

Studies on Porphobilinogen-Deaminase from *Saccharomyces cerevisiae*

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Porphobilinogen-deaminase from *Saccharomyces cerevisiae* has been isolated and partially purified 80- and 230-fold in the absence or presence of phenylmethylsulphonyl fluoride, respectively.

Some properties of the isolated enzyme were studied. Porphyrin formation was linear with time and protein concentration. Optimum pH was about 7.5–7.8. Molecular mass of the protein was $30,000 \pm 3000$ Dalton when the enzyme was purified in the presence of phenylmethylsulphonyl fluoride. A less active and unstable 20,000 Da molecular mass species was obtained when purification was performed in the absence of the protease inhibitor.

Porphobilinogen-deaminase exhibited classical Michaelis-Menten kinetics. The apparent K_m for uroporphyrinogen formation was $19 \mu\text{M}$; $V_{\max}$ was $3.6 \text{ nmol uroporphyrin/h}$ and the Hill coefficient was $n = 1$.

Also the action of several reagents on the activity was studied. Protective thiol agents had no effect. Heavy metals inhibited both porphyrin formation and porphobilinogen consumption, but known sulphhydryl inactivating chemicals inhibit the former without modifying the latter. Ammonium ions had no effect on the activity while hydroxylamine completely inhibited both porphyrin formation and porphobilinogen consumption.

Introduction

Porphobilinogen-deaminase (PBG-deaminase) (EC 4.3.1.8) also known as hydroxymethylbilane-synthase (HMB-S) joins four molecules of porphobilinogen (PBG) and releases a linear unbranched tetrapyrrole identified as a hydroxymethylbilane (HMB). This HMB can be converted with intramolecular rearrangement of ring D into the cyclic tetrapyrrole uroporphyrinogen III by uroporphyrinogen III-synthase (EC 4.2.1.75). In the absence of this enzyme, HMB is chemically ring closed to uroporphyrinogen I [1].

Although it was originally proposed that a lysine residue was directly involved in the covalent binding of the substrate to the enzyme [2], it has now been established that the first molecule of PBG actually binds to a dipyrromethane cofactor linked to the cysteine-242 of the enzyme [3, 4].

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; PBG-deaminase, porphobilinogen-deaminase; HMB-S, hydroxymethylbilane synthase; *S. cerevisiae*, *Saccharomyces cerevisiae*; PCMB, *p*-chloromercuribenzoate; NEMI, *N*-ethylmaleimide; DTNB, 2,2'-dinitro-5,5'-dithiobenzoic acid; GSH, glutathione.

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PBG-deaminase has been isolated from a wide range of prokaryotic and eukaryotic sources. All the deaminases appear to be monomeric enzymes with molecular mass values ranging from 33,000 to 44,000 dalton.

Multiple forms of the enzyme have been reported by Miyagy *et al.* [5], Anderson and Desnick [6], Jordan and Berry [7], and Williams [8] representing stable enzyme-substrate covalent intermediates.

In this paper we report on the isolation and partial purification of PBG-deaminase from a simple eukaryotic organism, *Saccharomyces cerevisiae* (*S. cerevisiae*). Some of its properties have also been determined.

Materials and Methods

PBG was obtained according to Sancovich *et al.* [9] from δ -amino levulinic acid (ALA) and was estimated by the method of Moore and Labbe [10]. Sephadex gels were obtained from Pharmacia Fine Chemicals Uppsala, Sweden. ALA and phenylmethylsulphonyl fluoride (PMSF) were from SIGMA Chem. Co. Tris-HCl buffer was used through this study unless otherwise indicated. All other reagents employed were of analytical grade obtained from several commercial sources.

Source of enzyme

All strains of *S. cerevisiae* were kindly supplied by Professor J. Mattoon from the University of Colorado, Denver, U.S.A. The D 27 strain used here derived from D 273-10 B and was auxotrophic for methionine.

Growth and harvesting conditions

The growth medium was as follows: 1% yeast extract (Difco); 2% peptone (Difco) and 2% dextrose (Merck). To the agar plates 2% Bacto-agar (Difco) was added. Cultures were shaken on a rotary shaker at 160 rpm and 28–30 °C. At the end of the exponential growth phase cells were harvested by centrifugation at $12,000 \times g$ for 10 min, washed with distilled water and stored at –20 °C until used.

Partial purification of yeast PBG-deaminase

All operations were carried out at 4 °C. Washed cells were suspended in 0.05 M Tris-HCl buffer pH 7.8 (1:1, g.wet. weight/ml) and disrupted by ultrasonic treatment (12 μ , 2 min, Soniprep 150 MSE) in the presence of sand (1:3 wet weight/g). The homogenate was then diluted with the same buffer at a 1:2 ratio (g.wet. weight/ml) and centrifuged at $23,000 \times g$ for 20 min. The supernatant was kept at 55 °C for 5 min and centrifuged at $25,000 \times g$ for 20 min to remove the precipitated protein. The resulting supernatant was then fractionated with solid ammonium sulphate (45–60%) and applied to a Sephadex G-100 column (1.7 cm $\times$ 80 cm) equilibrated with 0.05 M Tris-HCl buffer pH 7.8 and eluted with the same buffer at a flow rate of 18 ml/h. When purification was carried out in the presence of PMSF, this chemical was added at a final concentration of 1 mM to the Tris-HCl buffer for suspending the washed cells before sonic disruption; after heating again 0.5 mM PMSF was added to the $23,000 \times g$ supernatant. The buffer used for dissolving the 45–60% ammonium sulphate precipitate included 1 mM PMSF. The dissolved precipitate was then applied to the Sephadex G-100 column. PMSF in the same buffer was also used to equilibrate the column and for eluting protein (0.1 mM). In all eluates protein concentration and enzyme activity were determined.

Estimation of enzyme activity

The standard incubation system contained: enzyme preparation (usually 2 ml and 5–10 mg protein), 80 μ g of PBG, 0.05 mM Tris-HCl buffer, pH 7.8, in a final volume of 3 ml. Incubations were carried out aerobically in the dark with mechanical shaking at 37 °C for 2 h, unless otherwise specified. After incubation, 50% TCA was added to a final concentration of 5% (v/v) to inactivate the enzyme. The mixture was then illuminated with white light for 20 min, to oxidize the formed porphyrinogens. The protein precipitate was separated by centrifugation and total porphyrins determined in acid solution [11]. Protein concentration was determined by measuring the absorbance at 280 nm or by the Bradford method [12].

Enzyme unit

One enzyme unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 nmol of uroporphyrinogen I in 1 h from porphobilinogen under standard incubation conditions. Specific activity is expressed as the number of units per mg of protein (U/mg).

Determination of molecular mass

Molecular mass measurements were performed by gel filtration. A Sephadex G-100 column (1.7 cm $\times$ 80 cm) was prepared and then calibrated with protein standards of known molecular mass. Protein content and enzyme activity were determined in all eluate fractions.

Results and Discussion

Isolation and optimum assay conditions

Several procedures have been applied to disrupt the cells and to liberate the enzyme, such as ultrasonic treatment at varying intervals and input, in the presence or absence of glass beads or Triton X-100, or using a mortar with glass beads or sand. However, none of these attempts was as effective as ultrasonic disruption in the presence of sand to obtain an active enzyme preparation.

Preliminary studies were carried out to establish the optimum conditions for purifying the enzyme and measuring its activity, using the $23,000 \times g$ supernatant as source of enzyme. First, the effect of time and heating temperature on the enzyme activ-

ity was investigated (Fig. 1). Protein content decreases significantly after 5 min of heating at all temperatures assayed. Longer heating intervals up to 15 min produced no further decrease in protein content, however heating at temperatures over 50 °C, even for short periods, drastically diminished porphyrin yield and thus specific activity. These findings indicate that *S. cerevisiae* PBG-deaminase from D 27 strain appears to be a more temperature labile enzyme than that isolated from other sources. Heating at 55 °C for only 5 min is thus the best method for partially enriching the enzyme. Porphyrinogen formation was linear with increasing protein concentrations up to 20 mg per test, and with incubation times at least up to 4 h. However, as it has already been observed [13], PBG consumption soon reaches a plateau with low amounts of enzyme and most of it is very rapidly consumed within the first 30 min. We therefore decided to use about 20 mg of protein per test and an incubation time of 2 h (data not shown). As shown below (see kinetics) a concentration of 118 μ M PBG was routinely used for all experiments. An optimum pH of about 7.5–7.8 was found, when activity was measured in terms of porphyrin formation (Fig. 2), and the same profile emerged when PBG consumption was determined (data not shown). In either case very low or nil activity was detected below pH 6.5 and above pH 8.5. The same results were obtained when incubations were carried out in either aerobic or anaerobic conditions. Therefore aerobic conditions were

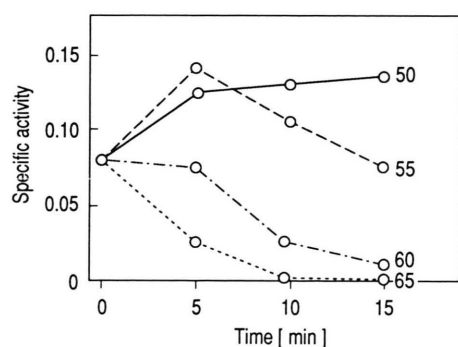


Fig. 1. The effect of heating on the specific activity. Supernatant fractions (S) were heated at those temperatures and for those periods of time as indicated. Incubation system and conditions as well as other methods are described in the text.

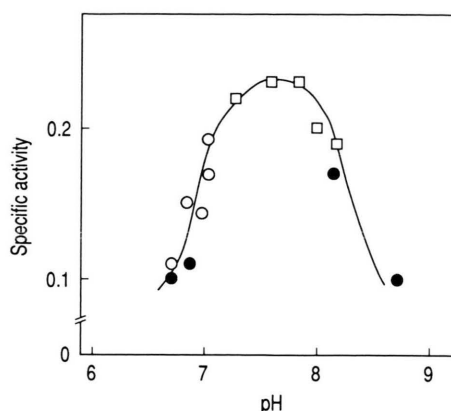


Fig. 2. The effect of pH on the enzyme activity. Specific activity was measured at the indicated pH values under standard incubation conditions. The 23,000 $\times$ g supernatant was used as enzyme source. Other experimental conditions were as described in the text. (○) Sodium phosphate buffer, (●) glycine-NaOH buffer and (□) Tris-HCl buffer.

normally used. Addition of sodium and magnesium ions to the incubation mixtures at concentrations known to stimulate PBG-deaminase activity in other sources [14] did not produce the expected effect.

Molecular mass determination

The enzyme obtained after Sephadex G-100 column was purified about 80- and 230-fold, with a specific activity of 33 and 97 units/mg protein and a yield of 30 and 73%, in the absence and presence of the protease inhibitor respectively. These enzyme preparations were not homogenous on SDS-PAGE. Fig. 3 shows a typical elution profile from a Sephadex G-100 column. PBG-deaminase activity appears in a single peak to which a molecular mass of $20,000 \pm 2000$ Da corresponds. However, it has to be mentioned that occasionally two peaks of molecular masses of $30,000 \pm 3000$ Da and $20,000 \pm 2000$ Da were found which were corroborated by SDS-PAGE (data not shown). Therefore, to determine whether the low molecular mass species could be the result of a proteolytic cleavage of a 30,000 Da form, purification was carried out in the presence of PMSF, a known protease inhibitor. The profile obtained when eluting the 45–60% $(\text{NH}_4)_2\text{SO}_4$ fraction prepared in the presence of PMSF only shows one active peak with a

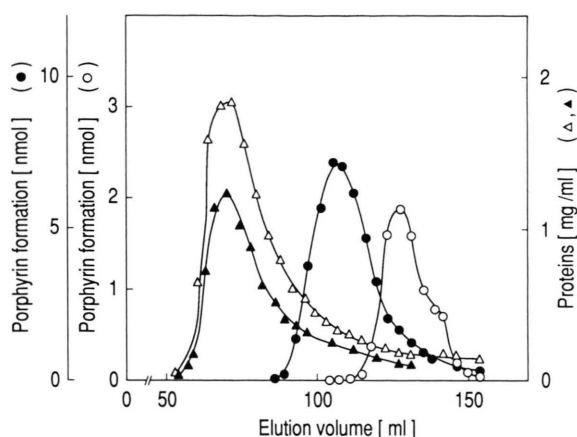


Fig. 3. Gel filtration of *S. cerevisiae* PBG-deaminase. A 16-fold enriched preparation of PBG-deaminase (obtained by 45–60% ammonium sulphate precipitation) was applied to a Sephadex G-100 column (1.7 cm $\times$ 80 cm) equilibrated and eluted with Tris-HCl buffer pH 7.8 with (●, ▲) or without (○, △) 0.1 mM PMSF. (○, ●) porphyrins formed (nmol); (△, ▲) protein (mg/ml). Experimental conditions are described in the text.

molecular mass 30,000 Da exhibiting a 3 to 4 times higher specific activity than obtained with fractions prepared in the absence of the protease inhibitor.

These results indicate that PMSF protects *S. cerevisiae* PBG-deaminase from the action of some proteases which seem to cleave a 10,000 Da inactive polypeptide from the highly active 30,000 Da species, rendering a 20,000 Da form, which is apparently more labile and/or less active, as can be seen from its final specific activity. Attempts to reconstitute the more active high molecular mass species by adding protein fractions eluting at molecular masses between 10,000 Da and 5000 Da to the 20,000 Da active peak were unsuccessful.

Nevertheless the molecular mass found for the *S. cerevisiae* PBG-deaminase is within the range of values reported for the enzyme from various sources (33,000–44,000 Da); however this deaminase appears to be more labile or unstable than others when it is purified, specially against heating. Other reasons for the appearance of less active fractions of *S. cerevisiae* PBG-deaminase at the final step of purification, depending on the presence or absence of PMSF, can be the following. The apo protein of PBG-deaminase might exist in

a conformationally unstable form which is stabilized to a more stable and active holoenzyme (molecular mass 30,000 Da) after binding to the dipyrromethane cofactor [15]. So, there might be an equilibrium mixture of various conformational variants of apo- and holoenzyme forms. During the purification procedure the holoenzyme might be decomposed yielding the less active or inactive apoenzyme by cleavage of a peptide which perhaps carries the dipyrromethane cofactor.

Kinetic studies

While the kinetic behaviour of porphobilinogenases from various sources indicate that this enzyme complex is an allosterically regulated protein [13], most PBG-deaminases exhibited Michaelis-Menten kinetics [16].

Plots of the reaction rate, measured in terms of total uroporphyrinogen formation, against substrate concentration shows for *S. cerevisiae* PBG-deaminase the classical Michaelis-Menten response (Fig. 4), in spite of the expected complexity of a reaction in which four identical substrate molecules are to be bound to the enzyme [17]. Slight substrate inhibition (15%) was observed at PBG concentrations above 200 μ M.

From linear double reciprocal and Hill plots, K_m , V_{max} and n values were calculated. K_m was found to be 19 μ M and V_{max} 3.6 nmol uroporphyrin/h. These values are of the same order as those reported for the enzyme from other sources [18].

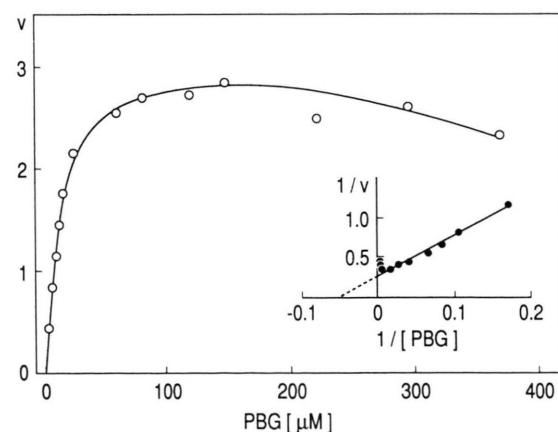


Fig. 4. Kinetics of PBG-deaminase. Experimental conditions are indicated in the text. v = nmol uroporphyrin/h. Inset: Double reciprocal plot (Lineweaver-Burk).

Correspondingly, the Hill coefficient of 1 indicates the existence of only one class of binding site for PBG per molecule of enzyme.

Effect of various reagents on PBG-deaminase activity

The involvement of sulphhydryl groups in the active site of PBG deaminases has been demonstrated since a long time [13]; however, their direct participation in catalysis was not elucidated until recently [19].

Studies on the group(s) involved in the covalent binding of the first PBG molecule have been carried out by different investigators. It was suggested that this group could correspond to a lysine [20] or cysteine residue [21]. It has also been demonstrated that an unusual cofactor identified as a dipyrromethane which is covalently linked through cysteine-242 to the enzyme is responsible for the binding of the first PBG molecule, so contributing to the synthesis of the tetrapyrrole at the catalytic site [3, 4, 19].

The effect of several known sulphhydryl reagents on the *S. cerevisiae* PBG-deaminase was therefore tested to get an insight into the presence of sulphhydryl and disulphide groups essential for activity.

Activity was assayed in the presence and absence of the chemicals under aerobic and anaerobic conditions and with and without preincubation. Essentially, identical results were found under different assay conditions. Thus only those results obtained in aerobiosis without preincubation are listed in Table I, except when reversion of thiol inhibition was studied.

Thiol alkylating reagents, such as *p*-chloro-mercuribenzoate (PCMB) and N-ethylmaleimide (NEMI) at a concentration of 1 mM completely inhibited porphyrin formation, but shows little or no action on PBG consumption. These inhibitions could be reversed by 5 mM cysteine. 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB) also affected only porphyrin formation. Reagents known to react with disulphide bonds such as potassium cyanide and thiourea again had no effect on PBG consumption, but inhibited by about 50% porphyrin formation. In contrast, metal ions inhibited both porphyrin synthesis and substrate consumption, as already found for the enzyme from other sources, very likely due to binding of the metal to essential thiol groups, as proved by the reversion of Pb^{2+} - and Hg^{2+} -effects with 5 mM cysteine.

Reducing and protective thiol agents, such as cysteine and glutathione (GSH), had no effect on

Table I. Effect of various reagents on PBG-deaminase activity.

Addition	1st Reagent		2nd Reagent		Activity [%]	
	Final Conc.	(mM)	CySH	(5 mM)	Porphyrin formed	Porphobilinogen consumed
Cysteine	5	—	—	—	94	100
GSH	5	—	—	—	102	100
PCMB	1	—	—	—	0	85
PCMB	1	+	—	—	90	100
NEMI	1	—	—	—	8	79
NEMI	1	+	—	—	95	100
DTNB	1	—	—	—	0	120
KCN	10	—	—	—	51	100
Thiourea	10	—	—	—	56	100
Hg^{2+}	0.2	—	—	—	1	20
Hg^{2+}	0.2	+	—	—	85	90
Cd^{2+}	0.2	—	—	—	1	20
Pb^{2+}	0.2	—	—	—	5	10
Pb^{2+}	0.2	+	—	—	88	92

A 16-fold enriched preparation was used as enzyme source. In the experiments where reversion by cysteine was examined, the thiol was added 20 min after the mixture had been preincubated with the inhibitor and the substrate. Metal ions were employed as chloride salts. Final concentration in the reaction mixture are indicated. All other experimental conditions are described in the text. Activities are expressed as % of the control (100%).

enzyme activity. These results thus indicate that, if sulphhydryl groups are essential for activity, they are either not located in exposed regions of the protein or they are well protected, as it has been demonstrated for the cysteine-242 in *Escherichia coli* PBG-deaminase, which is bound to the dipyrromethane cofactor [19].

The differential action of some chemicals on PBG consumption and porphyrin formation is more difficult to explain, if it is considered that the only essential thiol group in the enzyme is cysteine-242 to which the cofactor is covalently bound. But, if other sulphhydryl groups are necessary for assuring the right spacial orientation of the growing pyrrol chain which is indispensable for cyclization [13, 20, 22], their modification could then interfere with HMB synthesis. By this ring closure could be altered, this explaining diminished or totally suppressed porphyrin synthesis and unaffected PBG consumption. However, the drastic action of heavy metals on sulphhydryl groups is reflected in both, product synthesis and substrate consumption inhibition.

The effect of ammonium ions and hydroxylamine on the deaminase was first described by Bogorad [23] who found that they inhibited PBG consumption to a lesser extent than porphyrin formation. These findings were recently confirmed for the enzyme from several different organisms [16]. Unexpectedly, we found that ammonium ions nor inhibit PBG consumption neither porphyrin formation using a wide range of concentrations up to 150 mM (data not shown). This is in contrast to previous results including ours [14, 16, 24]. Hydroxylamine however, inhibited about 50–60% at 10 mM and 100% at 100 mM both, porphyrin syn-

thesis and PBG consumption. This is in agreement with the results of Jordan and Warren [19] who found that hydroxylamine releases bound substrate molecules, but not the dipyrromethane cofactor.

These findings indicate that in *S. cerevisiae* hydroxylamine seems to be a stronger inhibitor compared to ammonia, although it cannot be excluded either, that hydroxylamine acts at an early step cleaving the bond between the first PBG molecule to the apoenzyme or to the holoenzyme like that from *E. coli*. Further studies with the purified yeast PBG-deaminase are in progress to establish the presence or absence and the nature of the possible cofactor of the enzyme from this source.

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