Bioorganic Chemistry 91 (2019) 103117

Contents lists available at ScienceDirect

Bioorganic Chemistry

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

Mechanistic studies on *Pyrobaculum calidifontis* porphobilinogen synthase (5-aminolevulinic acid dehydratase)

Naseema Azim[a], Qurratulann Afza Gardner[a], Naeem Rashid[a], Muhammad Akhtar[a,b,*]

[a] School of Biological Sciences, University of the Punjab, New Campus, Lahore 54590, Pakistan
[b] Biological Sciences, University of Southampton SO17 1BJ, UK

ARTICLE INFO

Keywords:
Heme
Tetrapyrroles
Catalytic zinc
Schiff base
MALDI and ESI mass spectrometry
Affinity labeling
Active site residues

ABSTRACT

Porphobilinogen synthase (PBG synthase) gene from *Pyrobaculum calidifontis* was cloned and expressed in *E. coli*. The recombinant enzyme was purified as an octamer and was found by mass spectrometry to have a subunit M_r of 37676.59 (calculated, 37676.3). The enzyme showed high thermal stability and retained almost all of its activity after incubation at 70 °C for 16 h in the presence of β-mercaptoethanol (β-ME) and zinc chloride. However, in the absence of the latter the enzyme was inactivated after 16 h although it regained full activity in the presence of β-ME and zinc chloride. The protein contained 4 mol of tightly bound zinc per octamer. Further, 4 mol of low affinity zinc could be incorporated following incubation with exogenous zinc salts. The enzyme was inactivated by incubation with levulinic acid followed by treatment with sodium borohydride. Tryptic digest of the modified enzyme and mass spectrometric analysis showed that Lys^{257} was the site of modification, which has previously been shown to be the site for the binding of 5-aminolevulinic acid giving rise to the propionate-half of porphobilinogen. *P. calidifontis* PBG synthase was inactivated by 5-chlorolevulinic acid and the residue modified was shown to be the central cysteine (Cys^{127}) of the zinc-binding cysteine-triad, comprising $Cys^{125, 127, 135}$. The present results in conjunction with earlier findings on zinc containing PBG synthases, are discussed which advocate that the catalytic role of zinc in the activation of the 5-aminolevulinic acid molecule forming the acetate-half of PBG is possible.

1. Introduction

Pyrobaculum calidifontis strain VA1 is a hyperthermophilic archaeon belonging to the genus *Pyrobaculum*. This genus is known for the diversity of its members in terms of oxygen requirements, comprising from strict anaerobes to aerobes [3]. *P. calidifontis* is known to be a facultative anaerobe. Interest in the oxygen requirement of this organism led us to study the enzymes, responsible for the synthesis of heme; the latter is central to oxygen assimilation [4].

The genome sequence of *P. calidifontis* has been annotated to predict open reading frames for three of the enzymes of the tetrapyrrole pathway [36]. Two of the enzymes are porphobilinogen synthase (previously, 5-aminolevulinic acid dehydratase; hereafter referred to as the synthase or PBG synthase) and porphobilinogen deaminase, which catalyze the early steps in the biosynthesis of heme, chlorophylls and corrins; an example of the latter is vitamin B_{12} [33].

The synthase has been studied from several sources and catalyses the condensation of two molecules of 5-aminolevulinate (ALA) to produce porphobilinogen (PBG), the 5-membered ring of which, then becomes the pyrrole ring of all biological tetrapyrroles (Scheme 1) [52,57,26,53]. Single turnover experiments performed on PBG synthase confirm that both ALA molecules bind at two different sites [35]. Since the condensation of both the molecules is asymmetric both of them give rise to two different side chains of porphobilinogen that is, the propionate and the acetate side chain. The binding site for the substrate molecule that serves as the source for the propionate side chain is named as the P-site whereas the second molecule that is the source of the acetate side chain is termed as the A-site of substrate binding.

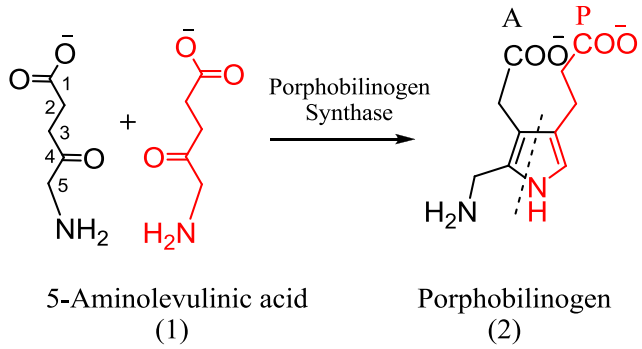
The Sequence alignment in Fig. S1 (A and B) represents PBG synthases from *Saccharomyces cervisiae, Homo sapiens, Drosophila melanogaster, Pyrobaculum calidifontis, Escherichia coli* and *Pisum sativum*. This alignment shows the *N*-terminal long arm of *P. sativum* PBG synthase, which is a characteristic of plant enzymes ([29]).

Almost all known PBG synthases have been described as metal

Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; A-site, binding site of ALA molecule which becomes the acetate side chain of PBG; P-site, binding site of the ALA molecule which becomes the propionate side chain of PBG

* Corresponding author at: Biological Sciences, University of Southampton SO17 1BJ, UK.
E-mail address: ma3@soton.ac.uk (M. Akhtar).

https://doi.org/10.1016/j.bioorg.2019.103117
Received 19 March 2019; Received in revised form 9 July 2019; Accepted 10 July 2019
Available online 13 July 2019



Scheme 1. Condensation of two molecules of 5-aminolevulinate to produce porphobilinogen. The condensation results with one ALA donating the acetate arm of porphobilinogen (denoted by A in **2**) while the propionate arm originates from the other molecule (denoted by P in **2**).

dependent, however, the identity of the interacting metal ions varied between bivalent zinc or magnesium [29]. There are reports that some enzymes respond to other metal ions like cobalt, potassium and sodium under laboratory conditions [18] or are totally metal independent [7]. Enzymes from mammalian, yeast and most bacterial sources are usually zinc dependent [22,51] while those from plant [40] and photosynthetic bacterial sources have been identified to interact with magnesium ion. The type of metal ion needed by the enzyme was used for some time to classify PBG synthase as being either magnesium or zinc ion dependent but later studies have identified enzymes from various sources that interact with both metal ions simultaneously like *E. coli*, which is a zinc dependent enzyme but interacts with magnesium allosterically [43,55]. Detailed reviews on this aspect are available for example, [29].

In this paper we describe, the cloning of the synthase from *P. calidifontis*; its purification, kinetic properties, identification of its active site residues and the role of zinc atoms necessary for catalysis.

2. Results

2.1. Cloning, purification and kinetic properties of PBG synthase from P. Calidifontis

The gene for the synthase was amplified from the genomic DNA of *P. calidifontis* as described in the Section 4.1. The PCR product was transferred to pET-21a, via pTZ57R, the latter sequenced, to show an open reading frame encoding a protein of M_r 37676.3 (Fig. S2). The plasmid, pET-21a, harbouring the synthase gene was then used to transform *E. coli* BL21-CodonPlus (DE3)-RIL which was grown in Luria broth and the protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells expressing the recombinant synthase were processed to purify the enzyme (Table S1A). The SDS-PAGE profiles at each stage of purification is presented Fig. S3A. Fig. S3D shows the ion exchange chromatography profile, when **peak a** was found to contain the PBG synthase activity; its reanalysis by HPLC (Fig. S3E) gave a single peak. MALDI-MS spectrum of the purified enzyme gave a $[M + 1H]^{+1}$ ion of 37677.1 (Fig. 1A) which corresponds to the predicted mass for the monomer of 37676.3 Da. The ESI –MS confirmed the value giving a mass of 37676.6 Da (Fig. 1B). In gel filtration the *P. calidifontis* PBG synthase eluted in the same fraction as the bovine enzyme which has been shown to be an octamer [57]. The octameric state of *P. calidifontis* PBG synthase was also shown by its crystallographic structure [42]. The ESI spectrum (Fig. 1C) showing the presence of oligomeric species containing up to four subunits, highlighted that the subunit-subunit interactions in this hyperthermophilic protein are very strong to withstand the harsh conditions (325 °C) of ESI mass spectrometry [2].

The activity of the synthase, measured in the presence Zn^{2+} and β-mercaptoethanol (β-ME) increased exponentially with temperature and the highest activity was recorded at 96 °C (Fig. S4A). The enzyme had high thermal stability and retained more than 45% of its activity after being incubated for 10 h at 100 °C (Fig. S4B). The enzyme as purified by the protocol in the Section 4.1, without any addition, had around 60–65% of its activity which was increased in the presence of Zn^{2+} and β-ME giving a K_m for ALA of 0.18 mM. When the activation is quantified in the presence of excess β-ME and varying concentrations of Zn^{2+}, a K_m for the latter of 2.5 μM was found. This reveals the low affinity Zn^{2+} compared to tightly bound Zn^{2+} which survives the harsh purification procedure and is responsible for the activity of the **stored-enzyme.**

2.2. Active site modifications

The active site of the enzyme was investigated, extending affinity labeling approaches used previously for the synthase from other sources [30,32,11,16,15]. Levulinate, an analogue of the substrate (ALA) is known to bind to one of the two substrate binding sites of the enzyme [44]. When the synthase from *P. calidifontis* (Fig. 2A) was incubated with levulinate and the expected Schiff base adduct (**3**, Scheme 2) trapped by $NaBH_4$ (Table 1), the enzyme was inactivated and its ESI spectrum showed the presence of a main species corresponding to the synthase conjugated to levulinate by a reduced Schiff base linkage (Fig. 2B). Following carboamidomethylation to protect the Cys thiol groups, the protein was digested with trypsin and the peptides analyzed by on-line ESI -LC/MS (Section 4.6.3). On separation, 23 tryptic peptides were identified giving sequence coverage of 81%. Amongst these was the peptide comprising residues 242 to 267, the mass of which was 100 Da more than that of the native peptide (**4**, Scheme 2) corresponding to modification by a levulinate group (see Table 2). Since the amino acid sequence of the peptide has a single Lys at position 257 this must have been modified. This is the P-site lysine equivalent to Lys^{252} in human synthase [23,14,13] (see Fig. S1 for the position of this residue in other PBG synthases).

Another active site directed inhibitor previously investigated for the synthases from other organisms is 5-chlorolevulinate [50,30,32,9]. In the present study, incubation of the synthase with 5-cholorolevulinate led to a time dependent loss of activity (Fig. S5). Mass spectrometric examination of the inactivated protein showed the presence of a major species corresponding to the incorporation of a single C_5 unit (Fig. 2C). It should be noted that the preparation which, following inactivation, contained 13.9% of the activity also showed a similar presence of unmodified protein in the mass spectrometric analysis (Fig. 2C). This shows that all the subunits of the enzyme were equally available for modification. In order to protect remaining Cys thiols, this 5-chlorolevulinic acid modified synthase was treated with iodoacetamide and the product processed for in-gel digestion using trypsin (Section 4.6.2). The resulting tryptic digest was analyzed by MALDI in search of a peptide containing the cluster of three cysteine residues, two of which should be carboamidomethylaed and the third containing the C_5 unit (Table S2). Such a peptide corresponding to residues 118 to 139 ($[M + 1H]^{+1}$ ionic peak of 2635.2) was identified and subjected to MS/MS analysis (Section 4.6.4). The salient features of its MS/MS fragmentation (Scheme 3; details in Table S3) are as follows.

The y_5 fragment arising from the cleavage of the bond between His^{134} and Cys^{135} gave a $[M + 1H]^{+1}$ ionic peak of 562.0 due to the pentapeptide residues 135–139, containing a carboamidomethyl moiety. In y-ion series, a difference between the masses of y13 and y12 of 217 units (theoretical; 217 amu) was observed which corresponds to Cys^{127} substituted by a C_5 unit (Scheme 3). Then the substitution status of the third cysteine, Cys^{125}, was shown by the mass difference of 159.8 (theoretical 160) between y15 and y14 ions, expected for the presence of a carboamidomethyl group. The results thus clearly identify the substitution pattern of the three cysteine residues and show that Cys^{127} was the predominant target for modification by 5-chlorolevulinate. The importance of the central cysteine of the cysteine-triad was also shown in human enzyme, where mutation of any of these cysteine residues

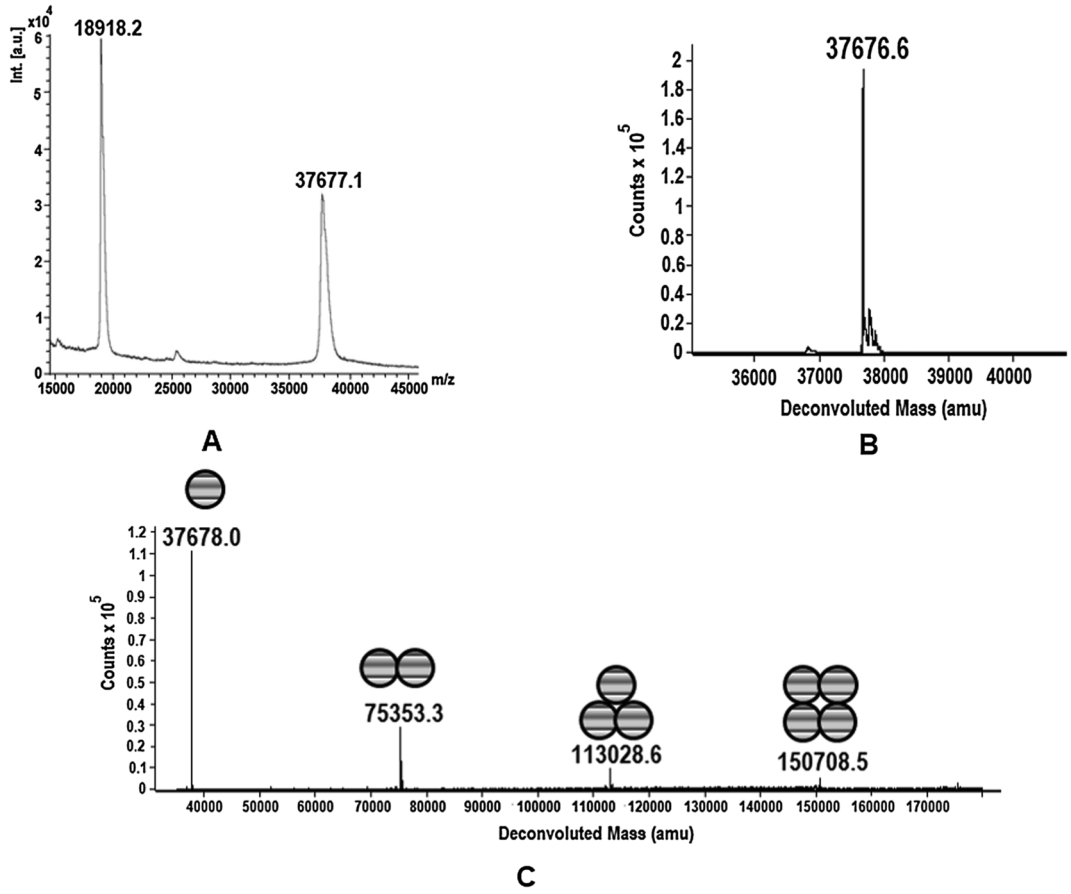


Fig. 1. Mass spectra of *P. calidifontis* PBG synthase. (**A**) MALDI-MS spectrum showing the $[M + 1H]^{+1}$ ion of 37677.1. (**B**) ESI mass spectrum showing a deconvoluted peak of 37676.6 amu. (**C**) Oligomeric state of PBG synthase (mono, di, tri and tetramer) seen in LC-ESI MS analysis is annotated by shaded circles.

diminished the activity of the enzyme but the adverse effect was greater when the central cysteine was mutated compared to the others [48].

2.3. Concerning the requirement of Zn^{2+} and thiols for the activity of the synthase

PBG synthase from non-plant sources show varying requirement for zinc and thiols. A somewhat similar but distinctive behavior was shown by the *P. calidifontis* synthase in the present study (see Table 3). Thus the purified enzyme, as such, or after storage, referred to as **stored-synthase**, contained 0.5 mol atoms of zinc per subunit of the octamer, and when assayed using ALA, without any other addition, had around 60–65% of the maximum activity. The full activity was obtained when the enzyme was pre-incubated with Zn^{2+} plus β-ME and assayed in their presence. Immediately following the removal of Zn^{2+} and β-ME by gel filtration, the **activated synthase** exhibited the maximum activity and contained 0.8 mol atoms of zinc per subunit. However, when the **activated-synthase** was incubated at 70 °C, there was a time dependent biphasic loss of activity, when the enzyme was assayed without Zn^{2+} plus β-ME. The first phase of inactivation, attended by a rapid loss of around 50% of the activity was 15 times faster than the second phase. After 16 h of incubation the enzyme had lost nearly 96% of the activity when assayed in the absence of Zn^{2+} plus β-ME but showed full activity in their presence (Fig. 3). The enzyme is designated as **inactivated-synthase**.

In the X-ray structure of PBG synthase from *P. calidifontis*, the cluster of three thiols belonging to $Cys^{125, 127, 135}$ converges on the Zn ion [42], as is the case with other synthases whose X-ray structures have been elucidated [17,16 15,12,31]. We hence considered the possibility that heat dependent inactivation of the enzyme shown in Fig. 3, may be due to the formation of disulphide bond between the juxta positioned thiols; for which there are three possible combinations ($Cys^{125-127}$, $Cys^{125-135}$, $Cys^{127-135}$). The thiol status of the three forms of synthase was investigated using the –SH specific reagent, *N*-ethylmaleimide (NEM). When the **stored**- or **activated-synthase,** was subjected to reaction with NEM, for 30 min at 37 °C, the mass spectrometric analysis of the reaction mixture showed that mono- plus di-modified species comprised 50–60% of the product, the remaining being the unmodified material. Surprisingly, the extent of modification was far greater (90%) with the heat **inactivated-synthase** (Figs. 4 and 5). Not only that the latter enzymic form had thiol groups, these were conformationally more accessible for modification compared to the thiols in **stored-** or **activated-synthase**. It would thus appear that the main effect of heat inactivation is to cause local changes to loosen zinc-ligand interactions. In the light of these considerations, it would appear that the requirement of both Zn^{2+} and β-ME for the conversion of **heat-inactivated** into **activated-synthase** could be that correct delivery of Zn^{2+} to the active site involves not Zn^{2+} but a zinc–β-ME complex.

3. Discussion

Seminal work on PBG synthase (then 5-aminolevulinate dehydratase) was reported by Shemin [52,49] and Neuberger [24] who established the stoichiometry of the reaction showing that two molecules of 5-aminolevulinate condense to produce the product, porphobilinogen, as shown in Scheme 1 [44]. Using bacterial and mammalian enzymes it was also shown that PBG synthase is an octamer of identical subunits containing 8 mol atoms of zinc per octamer. Subsequent extensive studies on the enzyme from other sources have, by and large, validated these features, except, that in enzymes from plants, zinc

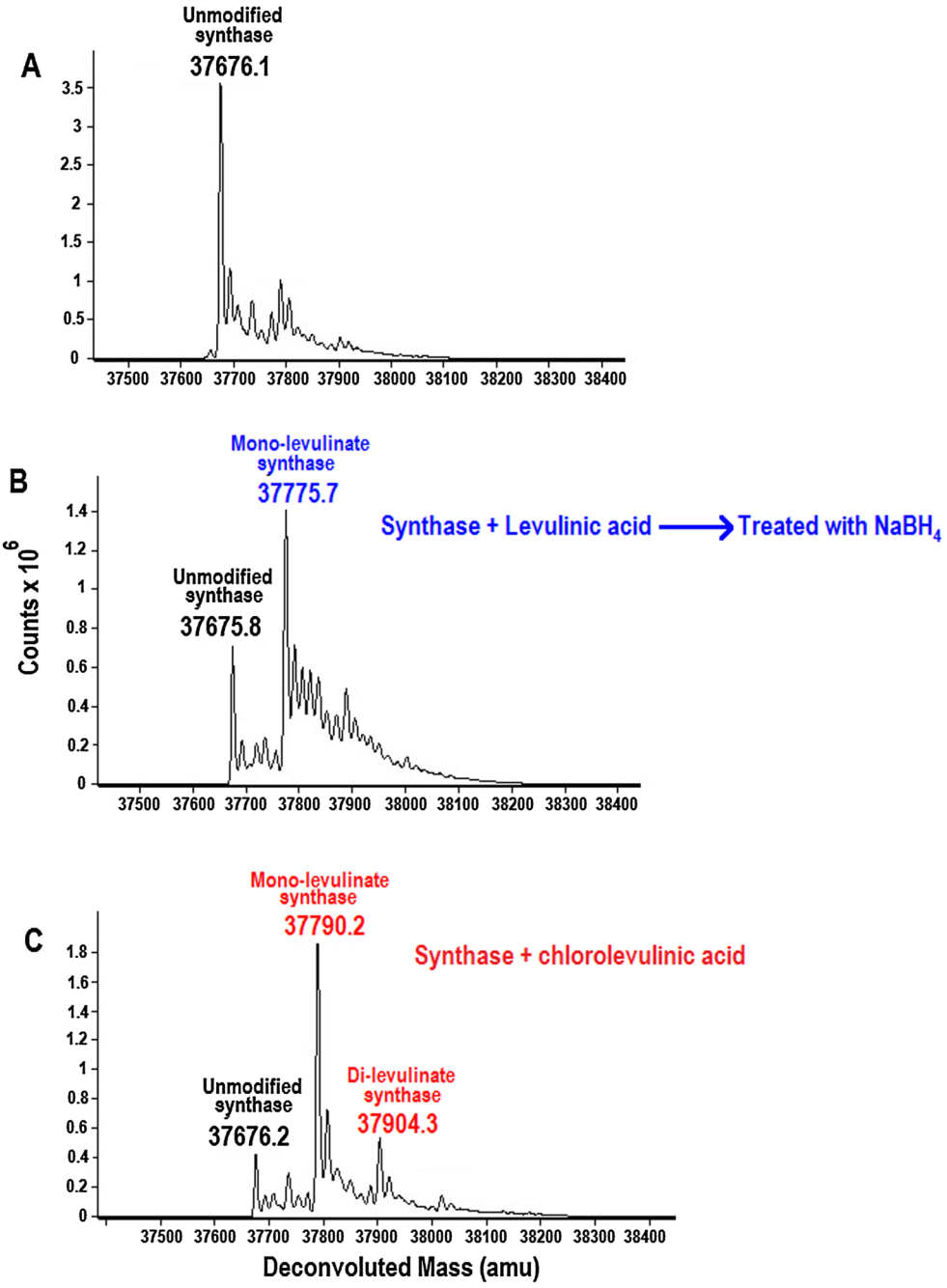
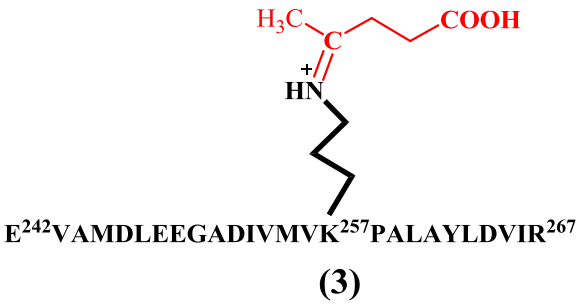


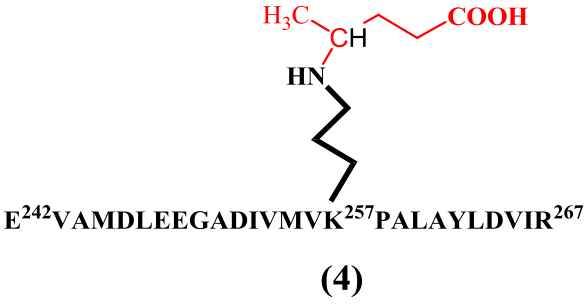
Fig. 2. ESI mass spectra of variously modified PBG synthase from *P. calidifontis*. (**A**) Unmodified control (peak labeled in black, theoretical mass, 37676.3 amu). (**B**) PBG synthase modified with levulinic acid plus $NaBH_4$, (peak labeled in blue, theoretical mass, 37776.3 amu) (**C**) 5-chlorolevulinic acid modified PBG synthase (peaks labeled in red, theoretical masses for the mono- and di-carboamidomethylaed species, 37790.3 and 37904.3 respectively).

requirement is replaced by magnesium [54,28]. In zinc dependent PBG synthases, a zinc ion (designated as catalytic Zn) is ligated to three conserved cysteine residues. Some synthases also contain another zinc ion (ligated to C^{223} along with four water molecules in the human enzyme), the precise role of which has not been clearly demonstrated. The latter zinc site is absent in *P. calidifontis* synthase, which in view of its close mechanistic similarity to synthases from other sources, would suggest that it is not directly involved in catalysis. In some synthases an 'allosteric' site involving Mg^{2+} has also been identified, although most residues involved in this interaction, as identified in allosterically active *E. coli* enzyme, are present in the *P. calidifontis* synthase, 1 mM Mg^{2+} had no effect on the activity of the enzyme (Table S1B). With respect to zinc content, PBG synthases have considerable variation i.e. 16 per octamer in yeast, 8 in *E. coli* and mammalian and 4 in *Drosophila* [27,38]. However, in general four zinc atoms per octamer give optimal enzymic activity. The latter feature is clearly shown by the *Drosophila* enzyme, in which the activity of the enzyme plateaued after the addition of four mole atoms of zinc per octamer, suggesting that the octamer comprises two asymmetric tetramers [38].

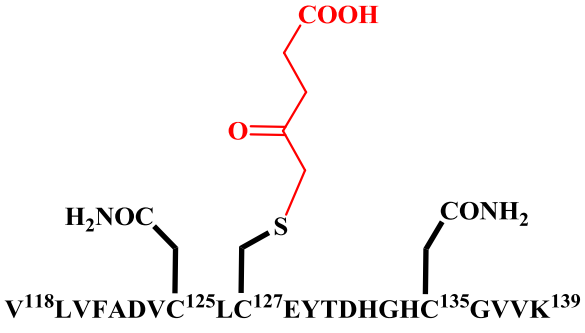
The *P. calidifontis* enzyme studied in this work, after multistage purification, in the absence of Zn^{2+}, contains four high affinity zinc ions, resistant to removal by EDTA, and the protein shows 60–65% of the maximum activity. Full activity is restored in the presence of exogenously added zinc and β-ME, the K_m for this low affinity zinc is 2.5 μM. Thus in this case also the octamer consists of two asymmetric tetramers, both of which are enzymically active but to a different extent



(3)



(4)



(5)

Scheme 2. Active site peptides of PBG synthase. Structure of levulinate attached to K^{257}, of the enzyme, via a Schiff bond (**3**) and the reduced adduct of the latter (**4**) (found M_r 2959.5; calculated, 2959.4). Structure of the peptide residues 118–139 (**5**), containing a C_5 moiety (red), from 5-chlorolevulinate and two carboamidomethylaed groups to protect the cysteine residues (found M_r, 2634.2; calculated 2634.1).

Table 1
Activity of PBG synthase from *P. calidifontis* modified with levulinic acid plus $NaBH_4$. The enzyme 0.4 mg was treated with levulinic acid and $NaBH_4$ in different permutations. The details are in the Section 4.5.1.

Sample	Activity (U/mg)
1. Stored-synthase	19
2. Stored-synthase treated with $NaBH_4$	19
3. Stored-synthase treated with $NaBH_4$ then with levulinate	17
4. Stored-synthase treated with levulinate then $NaBH_4$	3.5

(having activity in the ratio of 2:1). The asymmetry is also shown by the thermal inactivation experiments when the first-half of the activity was lost at a rate 15 times greater than the second half (Fig. 3).

The requirement of Zn^{2+} and thiols to activate PBG synthases has been often reported in the literature. With the *P. calidifontis* PBG synthase, it was shown that in the heat **inactivated-enzyme,** thiols were intact and, if at all, susceptible to more ready modification by NEM than those in the fully activated enzyme, indicating the opening of the active site in the inactive protein. In synthases from other sources, the octamer has been found to dissociate into dimers that can change conformation and reassemble to form a hexameric species with reduced activity. The possibility was considered whether the **inactivated-synthase** had also undergone transformation into smaller ensembles. This aspect was studied by non-denaturing PAGE analysis which showed that the **inactivated-synthase** migrated in the same region as the **stored-synthase**. If at all, the former showed signs of marginal retardation indicative of loss of globular elements but retaining the same structure as the **activated-synthase** (Fig. S3B and C). In passing it may be mentioned that unlike SDS PAGE, where proteins migrate according to their masses, the mobility in non-denaturing PAGE depends on several factors, such as mass, shape and charge. The main value of the latter technique is that it may maintain proteins in their active form, without giving information on their size. This feature is shown in Fig. S3B and C, where two oligomeric proteins, phosphorylase b dimer (194,578 Da) and lactate dehydrogenase tetramer (136,700 Da), were used as reference and it was found that the latter, with smaller size, moved slower, presumably, because of its low PI value, while the migration of PBG synthase (octamer 301,410.4 Da) was retarded relative to phosphorylase b dimer (194,578 Da). Thus, all the three forms of PBG synthase migrated in the same region as these two reference proteins and, more importantly, the migration was not affected by the presence of ALA in the samples (see Fig. S3C), which is claimed to stabilize the octameric structure [29]. Because of the constraint mentioned above, we cannot tell the subunit-number of the synthase, during non-denaturing PAGE, however, it is prudent to conclude that the **inactivated-synthase** has the same subunit structure as the other forms[1], except that its thiols are conformationally more accessible to the solvent. Given that the thiols are intact in the **inactivated-synthase,** the requirement of zinc as well as β-ME for the activation of the **inactivated-synthase** could be attributed to the possibility that correct delivery of zinc to the active site involves not zinc ion but a zinc–β-ME complex.

Two aspects of the mechanism of PBG synthase are well established. The first is the seminal finding of Jordan (now Shoolingin-Jordan), who using human and bovine PBG synthases showed that of the two molecules of the substrate, the first molecule of ALA binds to the enzyme by a Schiff base linkage, involving Lys^{252} (in human) and becomes the propionate side-chain of porphobilinogen (**7**, Scheme 4) [35,34,23]. The second, that in the further processing of the P-site ALA its H_{Re} is stereospecifically removed (**9**, Scheme 4) [1,10]. Lysine residue, corresponding to Lys^{252} (in human) are conserved in the sequences of other synthases discovered subsequently and are considered to be the Schiff base forming residue (Fig. S1). Here, we have directly identified the residue to be Lys^{257} in the sequence of *P. calidifontis* PBG synthase.

The next step, in the conversion of the initially formed Schiff base intermediate (**7**, Scheme 4), is the addition of the second molecule of ALA, which, in the earliest conjuncture on this aspect, was assumed to bind to the tri-cysteine binding catalytic zinc which acts as a Lewis acid to polarize the carbonyl group of the A-site ALA [15,16] Using the *P. calidifontis* PBG synthase, we have shown in the present paper that 5-chlorolevulinate, a substrate analogue of ALA, alkylates Cys^{127}, suggesting that an ALA binding site is closely positioned with respect to the cysteine, hence by implication to the zinc ion. Such a scenario is

[1] It should be remembered that using other techniques the subunit structure of the stored synthase has been established to be octameric.

Table 2
Theoretical and observed ESI masses for the tryptic peptide of PBG synthase from *P. calidifontis* modified with levulinic acid (LA) after $NaBH_4$ reduction.

Positions	Sequence	Modification	Theoretical mass (amu)	Observed mass (amu)
242–267	EVAMDLEEGADIVMV$\mathbf{K^{LA}}$PALAYLDVIR	K^{257} modified by LA	2959.48	2959.53
242–267	EVAMDLEEGADIVMV**K**PALAYLDVIR	None	2859.48	Not seen

supported by X-ray structures, in which A-site ALA can be modeled adjacent to the zinc, to polarize its carbonyl group [32,13]. The X-ray structures show that the fourth ligand for zinc is a water molecule, which as hydroxide can act as base for removal of one of the hydrogen atoms from C-3 as shown in structure **7** (Scheme 4), producing an enolate **8** ready to attack the Schiff base at C-4 of the P-site ALA moiety. The further conversion of the resulting intermediate **9** into porphobilinogen involves three manipulations; (a) removal by the aforementioned hydroxide, of H* from C-3, of the A-site ALA, coupled to elimination of the enzyme (b) formation of the C=N bond between the A-site carbonyl and P-site $–NH_2$ (c) and deprotonation of H_{Re} from C-5 of the P-site, presumably by the amino group of P-site lysine, leading to the formation of the pyrrole ring. The order in which these events occur has been speculated [15,16].

In passing, it should be mentioned that the centrally located Cys^{127}, modified by 5-chlorolevulinate, being the most nucleophilic of the three cysteine residues is orthogonally located to the fourth water ligand. The side chain of Cys^{127} is the most solvent exposed of the three cysteines at the active site and it points towards the A-site of the enzyme. We cannot tell whether the nucleophilicity is merely a reflection of the geometric relationship, or it is the result of interaction with neighbouring side chains and how the negative charge contributes to catalysis.

The aforementioned mechanistic view, however, underwent gradual modification in the light of X-ray crystallographic observations made on PBG synthase-inhibitor complexes [37,20,13]. When yeast PBG synthase was co-crystallised with a diketo acid inhibitor, 4, 7-dioxosebacic acid, the diffraction data showed that the inhibitor was bound to the enzyme, not only, by the P-site lysine but also another invariant lysine [37]. Contemporaneously, it was shown that D139N mutant of a magnesium-dependent PBG synthase from *Pseudomonas aeruginosa* in the presence of 5-fluorolevulinic acid, gave crystals in which two molecules of the inhibitors were bound to two conserved lysine residues, Lys^{260} and Lys^{205} [20,19]. The role of P-site lysine (Lys^{260}) in Schiff base formation had already been established with zinc-dependent PBG synthases, now Lys^{205} was assigned a similar function in binding A-site ALA, culminating in the proposal of a double Schiff base mechanism [37,20,13,19].

In our view, such a mechanism seems plausible for magnesium-dependent PBG synthases where a metal ion is not located at the active site and thus unable to activate the A-site ALA molecule for condensation. In the X-ray map, the Mg^{2+} ion is present 10 Å from the active site [20], apart from its remoteness, magnesium ion is rarely, if at all, used as Lewis acid, for carbonyl activation, in enzymology. However, in the case of zinc dependent PBG synthases, where a double Schiff base is invoked, the inhibitor contained two electrophilic groups, one occupying the position of P-site ALA (as well as inhibitors, for example levulinic acid, as shown here) which covalently binds to P-site lysine. Once this reaction has occurred there is no assurance that the second carbonyl group of the inhibitor, during the long crystallization process will not react with another amino group present in the active site. The evidence for a double Schiff base mechanism for zinc dependent PBG synthases is thus weak. In contrast a number of X-ray structures have shown that in zinc dependent PBG synthases the metal ion is

Table 3
Effect of Zn^{2+} and βME on the activity of various forms of synthase. Stored-synthase is the enzyme following purification. **Activated-synthase** is the enzyme after the incubation of the stored synthase with Zn^{2+} and β-ME followed by their (excess) removal using gel filtration. **Heat-inactivated enzyme** is that where the activated synthase is incubated for 16 h at 70 °C (Fig. 3). The assays were performed as outlined in the Section 4.4.

Enzyme-form and assay conditions	Activity (%)
Stored-synthase	
No addition	**62.9**
Plus Zn^{2+}	**60**
Plus β-ME	**77.4**
Plus Zn^{2+} and β-ME	**100**
Activated- synthase	
No addition	**99.4**
Plus Zn^{2+}	**92**
Plus β-ME	**99.6**
Plus Zn^{2+} and β-ME	**100**
Heat-inactivated- synthase	
No addition	**4.43**
Plus Zn^{2+}	**4.8**
Plus β-ME	**80.1**
Plus Zn^{2+} and β-ME	**100**

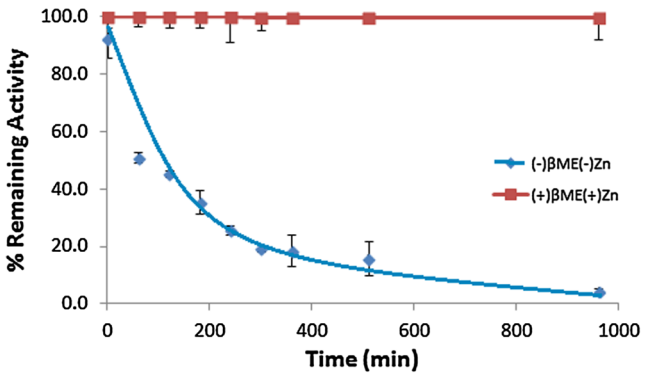
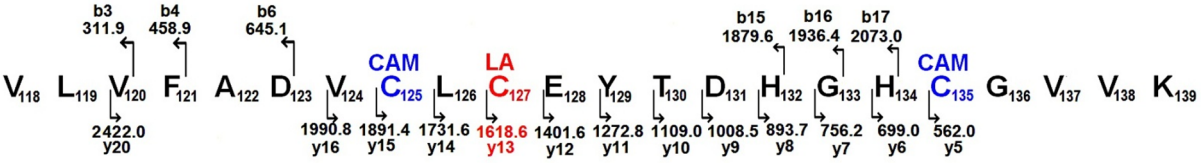


Fig. 3. Heat inactivation of activated-sythase. Stored-synthase was activated by incubation with $ZnCl_2$ and β-ME, which following gel filtration was incubated at 70 °C. Aliquots of 10 μL were removed (in triplicate) at different time intervals and assayed for the enzyme activity either in the presence (red) or absence of $ZnCl_2$ and β-ME (blue).



Scheme 3. MS/MS fragmentation of tryptic peptide, residues 118–139 from the modification of PBG synthase with 5-chlorolevulinic acid. The observed $[M + 1H]^{+1}$ ion of the modified peptide was 2635.2 whereas that of the naked peptide was 2407.1 (not seen;, Table S3).

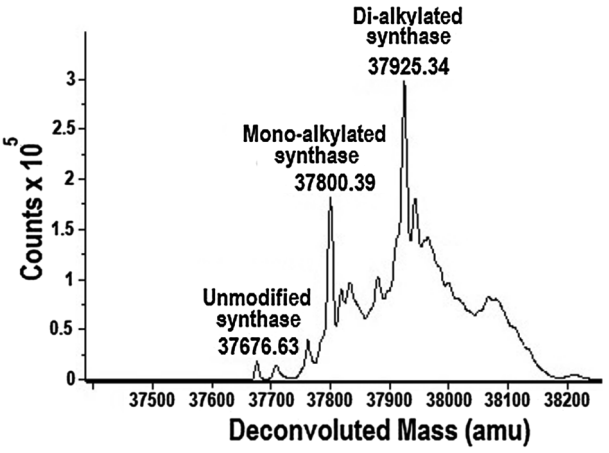


Fig. 4. ESI mass spectrum of inactivated-synthase modified by NEM at 37 °C for 30 min showing peaks for unmodified (theoretical; 37676.3 amu), mono- (theoretical; 37801.3 amu) and di-alkylation (theoretical; 37926.3 amu) modification.

located where it is available to comfortably coordinate with the carbonyl group of A-site ALA. Furthermore, in these cases the zinc ion is coordinated to three amino acid side chains and the fourth ligand being a water molecule. In 1990, X-ray crystallographic structures of 12 zinc enzymes was known [56,28], using the data Vallee and Auld generalized that catalytic zinc would normally be coordinated to three amino acid ligands and the fourth ligand being water, as is the case with zinc dependent PBG synthases. In contrast structural zinc containing proteins would have all the four amino acid ligands. The spacing of the three cysteines of PBG synthase from *P. calidifontis* is one and seven residues, which partly fulfils another Vallee-Auld requirement that two of the amino acid ligands will be closely located (one residue apart here) and the third by a 'longer spacer' of 20–120 amino acids. In the present example the space being 7 residues is less optimal, though similarly short spacing is found for a group of zinc dependent proteases, astacin [6]. Thus, despite authoritative advocacy of a double Schiff base mechanism for the formation of PBG by zinc dependent PBG synthases, possibility should remain that with the latter enzymes the mechanism originally considered by the Southampton School [15,16] where zinc had played a catalytic role is a serious contender. If it is for no other reason, than the fact that zinc with an accepted coordination geometry is located at the centre of the active site, as is shown by the 5-chlorolevulinate inactivation experiments above, and by precedent is suitable for the key step in the condensation process (**8** to **9**, Scheme 4).

What then could be the role of the second lysine, corresponding to Lys^{204} of *P. calidifontis* PBG synthase. This residue as well as the P-site lysine (K^{257}), are located close to an aspartate-serine pair. It is conceivable that the closeness facilitates that Lys^{204} is in a protonated form lowering the pK_a of the neighbouring P-site lysine, keeping it in a basic form, suitable for Schiff base formation (reaction **6** to **9**) and proton removal (reaction **11** to **2**).

If it turns out that zinc and magnesium dependent PBG synthases operate by different mechanisms, this will mirror the situation with aldolases, where Schiff base and zinc dependent catalysis exist.

4. Materials and methods

4.1. Materials

The chemicals used in this study were analytical grade, purchased from Fisher Scientific (Leicestershire, UK), Fluka (Buchs, Switzerland), Merk (Germany) and Sigma-Aldrich (USA). Restriction endonucleases, InsTAclone PCR cloning kit, DNA extraction kit, T4 DNA ligase, DNA and protein markers and *Taq* DNA polymerase were all purchased from Fermentas Life Sciences (Maryland, USA) while the vector pET-21a was from Novagen (USA).

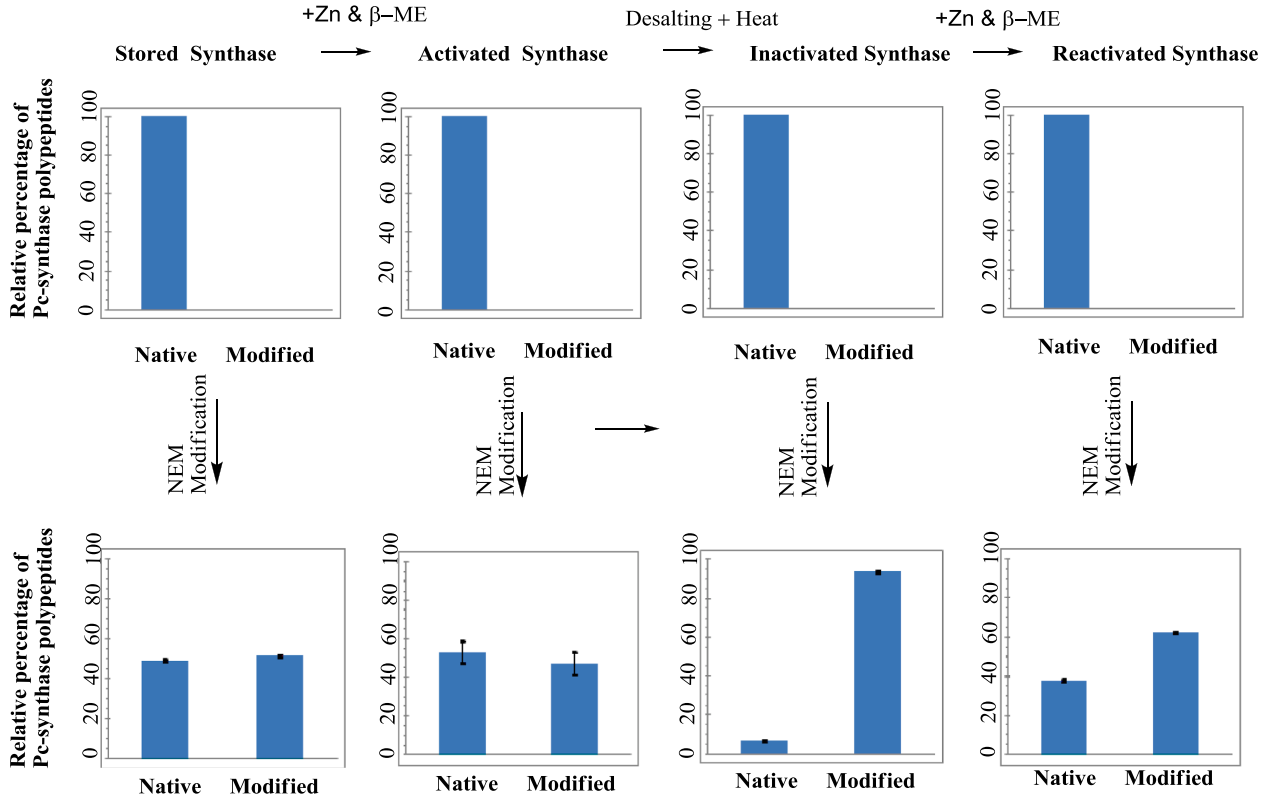
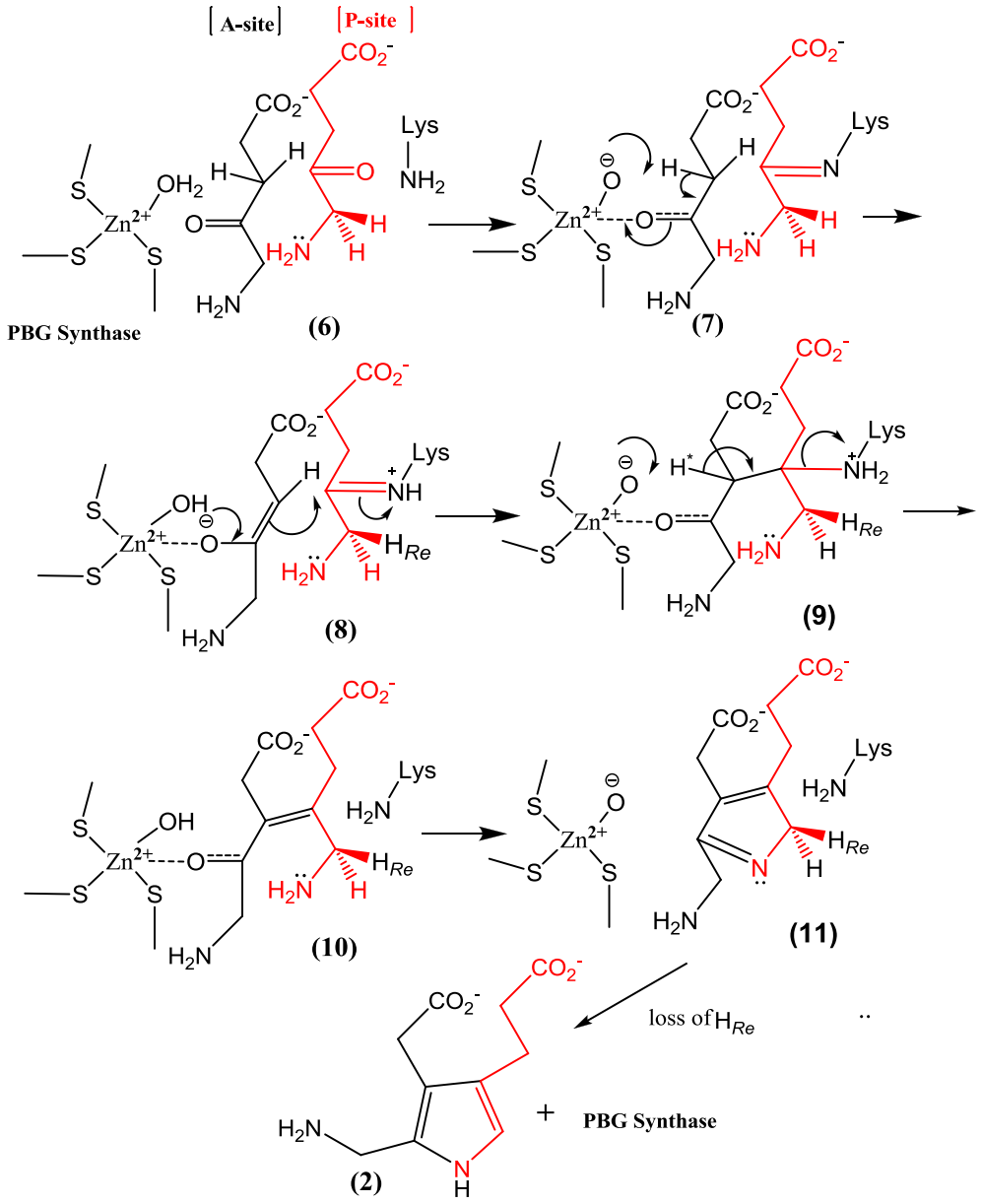


Fig. 5. NEM modifications under variously treated samples of PBG synthase from *P. calidifontis*. The histograms show percent distribution of native and mono- plus di-modified PBG synthase as quantified by ESI-mass spectrometry (see Fig. 4).



Scheme 4. Reaction sequence for the action PBG synthase using zinc catalysis. Key steps are: activation of A-site ALA by zinc as in **7**, removal of C-3 hydrogen by hydroxide and condensation by the resulting enol **8** to give **9.** The reactions for the further conversion of **9** into PBG (**2**) involve three manipulations as described in the text.

E. coli strain; DH5α was kindly provided by Madam Monika Akhtar and BL21-CodonPlus (DE3)-RIL strain was bought from Stratagene, La Jolla, Calif USA. Prof. Dr. Naeem Rashid kindly provided *Pyrobaculum calidifontis* strain VA1.

4.2. Cloning and purification of PBG synthase from P. Calidifontis

P. calidifontis genome was isolated from live culture grown at 96 °C under conditions given by [4]. The basic genomic DNA protocol for bacterial cells was successfully followed in this case [47]. The PBG synthase gene was then amplified using oligonucleotides 5′***CATATG*** CGCGTACAGTTTCCAACGAC3′ and 5′GCGCAAGGAGTAGCGCTGT TCG3′ as forward and reverse primers respectively. The reverse primer site was 8 nucleotide downstream to the gene stop codon. The gene was finally cloned into expression vector pET-21a. The recombinant plasmid was then transformed into expression strain, BL21-CodonPlus (DE3)-RIL cells which were used for large scale protein production.

Bacterial cells (3.5 g) from 2 L culture were harvested and resuspended in 50 mL of 20 mM potassium phosphate buffer (pH 6.8) containing 5 mM β-ME and 100 μM $ZnCl_2$. This cell suspension was then sonicated for 15 min giving 30 s pulses followed by 1 min rest period using a Sonics Vibra Cell™ sonicator. During sonication, the cell lysate was kept in an ice-bath to maintain a low temperature. After sonication, the total cell lysate was centrifuged at 7500 × *g* to remove cellular debris and to give a clear supernatant. The supernatant was then subjected to heat shock at 80 °C for 20 min and centrifuged at 40,000 × *g* for 15 min to remove all the coagulated proteins. The protein was then concentrated and partly fractionated by precipitating with 0–35% $(NH_4)_2SO_4$. The precipitates were spun down by centrifugation at 40,000 × *g* and dissolved in 20 mL of 50 mM potassium phosphate buffer (pH 7.9) containing 5 mM β-ME and 100 μM $ZnCl_2$. The dissolved protein was dialyzed against the same buffer to remove all traces of residual $(NH_4)_2SO_4$.

The protein solution was then subjected to anion exchange column chromatography. A pre-packed HiTrapQ FF 5 mL column attached to an AKTA purifier FPLC system was used. The column was equilibrated

with 10 column volumes of buffer A (50 mM potassium phosphate buffer (pH 7.9) containing 5 mM β-ME and 100 μM $ZnCl_2$) before loading the sample. The unbound proteins were washed out with 2 column volumes of buffer A before starting the 0–100% gradient of buffer B (50 mM potassium phosphate buffer (pH 7.9) containing 1 M NaCl, 5 mM β-ME and 100 μM $ZnCl_2$) which was completed in 5 column volumes. The protein was collected in 1 mL fractions. Fractions obtained from a single peak were pooled and dialyzed against 3 L of 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM β-ME and 100 μM $ZnCl_2$ in 4–5 batches and stored at 4 °C. This protein is referred to as **stored-synthase** and had approximately 60–65% of the optimal activity when assayed without the addition of $ZnCl_2$ and β-ME (the concentrations of the latter already present in the protein solution had been diluted 15–25 times in the assay mixture). The **stored-synthase** when incubated with 5 mM β-ME and 100 μM $ZnCl_2$ at 70 °C for 10 min, after passing through a PD-10 column (GE Healthcare), gave the **activated-synthase,** which when assayed immediately after gel filtration had maximum activity of 21 U/ mg (Table S1A). This activated synthase when incubated overnight at 70 °C in the absence of β-ME and $ZnCl_2$ goes through a time dependent loss of activity and becomes the **inactivated-synthase**. This heat induced inactivation can be reversed by re-incubation with 5 mM β-ME and 100 μM $ZnCl_2$ at 70 °C.

4.3. Protein quantification

Protein was quantified by the Bradford protein assay method [8] or by measuring the optical density (OD) at 260 and 280 nm. Using the molar extinction coefficient (47330 $M^{-1} cm^{-1}$) the absorbance of 1.256 at λ 280 nm was equal to 1 mg of protein. Correction for the absorbance due to presence of nucleic acids was made by using the equation below [39,25]:

$$\text{Concentration}\,(\text{mg/mL}) = (1.55 \times OD_{280}) - (0.76 \times OD_{260})$$

4.4. Assay for PBG synthase

4.4.1. Definition of the unit of PBG synthase activity

One unit of PBG synthase activity is defined as the enzyme activity, which forms one micromole of porphobilinogen per hour. During routine assay 40–60 μg of protein/ml of the assay mixture was used. However, in inactivation experiments, when it was important, to accurately measure the low-remaining-activity, larger amounts of enzyme (80–120 μg/ml) was used. This was necessary, to conduct assays for a shorter time, to avoid the formation of coloured compounds, arising from the degradation of the substrate ALA) as well the product (PBG) at 70 °C, at which the assays were conducted. Using linear part of the activity versus time graph, the effect of protein concentration on the specific activity of the enzyme is tabulated in Table 4.

4.4.2. Assay procedure

For the measurement of the enzyme activity, the reaction mixture 0.25 mL contained 100 mM potassium phosphate buffer (pH 6.8), 20 mM β-ME, 100 μM $ZnCl_2$ and 5 mM 5-aminolevulinic acid and the synthase (20–30 μg). The enzyme was activated by pre-incubation without substrate for 10 min at 70 °C followed by the addition of 5 mM ALA and incubation at the same temperature. At various time intervals aliquots were removed and mixed with an equal volume of the stopping solution (10% trichloroacetic acid in 0.1 M mercuric chloride) at room temperature. Following centrifugation the reaction mixture was treated, at room temperature, with twice the volume of Modified Ehrlich's reagent (0.02 g N, *N*-aminobenzaldehyde dissolved in 160 μL 20% perchloric acid and 840 μL glacial acetic acid) for about 20 min and then absorbance at λ 555 nm was measured [41]. The amount of porphobilinogen formed was quantified from a standard curve which was made by taking known amounts of porphobilinogen and developing its colour in the same fashion as for the assay.

To study the effect of Zn^{2+} and thiols on the activity of PBG synthase, these components were not present in the above assay mixture and added as appropriate.

Effect of temperature was determined by varying the pre-incubation and assay temperatures between 37 and 100 °C. The temperature endurance of the enzyme was measured by incubating it at 100 °C and then assaying (under standard conditions for PBG synthase from *P. calidifontis* described above) aliquots at different time intervals to measure the percent remaining activity.

In order to determine the zinc content of the protein, samples were prepared by removing all exogenous zinc either by extensive dialysis or via a desalting column followed by subjection to atomic-absorption spectrophotometer equipped with an air-acetylene flame at primary wavelength of 213.9 nm. The resulting absorptions were compared to that of a standard curve for zinc ion between 0 and 0.03 mM to determine the concentration.

Table 4
Specific activity at various concentrations of PBG synthase from *P. calidifontis* in assay mixture at 70 °C.

Concentration of enzyme in assay mixture (μg/mL)	Specific activity (U/mg)
0	0
2	20
4	21
20	18
200	19
320	13
400	13
640	11

4.5. Covalent modifications of PBG synthase from P. Calidifontis

4.5.1. Levulinic acid modification

Modification with levulinic acid was performed using the method described by Gibbs and Jordan [23]. For this purpose, in 0.1 mL 100 mM potassium phosphate buffer (pH 6.8), 0.4 mg of the **stored-synthase** was treated with levulinic acid (final concentration 3.57 mM) at 37 °C for five minutes followed by 5 min treatment with sodium borohydride (final concentration 57.14 mM) at the same temperature. After which the reaction mixture was passed through sephadex G-25 spin columns (GE Healthcare) also equilibrated with 100 mM potassium phosphate buffer (pH 6.8) to remove the reactant salts leaving only the synthase polypeptide as eluate. Aliquots with 0.25 mg of the modified synthase were then assayed using the protocol above (Section 4.4.2). The other control experiments in Table 1 were performed similarly.

4.5.2. 5-Chlorolevulinic acid modification

5-Chlorolevulinic acid, for modification of PBG synthase, was prepared in our laboratory according to the method described by Bloxham and Chalkley [5]. In order to modify PBG synthase, 10 mg of the enzyme in 2.5 mL of 100 mM potassium phosphate buffer (pH 6.8) was incubated with a final concentration of 5 mM β-ME and 100 μM $ZnCl_2$ at 70 °C for 15 min followed by their removal by gel filtration using a PD-10 column. The eluted synthase was immediately incubated with 5 mM freshly prepared solution of neutralized 5-chlorolevulinic acid (prepared by treating freshly dissolved 5-chlorolevulinic acid with equimolar concentration of sodium bicarbonate) for 5 min at 37 °C before assaying it at various time intervals for a time course and subjecting it to mass spectrometric and proteomics studies (Section 4.6).

4.5.3. N-ethylmaleimide (NEM) modification

In order to alkylate the synthase with *N*-ethylmaleimide (NEM) the three different forms described earlier i.e. **stored-, activated- and inactivated- synthase** were subjected to a buffer exchange by desalting columns equilibrated with 50 mM ammonium bicarbonate buffer (pH

8.4). Special care was taken for activated synthase by chilling it in an ice-bath before the exchange to minimize any heat induced change before analysis. The samples were treated with a final concentration of 15 mM NEM (solution prepared in absolute ethanol) in a reaction mixture of 200 μL containing 400–500 μg of synthase at 37 °C for approximately 30 min after which aliquots were taken and passed through a spin column equilibrated with distilled water for removal of other reactants from protein and analyzed for kinetics and mass spectrometric studies.

4.6. Mass spectrometric studies

4.6.1. Intact protein MS analysis

Mass spectrometric analyses of PBG synthase were performed by on-line ESI-LC MS 6224 (Agilent, USA) and also on Autoflex™ MALDI-TOF TOF (Bruker Daltonics, Germany). The intact protein analyses were performed in positive polarity as described earlier in [21,2] by optimizing the various mass spectrometric parameters for PBG synthase.

4.6.2. In-gel digestion processing of PBG synthase

In-gel tryptic digestion was performed for proteomics analysis of modified and unmodified PBG synthase [45]. The protein sample to be analyzed was run on 12% SDS-PAGE, visualized by Coomassie blue R-250 staining. The required protein band was excised from the gel using a sharp blade, diced into small cubes, soaked in 200 μL of 50 mM ammonium bicarbonate solution (pH 8.4) followed by the addition of 200 μL of acetonitrile and incubated at room temperature for 5 min. Tubes were vortexed so that dye is completely dispersed in a solution and supernatant was removed. The procedure was repeated to remove completely the stain from the gel pieces (at least thrice). The vacuum dried gel pieces were then soaked in 200 μL of freshly prepared 10 mM DTT solution in 50 mM ammonium bicarbonate (pH 8.4) and incubated at 56 °C. After half an hour the DTT solution was removed; the gel pieces were washed with an equal volume of acetonitrile and vacuum dried. The pieces were swollen in 200 μL of 55 mM iodoacetamide in 50 mM ammonium bicarbonate (pH 8.4) at 37 °C in dark. The solution was removed after 30 min and the gel pieces were washed and dried as described earlier. Gel pieces were swollen in 20 μL of working solution of sequencing grade porcine trypsin from Promega (25 ng/μL), incubated at room temperature for 10–15 min, and 50–60 μL of ammonium bicarbonate buffer (pH 8.4) was added to completely cover the gel pieces. The in-gel tryptic digestion mixture was incubated at 37 °C for overnight. Next day, after 16–18 h of overnight digestion, the reaction was quenched by the addition of 10 μL of 10% trifluoroacetic acid (TFA) in water so that pH of the supernatant was acidic (pH to 2–3). The supernatant containing tryptic peptides, was collected in a separate microfuge tube. Further extraction of peptides from gel pieces, was made by the addition of 50 μL of 0.1% TFA, incubated at 37 °C for 10 min, followed by the addition of 100 μL of acetonitrile and again incubated at 37 °C for 15 min. The supernatant was removed and pooled with the previous aliquot of tryptic peptides, and the extraction process was repeated again. The volume of the eluted peptides in the pooled supernatant was reduced in a vacuum concentrator to 10–20 μL before mass spectrometric analysis [2].

4.6.3. First dimension MALDI-MS analysis of peptides

Proteomics analysis by on-line LC MS was carried out as described previously [2]. For MALDI-MS analysis of peptide mixture from in-gel digestion, 3 μL of sample was mixed with 8 μL of α-cyano-4-hydroxycinnamic acid (CHCA), solution (prepared from 12 to 14 mg/mL dissolved in 1 mL of 33% of acetonitrile in 0.1% TFA water) and 1–2 μL sample was spotted either on a ground steel or Anchor chip plate in triplicate, using dried droplet method [21]. The spots were analysed on MALDI-TOF, in a positive reflectron mode using RP_PEPMIX in-built Flex Control method (version 3.4.135.0), by adjusting the laser intensity slider value (60–70%), 200 Hz, accumulated 8000 shots and acquired first dimension MS spectrum. The spectrum was analysed and processed in Flex analysis method (version 3.4.76.0.). The *m/z* list obtained from the analysis was then processed for protein identification, sequence coverage of peptides and data was analysed for the peptide of interest.

4.6.4. MS/MS fragmentation analysis (second dimension MALDI MS analysis)

In case of 5-chlorolevulinate modified PBG synthase, following the MALDI-MS analysis of in-gel tryptic digest, a peptide (118 – 139) of ionic peak $[M + 1H]^{+1}$ 2635.2 having mono-levulinate and di-carboamidomethylaed modifications, was selected as parent ion peak in Flex analysis method and sent to Flex control acquisition programme for fragmentation. The fragmentation was performed in LIFT fragmentation method under positive polarity. The conditions for fragmentation were; laser slider value of 93%, laser frequency of 100 Hz, pulse ion extraction delay was 20 ns, ion source voltage 1 (IS 1) 6 kV, ion source voltage 2 (IS 2) 5.3 kV, lens voltage 2.9 kV, reflector voltage 1 (Ref 1) 27 kV, reflector voltage 2 (Ref 2) 11.6 kV, reflector detector voltage of 1.948 kV, peak detection by centroid algorithm method, and accumulated spectrum was acquired by accumulating 12,000 – 14,000 shots. The MS/MS data acquired was then examined for possible b- and y-ions [46] generated for ionic peak $[M + 1H]^{+1}$ 2635.2.

4.6.5. Method for denaturing and native PAGE analysis

SDS and Native PAGE analyses of PBG synthase were carried out using CBS MGV-102 vertical gel electrophoresis system. For denaturing SDS-PAGE analysis 12% acrylamide resolving gel along with 5% stacking gel on top was cast according to standard protocol [47]. The protein samples for the SDS-PAGE were prepared by mixing 1 part of the 5 × SDS loading buffer (60 mM Tris- Cl pH 6.8, 40% glycerol, 2% SDS, 0.1% bromophenol blue and 1.4 mM β-mercaptoethanol) with four parts of the sample in a microfuge tube, followed by heating them for 5 min in boiling water bath. The samples were centrifuged at 12, 000 × *g* for one minute, and loaded into the wells of the gel, using 1X Tris-glycine buffer (0.025 M Trisma base, 0.25 M glycine and 0.1% SDS pH 8.3). The gel was run on 80 V until the samples entered the resolving gel and then on 120 V. The progress of the samples was monitored by observing the band of bromophenol blue present in the loading buffer. The protein samples on the gel were visualized by Coomassie blue R-250 staining followed by destaining [47].

For native-PAGE analysis, 8% non-denaturing acrylamide resolving gel was prepared according to standard protocol [47]. Sample preparation and running the gel followed the same protocol as mentioned above but the solutions were devoid of any SDS, reducing agent and heating.

Acknowledgements

We thank Professors P.M. Shoolingin-Jordan and J.B. Cooper for encouragement and valuable suggestions. We also thank Higher Education Commission (HEC; Islamabad, Pakistan) for generous funding of the project.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103117.

References

[1] M.M. Abboud, M. Akhtar, Stereochemistry of hydrogen elimination in the enzymic

formation of the C-2–C-3 double bond of porphobilinogen, J. Chem. Soc., Chem. Commun. 24 (1976) 1007–1008.
[2] F. Ahsan, A. Arif, N. Mahmood, Q.A. Gardner, N. Rashid, M. Akhtar, Characterization and bioassay of post-translationally modified interferon α-2b expressed in *Escherichia coli*, J. Biotechnol. 184 (2014) 11–16.
[3] J.P. Amend, E.L. Shock, Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and bacteria, FEMS Microbiol. Rev. 25 (2) (2001) 175–243.
[4] T. Amo, M.L. Paje, A. Inagaki, S. Ezaki, H. Atomi, T. Imanaka, *Pyrobaculum calidifontis* sp. nov., a novel hyperthermophilic archaeon that grows in atmospheric air, Archaea 1 (2002) 113–121.
[5] D.P. Bloxham, R.A. Chalkley, Selective modification of rabbit muscle pyruvate kinase by 5-chloro-4-oxopentanoic acid, Biochem. J. 159 (2) (1976) 201–211.
[6] W. Bode, F.-X. Gomis-Rüth, W. Stöckler, Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins', FEBS Lett. 331 (1–2) (1993) 134–140.
[7] D.W. Bollivar, C. Clauson, R. Lighthall, S. Forbes, B. Kokona, R. Fairman, L. Kundrat, E.K. Jaffe, Rhodobacter capsulatus porphobilinogen synthase, a high activity metal ion independent hexamer, BMC Biochem. 5 (1) (2004) 17.
[8] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.
[9] H.L. Carrell, J.P. Glusker, L. Shimoni, L.J. Keefe, C. Afshar, M. Volin, E.K. Jaffe, Crystallization and preliminary X-ray diffraction studies of 5-chlorolevulinate-modified bovine porphobilinogen synthase and the PbII-complexed enzyme, Acta Crystallogr. D Biol. Crystallogr. 52 (2) (1996) 419–421.
[10] A.G. Chaudhry, P.M. Jordan, Stereochemical studies on the formation of Porphobilinogen, Biochem. Soc. Trans. 4 (4) (1976) 760–761.
[11] K.-M. Cheung, P. Spencer, M.P. Timko, P.M. Shoolingin-Jordan, Characterization of a recombinant pea 5-aminolevulinic acid dehydratase and comparative inhibition studies with the *Escherichia coli* dehydratase, Biochemistry 36 (5) (1997) 1148–1156.
[12] L. Coates, G. Beaven, P.T. Erskine, S.I. Beale, Y.J. Avissar, R. Gill, F. Mohammed, S.P. Wood, P. Shoolingin-Jordan, J.B. Cooper, The X-ray structure of the plant like 5-aminolaevulinic acid dehydratase from *Chlorobium vibrioforme* complexed with the inhibitor laevulinic acid at 2.6 Å resolution, J. Mol. Biol. 342 (2) (2004) 563–570.
[13] P.T. Erskine, L. Coates, D. Butler, J.H. Youell, A.A. Brindley, S.P. Wood, M.J. Warren, P.M. Shoolingin-Jordan, J. Cooper, X-ray structure of a putative reaction intermediate of 5-aminolaevulinic acid dehydratase, Biochem. J. 373 (2003) 733–738.
[14] P.T. Erskine, R. Newbold, A.A. Brindley, S.P. Wood, P.M. Shoolingin-Jordan, M.J. Warren, J.B. Cooper, The x-ray structure of yeast 5-aminolaevulinic acid dehydratase complexed with substrate and three inhibitors, J. Mol. Biol. 312 (1) (2001) 133–141.
[15] P.T. Erskine, R. Newbold, J. Roper, A. Coker, M.J. Warren, P.M. Shoolingin-Jordan, S.P. Wood, J.B. Cooper, The Schiff base complex of yeast 5-aminolaevulinic acid dehydratase with laevulinic acid, Protein Sci. 8 (6) (1999) 1250–1256.
[16] P.T. Erskine, E. Norton, J.B. Cooper, R. Lambert, A. Coker, G. Lewis, P. Spencer, M. Sarwar, S.P. Wood, M.J. Warren, X-ray structure of 5-aminolevulinic acid dehydratase from *Escherichia coli* complexed with the inhibitor levulinic acid at 2.0 Å resolution, Biochemistry 38 (14) (1999) 4266–4276.
[17] P.T. Erskine, N. Senior, S. Awan, R. Lambert, G. Lewis, I.J. Tickle, M. Sarwar, P. Spencer, P. Thomas, M.J. Warren, P.M. Shoolingin-Jordan, S.P. Wood, J.B. Cooper, X-ray structure of 5-aminolaevulinate dehydratase, a hybrid aldolase, Nat. Struct. Mol. Biol. 4 (12) (1997) 1025–1031.
[18] N. Frankenberg, D.W. Heinz, D. Jahn, Production, purification, and characterization of a Mg2+-responsive porphobilinogen synthase from *Pseudomonas aeruginosa*, Biochemistry 38 (42) (1999) 13968–13975.
[19] F. Frère, M. Nentwich, S. Gacond, D.W. Heinz, R. Neier, N. Frankenberg-Dinkel, Probing the active site of *Pseudomonas aeruginosa* Porphobilinogen synthase using newly developed inhibitors, Biochemistry 45 (27) (2006) 8243–8253.
[20] F. Frère, W.-D. Schubert, F. Stauffer, N. Frankenberg, R. Neier, D. Jahn, D.W. Heinz, Structure of porphobilinogen synthase from *Pseudomonas aeruginosa* in complex with 5-fluorolevulinic acid suggests a double schiff base mechanism, J. Mol. Biol. 320 (2) (2002) 237–247.
[21] Q.A. Gardner, H. Younas, M. Akhtar, Studies on the regioselectivity and kinetics of the action of trypsin on proinsulin and its derivatives using mass spectrometry, Biochim. Biophys. Acta (BBA)-Prot. Proteom. 1834 (1) (2013) 182–190.
[22] P.N. Gibbs, A.-G. Chaudhry, P.M. Jordan, Purification and properties of 5-aminolaevulinate dehydratase from human erythrocytes, Biochem. J. 230 (1985) 25–34.
[23] P.N. Gibbs, P.M. Jordan, Identification of lysine at the active site of human 5-aminolaevulinate dehydratase, Biochem. J. 236 (1986) 447–451.
[24] K.D. Gibson, A. Neuberger, J.J. Scott, The purification and properties of δ-aminolaevulic acid dehydrase, Biochem. J. 61 (4) (1955) 618–629.
[25] W.E. Groves, F.C. Davis Jr, B.H. Sells, Spectrophotometric determination of microgram quantities of protein without nucleic acid interference, Anal. Biochem. 22 (2) (1968) 195–210.
[26] E.K. Jaffe, S.P. Salowe, N.T. Chen, P.A. DeHaven, Porphobilinogen synthase modification with methylmethanethiosulfonate. A protocol for the investigation of metalloproteins, J. Biol. Chem. 259 (8) (1984) 5032–5036.
[27] E.K. Jaffe, The porphobilinogen synthase family of metalloenzymes, Acta Crystallogr. D Biol. Crystallogr. 56 (2) (2000) 115–128.
[28] E.K. Jaffe, The porphobilinogen synthase catalyzed reaction mechanism, Bioorg. Chem. 32 (5) (2004) 316–325.
[29] E.K. Jaffe, The remarkable character of porphobilinogen synthase, Acc. Chem. Res. 49 (11) (2016) 2509–2517.
[30] E.K. Jaffe, W.R. Abrams, H.X. Kaempfen, K.A. Harris Jr, 5-Chlorolevulinate modification of porphobilinogen synthase identifies a potential role for the catalytic zinc, Biochemistry 31 (7) (1992) 2113–2123.
[31] E.K. Jaffe, D. Shanmugam, A. Gardberg, S. Dieterich, B. Sankaran, L.J. Stewart, P.J. Myler, D.S. Roos, Crystal, structure of *Toxoplasma gondii* porphobilinogen synthase: insights on octameric structure and porphobilinogen formation, J. Biol. Chem. 286 (17) (2011) 15298–15307.
[32] E.K. Jaffe, M. Volin, C.B. Myers, W.R. Abrams, 5-Chloro [1, 4–13C] levulinic acid modification of mammalian and bacterial porphobilinogen synthase suggests an active site containing two Zn (II), Biochemistry 33 (38) (1994) 11554–11562.
[33] P.M. Jordan, Biosynthesis of tetrapyrroles, Elsevier, 1991.
[34] P.M. Jordan, P.N. Gibbs, Mechanism of action of 5-aminolaevulinate dehydratase from human erythrocytes, Biochem. J. 227 (3) (1985) 1015–1020.
[35] P.M. Jordan, J.S. Seehra, ^{13}C NMR as a probe for the study of enzyme-catalysed reactions: mechanism of action of 5-aminolevulinic acid dehydratase, FEBS Lett. 114 (2) (1980) 283–286.
[36] M. Kanehisa, S. Goto, KEGG: kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (1) (2000) 27–30.
[37] J. Kervinen, E.K. Jaffe, F. Stauffer, R. Neier, A. Wlodawer, A. Zdanov, Mechanistic basis for suicide inactivation of porphobilinogen synthase by 4, 7-dioxosebacic acid, an inhibitor that shows dramatic species selectivity, Biochemistry 40 (28) (2001) 8227–8236.
[38] L. Kundrat, J. Martins, L. Stith, R.L. Dunbrack, E.K. Jaffe, A structural basis for half-site metal binding revealed in *Drosophila melanogaster* porphobilinogen synthase, J. Biol. Chem. 278 (33) (2003) 31325–31330.
[39] E. Layne, [73] Spectrophotometric and turbidimetric methods for measuring proteins, Methods Enzymol. 3 (1957) 447–454.
[40] W. Liedgens, C. Lutz, H.A. Schneider, Molecular properties of 5-aminolevulinic acid dehydratase from *Spinacia oleracea*, Eur. J. Biochem. 135 (1) (1983) 75–79.
[41] D. Mauzerall, S. Granick, The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine, J. Biol. Chem. 219 (1) (1956) 435–446.
[42] N. Mills-Davies, D. Butler, E. Norton, D. Thompson, M. Sarwar, J. Guo, R. Gill, N. Azim, A. Coker, S.P. Wood, P.T. Erskine, L. Coates, J.B. Cooper, N. Rashid, M. Akhtar, P.M. Shoolingin-Jordan, Structural studies of substrate and product complexes of 5-aminolaevulinic acid dehydratase from humans, *Escherichia coli* and the hyperthermophile *Pyrobaculum calidifontis*, Acta Crystallogr. Sect. D 73 (1) (2017) 9–21.
[43] L.W. Mitchell, E. Jaffe, Porphobilinogen synthase from *Escherichia coli* is a Zn (II) metalloenzyme stimulated by Mg (II), Arch. Biochem. Biophys. 300 (1) (1993) 169–177.
[44] D.L. Nandi, K.F. Baker-Cohen, D. Shemin, δ-aminolevulinic acid dehydratase of *Rhodopseudomonas spheroides* I. Isolation and properties, J. Biol. Chem. 243 (6) (1968) 1224–1230.
[45] S.J. North, J. Jang-Lee, R. Harrison, K. Canis, M.N. Ismail, A. Trollope, A. Antonopoulos, P.-C. Pang, P. Grassi, S. Al-Chalabi, A.T. Etienne, A. Dell, S.M. Haslam, Mass spectrometric analysis of mutant mice, Methods Enzymol., Elsevier. 478 (2010) 27–77.
[46] P. Roepstorff, J. Fohlman, Proposal for a common nomenclature for sequence ions in mass spectra of peptides, Biomed. Mass Spectrom. 11 (11) (1984) 601.
[47] J.R. Sambrook, D.W. Russell, Molecular Cloning; A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York, 2001.
[48] N. Sawada, N. Nagahara, T. Sakai, Y. Nakajima, M. Minami, T. Kawada, The activation mechanism of human porphobilinogen synthase by 2-mercaptoethanol: intrasubunit transfer of a reserve zinc ion and coordination with three cysteines in the active center, J. Biol. Inorg. Chem. 10 (2) (2005) 199–207.
[49] R. Schmid, D. Shemin, The enzymatic formation of porphobilinogen From δ-aminolevulinic acid and its conversion to protoporphyrin1, J. Am. Chem. Soc. 77 (2) (1955) 506–507.
[50] J.S. Seehra, P.M. Jordan, 5-Aminolevulinic acid dehydratase: alkylation of an essential thiol in the bovine-liver enzyme by active-site-directed reagents, Eur. J. Biochem. 113 (3) (1981) 435–446.
[51] N.M. Senior, K. Brocklehurst, J.B. Cooper, S.P. Wood, P.T. Erskine, P.M. Shoolingin-Jordan, P.G. Thomas, M.J. Warren, Comparative studies on the 5-aminolaevulinic acid dehydratases from *Pisum sativum, Escherichia coli* and *Saccharomyces cerevisiae*, Biochem. J. 320 (1996) 401–412.
[52] D. Shemin, C.S. Russell, δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines, J. Am. Chem. Soc. 75 (19) (1953) 4873–4874.
[53] P.M. Shoolingin-Jordan, Structure and mechanism of enzymes involved in the assembly of the tetrapyrrole macrocycle, Biochem. Soc. Trans. 26 (3) (1998) 326–336.
[54] P.M. Shoolingin-Jordan, P. Spencer, M. Sarwar, P.T. Erskine, K.-M. Cheung, J.B. Cooper, E.B. Norton, 5-Aminolaevulinic acid dehydratase: metals, mutants and mechanism, Biochem. Soc.Trans. 30 (4) (2002) 584–590.
[55] P. Spencer, P.M. Jordan, Purification and characterization of 5-aminolaevulinic acid dehydratase from *Escherichia coli* and a study of the reactive thiols at the metal-binding domain, Biochem. J. 290 (1993) 279–287.
[56] B.L. Vallee, D.S. Auld, Active-site zinc ligands and activated H_2O of zinc enzymes, PNAS 87 (1) (1990) 220–224.
[57] W.H. Wu, D. Shemin, K.E. Richards, R.C. Williams, The quaternary structure of δ-aminolevulinic acid dehydratase from bovine liver, Proc. Natl. Acad. Sci. 71 (5) (1974) 1767–1770.