic residue could be labeled by the same or different enzyme inhibitors leading to enzyme inactivation.

Compound **26** was found to be a moderate irreversible inhibitor of rat PBG synthase, and its time-dependent inactivation of the enzyme is shown in Fig. 7d. In comparison, compound **28** is the corresponding saturated derivative of compound **26** and was found to be a moderate competitive inhibitor. This indicates that the β position of compound **26** could be attacked by a nucleophilic residue of rat PBG synthase through conjugate addition reaction leading to enzyme inactivation.

All these compounds could inactivate rat PBG synthase by reacting with one or more nucleophilic residues. There are four cysteines in the active site of PBG synthase (Fig. 4), which can substitute the bromine atom of the substrate analog or react with the α , β -unsaturated analog as good nucleophilic reagents. If an analog is bound to the active site tightly, the compound may only be able to react with one of these cysteines. But if an analog is bound to the active site loosely, it may react with more cysteines leading to stronger inactivation. This study further increased our understanding of the active site of PBG synthase.

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