Bioorganic Chemistry 37 (2009) 33–40

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

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

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ARTICLE INFO

Article history:
Received 1 October 2008
Available online 17 December 2008

Keywords:
Porphobilinogen synthase
PBG synthase
5-Aminolaevulinic acid dehydratase
ALA dehydratase
5-Aminolaevulinic acid
Porphobilinogen
Tetrapyrrole

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 give stronger inactivation. The amino group and carboxyl group of 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 (ALA[1] 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 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 nonadditive 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

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[1] *Abbreviations used:* ALA, 5-aminolaevulinic acid; DHP, 1,2-dihydropyrane; DMF, dimethyl formamide; DMSO, dimethyl sulfuroxide; DTT, dithiothreitol; IPTG, isopropyl-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, tetrahydropyran; UV/vis, ultraviolet–visible spectroscopy.

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doi:10.1016/j.bioorg.2008.11.001

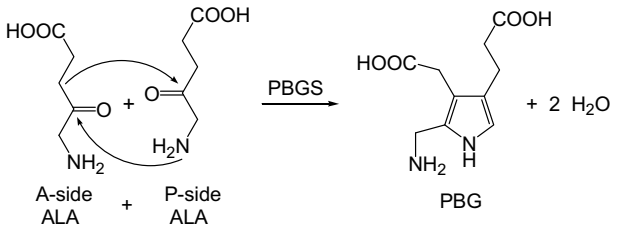

Fig. 1. Reactions catalyzed by PBG synthase.

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 Pb^{2+} [34,35]. Al^{3+}, Ga^{3+}, and In^{3+} inhibit bovine liver PBG Synthase by competing with Zn^{II}, whereas Tl^{3+} and In^{3+} 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 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 SalI 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 aag ctt tta 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