Studies on Uroporphyrinogen Biosynthesis in Pig Liver

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Porphobilinogen-deaminase (PBG-D) and PBG-D-isomerase complex (PBG-D-I) from pig liver were isolated and partially purified. Uroporphyrinogen I and III formation was found to be linear with time and protein concentration. Optimal pH was about 7.4 and 7.6–7.8 for PBG-D and PBG-D-I complex, respectively.

Some properties of the isolated enzymes were studied. Molecular mass determination gave a value of 40,000 Da for PBG-D and 50,000 Da for the complex. Both enzymes exhibited classical Michaelis-Menten kinetics. K_m and V_{max} parameters were estimated. The effect of several divalent cations, ammonia and thiol reagents was also investigated. The differential action of some of these chemicals on PBG-D and PBG-D-I system would suggest that PBG-D and isomerase may not be only physically adjacent but actually associated.

Introduction

The biosynthesis of uroporphyrinogen III, the precursor of hemes, chlorophylls, corrins and related structures, requires the sequential participation of two enzymes, porphobilinogen-deaminase (PBG-D; EC 4.3.1.8) and uroporphyrinogen III synthase (Isomerase; EC 4.2.1.75). PBG-D catalyzes the condensation of four molecules of porphobilinogen (PBG) to form the linear tetrapyrrole hydroxymethylbilane (HMB) which is rapidly converted into uroporphyrinogen III by the isomerase. In the absence of the latter, HMB cyclizes to give uroporphyrinogen I [1, 2].

PBG-D has been extensively studied in numerous sources [3–8]. The existence of a dipyrromethane cofactor linked covalently to *Escherichia coli* PBG-D involved in the HMB synthesis has been reported [9, 10].

On the other hand, uroporphyrinogen III synthase has been purified from rat liver [11, 12], *Euglena gracilis* [13], *Escherichia coli* [14] and human erythrocytes [15].

A good deal of information now exists about the separated enzymes and about the substrate for the isomerase, but less work has been carried out in recent years on the properties of the complex of the two enzymes [16–20]. Without doubt PBG-D and isomerase should be associated *in vivo* in some way to assure the exclusive formation of the physiological intermediate uroporphyrinogen III; it is therefore of interest to study the interactions between both enzymes as well as the properties of the PBG-D-I system.

We will describe here some results on the behaviour of partially purified preparations of both, the complex and the PBG-D from pig liver.

Materials and Methods

Fresh pig liver was kindly supplied by Frigorífico INGA (Buenos Aires) and was stored frozen at −20 °C. PBG was obtained according to Sancovich *et al.* [21] and assayed as described by Moore and Labbe [22]. Sephadex gels were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Partial purification of PBG-D

All operations were carried out at 4 °C unless otherwise stated. Pig liver homogenate (10%, w/v) was prepared in 0.25 M sucrose [21] and centrifuged at 10,000 × *g* for 20 min. Glacial acetic acid was added to the supernatant to adjust it to pH 5. After stirring for 30 min the preparation was centrifuged for 15 min at 10,000 × *g*. The supernatant was treated with ammonium sulphate. The

Abbreviations: PBG, porphobilinogen; PBG-D-I, porphobilinogen-deaminase-isomerase; PBG-D, porphobilinogen-deaminase; HMB, hydroxymethylbilane; PCMB, *p*-chloromercuribenzoate; NEMI, N-ethyl-maleimide; BAL, 2,3-dimercaptopropanol; DTNB, 3′,5′-dithionitrobenzoic acid; GSH, glutathion.

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35–55% fraction was collected by centrifugation and desalted by Sephadex G-25 filtration. The eluted protein was heated at 65 °C for 10 min, centrifuged and the supernatant concentrated by adding ammonium sulphate up to 55% saturation. The precipitate, dissolved in a small volume of buffer, was then applied to a Sephadex G-100 column (2.5 × 57 cm) which was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.4.

Partial purification of pig liver PBG-D-Isomerase (PBG-D-I) system

Purification steps were identical with those described for PBG-D excluding the heat treatment.

Estimation of enzyme activities

The standard system contained: the enzyme preparation (2 ml) together with 88.5 μM PBG and 50 mM Tris-HCl buffer (pH 7.4 for PBG-D or 7.6–7.8 for the PBG-D-I complex) in a final volume of 3 ml. Incubations were carried out in the dark at 37 °C for 2 h under aerobic or anaerobic conditions for the PBG-D and the PBG-D-I system, respectively. The reaction was stopped by adding concentrated HCl to a final concentration of 5% (w/v). Porphyrin determinations as well as other methods not specified here were described earlier [6]. An enzyme unit is defined as the amount of enzyme that catalyses the synthesis of 1 nmol of uroporphyrin per 2 h under standard conditions.

Results and Discussion

Assay conditions

Preliminary assays were carried out in order to determine the optimal incubation system, employing the heat-treated protein supernatant and the desalted 35–55% ammonium sulphate fraction as enzyme source for the PBG-D and the PBG-D-I system, respectively.

Uroporphyrinogen I and III formation was found to be linear with time up to 5 h and with protein concentrations up to 3 and 12 mg, respectively. PBG-D showed a sharp pH optimum at 7.4 in 50 mM Tris-HCl buffer while the complex exhibited its maximum activity in the range between pH 7.6–7.8 in the same buffer. No differences in uroporphyrinogen I synthesis were found under either aerobic or anaerobic incubation conditions. However, when the activity of the PBG-D-I system was assayed, the isomeric composition changed with the incubation atmosphere. In aerobiosis 70% uroporphyrinogen I was synthesized at the expense of type III, due to the inhibitory effect of oxygen on the isomerase.

Effect of heating on the enzyme activity

In order to obtain a PBG-D preparation free of isomerase activity, the desalted 35–55% ammonium sulphate fraction was heated for different time intervals and at various temperatures (Fig. 1). As shown in Fig. 1 b, the maximum specific activity was obtained by heating to 65 °C for 10 min. A 4-fold activity increase with respect to the control was obtained. Under these conditions 70% uroporphyrinogen I was synthesized, indicating that pig liver isomerase is more stable against heat treatment than other known isomerases, as 20% uroporphyrinogen III were still formed when the protein was heated to 75 °C for 20 min.

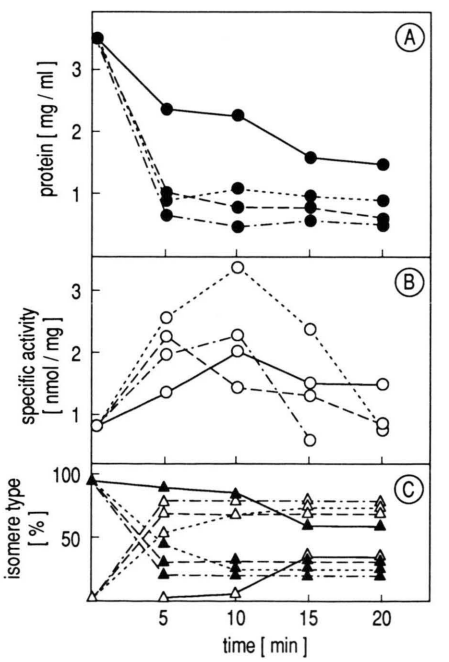

Fig. 1. Effect of heating on protein concentration (●); specific activity (○) and formation of uroporphyrinogen I (△) and III (▲). Experimental conditions are indicated in the text. — 60 °C; ---- 65 °C; – – – 70 °C; –·–·– 75 °C.

Molecular masses of PBG-D and its isomerase complex

Employing Sephadex G-100 chromatography, pig liver PBG-D and PBG-D-I complex showed molecular masses of 40,000 ± 4000 Da and 50,000 ± 5000 Da, respectively. This is in good agreement with the values reported for the enzymes from other sources [3, 8, 16, 17, 19, 20, 23–26]. A typical elution profile is shown in Fig. 2 for the PBG-D-I system. This complex was purified about 50-fold with a specific activity of 4.3 units/mg protein and a yield of 47%. Isomeric analysis of uroporphyrins formed revealed 95% of uroporphyrin III. For PBG-D a 150-fold purified enzyme producing 90% of uroporphyrinogen I with a specific activity of 13.3 units/mg protein and a yield of 40% was obtained. When eluate fractions were subjected to SDS polyacrylamide electrophoresis [27, 28], although these fractions were not yet homogeneous, main active bands at M_r 40,000 and M_r 50,000 for PBG-D and the complex, respectively, were observed.

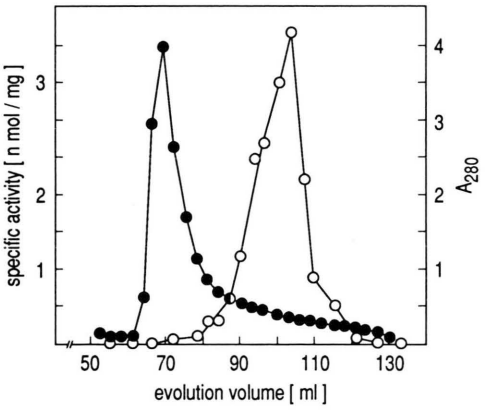

Fig. 2. Sephadex G-100 elution profile of the pig liver PBG-D-I complex; (●) protein concentration; (○) specific activity.

Kinetic studies on urophorphyrinogen I and III formation

When plots of velocity against substrate concentration were performed, it was found that the curves for both, uroporphyrinogen I and uroporphyrinogen III formation, were hyperbolic. Double reciprocal plots (Fig. 3) and Eadie plots were

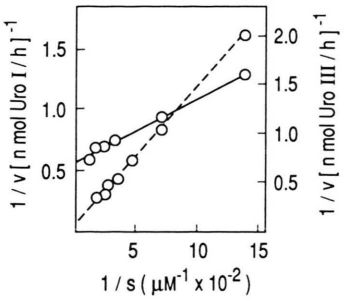

Fig. 3. Double reciprocal plots for uroporphyrinogen I (—) and uroporphyrinogen III synthesis (– – –).

linear with n being 1, indicating Michaelis-Menten kinetics for both, the PBG-D and the PBG-D-I system. From these plots kinetic parameters were calculated. For PBG-D V_M was found to be 1.78 nmol/h, the K_m being 10 μM. For the complex V_M was 12.5 nmol/h at a K_m of 167 μM.

Reaction stoichiometry of uroporphyrinogen formation

In contrast to observations in soybean-callus [24], *Euglena gracilis* [25] and *Rhodopseudomonas palustris* [7, 20] uroporphyrinogen synthesis in pig liver did not follow the stoichiometry ratio of 4 mol of PBG to 1 mol of product.

Employing pig liver PBG-D (Fig. 4), a 2.5-fold excess in PBG consumption up to 15 μM substrate concentration was found. This deviation could be attributed to the existence of polypyrrole intermediates in a appropiate length for cyclization. Higher PBG concentrations (15 up to 88.5 μM) produced an increase in HMB synthesis which in turn could possibly inhibit PBG-D activity by binding

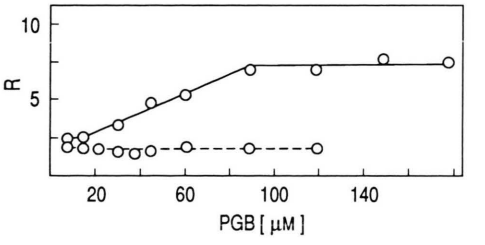

Fig. 4. Stoichiometry of the reaction catalyzed by PBG-D (—) and the PBG-D-I complex (– – –). R is the ratio of theoretical and experimental porphyrin formation in nmol.

to other sites than the catalytic ones as it has been reported by Battersby *et al.* [30]. At saturating substrate concentrations a constant R value (R is the ratio of theoretical and experimental porphyrin formation) of 7–8 was found, showing that nor more enzyme for inhibition is available.

These results are in contrast to those reported by Williams *et al.* [31] for *Euglena gracilis* PBG-D; it has, however, to be emphasized that experimental conditions were very different in both cases. A great excess of substrate (320 μM) and a very low amount of protein were employed by Williams's group, so that the competitive inhibition exerted by HMB on PBG-D could never be observed.

Studying the total PBG-D-I complex only a constant 2-fold excess in substrate consumption for all the concentrations tested was observed (Fig. 4), indicating that almost all the HMB formed is converted to uroporphyrinogen III by the isomerase.

Effect of different cations on uroporphyrinogen biosynthesis

The effect of several divalent cations on pig liver uroporphyrinogen formation was also studied (Table I). As can be seen, Pb^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+} inhibited enzyme activities almost completely, as already reported for the enzymes from other sources [5, 15, 32]. Their action was stronger on uroporphyrinogen synthesis than on substrate consumption (inhibition about 50%, data not shown), indicating the importance of thiol groups at the active sites of these enzymes, as previously proposed [24, 25, 31]. Ca^{2+} ions at a concentration of 10 mM did not show any effect. As expected increasing concentrations of ammonia inhibited both, PBG-D and the complex activities (Fig. 5). Ammonia only slightly modified PBG consumption when uroporphyrinogen I synthesis was measured, whereas it was inhibited to about 50% when PBG-D-I system activity was studied. Porphyrin formation was also inhibited in both cases, how-

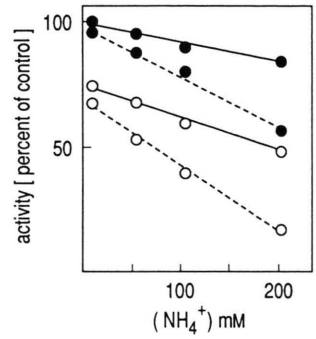

Fig. 5. The effect of ammonium ions on PBG-D (—) and the PBG-D-I complex (– – –): (●) PBG consumed; (○) porphyrins formed. Results with and without preincubation were identical.

Table I. Effect of different divalent cations on uroporphyrinogen synthesis. The effect of divalent cations was assayed at the concentrations indicated. 150-fold (0.5 mg protein/ml) and 35-fold (0.3 mg protein/ml) enriched PBG-D and PBG-D-I complex were used as enzyme source. Activity of the control without any addition was taken as 100%. All other experimental conditions are indicated in the text.

Compound	Concentration [mM]	Enzyme activities	
		Uroporphyrinogen I [%]	Uroporphyrinogen III [%]
Pb^{2+}	1	4	4
	10	2	15
Hg^{2+}	1	6	5
	10	4	10
Cd^{2+}	1	6	6
	10	2	12
Zn^{2+}	1	9	7
	10	5	15
Ca^{2+}	1	90	90
	10	85	90

ever, the effect of ammonia on the complex was much more drastical. In the presence of ammonia polypyrrol intermediates accumulated and uroporphyrinogen synthesis decreased compared to the control.

These results suggest that both enzymes, PBG-D and the isomerase, are not only adjacent to each other, but are actually associated to form a complex.

Effect of sulphydryl reagents on pig liver uroporphyrinogen biosynthesis

PBG-D and the PBG-D-I system seem to be sulphydryl groups containing enzymes. This was shown for all systems so far examined [3, 6]. Therefore it was of great interest to study the effect of different thiol protecting compounds also on these enzymes from pig liver.

As can be seen from Table II, PBG-D as well as the complex are not dependent on the presence of thiol protecting reagents to show maximum activity. Moreover, neither preincubation nor incubation under different atmospheres had any effect. These results indicate that if –SH groups are essential for activity they are not located in exposed regions of the protein or that they are well protected, as it has been demonstrated for the cysteine-242 from *Escherichia coli* PBG-D, to which the dipyrromethane cofactor is bound [9]. The isomerase from this source might not need thiol groups for activity as it was suggested for *Euglena gracilis* and human erythrocytes enzymes [13, 15], since the isomer composition remains practically unchanged in the presence of applied reagents.

When sulphydryl inactivating chemicals were employed, essentially identical results were obtained for both enzymes with or without preincubation and under aerobic or anaerobic conditions. Therefore, only data obtained in anaerobiosis and without preincubation, except when the reversion of inactivation was studied, are presented (Table III).

Group I includes thiol alkylating reagents such as PCMB and NEMI which significantly inhibited enzyme activity; these inhibitions were reversed by cysteine and BAL. Again, inactivation was more pronounced on porphyrin formation than on PBG consumption. The second group (II) includes agents which oxidize monothiols; they markedly but differentially inhibited enzyme activity. Group III is represented by arsenite which at concentrations of 1 mM or lower reacts with only vicinal thiol groups while at concentrations above 10 mM it reacts with monothiols. We could only observe a

Table II. Effect of sulphydryl reagents on PBG-D and PBG-D-I complex activities. 150-fold and 35-fold enriched preparations were employed for measuring PBG-D and the complex activities. Results obtained in aerobic and anaerobic conditions for PBG-D as well as with or without preincubation for both enzymes were identical; thus only data obtained with both enzymes under aerobiosis and without preincubation are listed. Activity of the control without any addition was taken as 100%.

Addition		PBG-D		PBG-D-I complex	
Reagent	Final conc. [mM]	PBG consumed [%]	Urogen I formed [%]	PBG consumed [%]	Urogen III formed [%]
GSH	50	93	40	95	55
	10	100	88	98	70
	1	100	98	100	95
	0.1	100	100	100	100
Cysteine	50	105	100	104	100
	10	110	100	105	100
	1	110	103	100	100
	0.1	100	98	100	100
DTE	10	95	78	90	78
	1	100	98	100	95
	0.1	100	100	100	100
Thioglycolate	10	85	68	95	65
	1	100	95	100	95
	0.1	105	100	100	100

Table III. Effect of sulphydryl inactivating chemicals on enzyme activities. Experimental conditions are identical with those indicated in the legend of Table II.

Group	1st Reagent		2nd Reagent		PBG-D		PBG-D-I complex	
	Addition	Final conc. [mM]	Addition	Final conc. [mM]	PBG consumed [%]	Urogen I formed [%]	PBG consumed [%]	Urogen III formed [%]
I	PCMB	1	–	–	40	2.5	30	2.7
		1	BAL	5	100	98	100	100
		1	Cysteine	5	100	104	100	100
	NEMI	5	–	–	70	20	71	22
		1	BAL	5	100	96	100	100
		1	Cysteine	5	105	92	100	100
II	DTNB	1	–	–	70	35	75	64
		5	–	–	50	8	60	34
	Cysteine	1	–	–	80	40	85	50
		5	–	–	70	20	75	30
	Oxidized GSH	1	–	–	75	30	70	20
		5	–	–	50	4	50	5
	I-benzoate	1	–	–	70	30	70	25
		5	–	–	40	5	40	5
III	Arsenite	1	–	–	105	104	100	102
		10	–	–	88	86	90	85
		100	–	–	90	80	80	80
		10	BAL	10	92	80	100	75
		1	BAL	1	100	96	100	90
IV	Thiourea	1	–	–	110	110	110	112
		10	–	–	100	100	100	79
	NaCN	1	–	–	110	110	120	116
		10	–	–	115	112	120	122
	Na_2SO_3	1	–	–	100	88	100	97
		10	–	–	100	85	100	90

slightly inhibitory effect on monothiol groups. The reagents in group IV known to react with disulphide bonds had no effect on either of the enzymes like it has been observed for *Euglena gracilis* isomerase [33]. From these results it is concluded that in PBG-D as well as in its isomerase complex only monothiol groups are active.

Conclusions

Table IV compiles the properties of pig liver PBG-D and the PBG-D-I complex as reported in the present paper.

PBG-D follows classical Michaelis-Menten kinetics which is in agreement with reports about these enzymes from other sources [6, 23–25, 31]. However, the complex classified as an allosterically regulated protein in other species [20, 23–25, 29, 34] does not exhibit such properties in preparations from pig liver.

From all systems so far studied PBG-D is reported to be a heat stable enzyme. However, the pig liver enzyme only retains complete activity when incubated for 15–30 min at 55 °C or for 10 min at 60 °C. With incubations above these temperatures or for longer intervals inactivation occurs. For instance there was a 76% inactivation obtained when the fraction was heated for 10 min at 65 °C. The complex, however, appears to be rather stable to heating retaining 50% of its activity after being heated for 10 min at 75 °C.

A differential action of some chemicals (heavy metals, thiol reagents) on PBG consumption and porphyrin formation was also observed. Recent investigations have demonstrated that the tetrapyrrolic chain is bound to the protein at the free α-position of a substrate-derived dipyrromethane cofactor and is then released as HMB [35]. It was also established that this cofactor is bound to the enzyme at the S atom of cysteine-242 [10, 36]. If then, an equilibrium between the apo- and holoenzyme really existed, some chemicals could block PBG binding to the protein. This would then be re-

Table IV. Summary of the properties of pig liver PBG-D and PBG-D-I complex.

Properties	PBG-D	PBG-D-I complex
Incubation atmosphere	aerobic	anaerobic
pH	7.4	7.6–7.8
Thermostability (t 1/2; min)	17 (65 °C)	10 (75 °C)
Molecular mass (Da)	40,000	50,000
Specific activity (units/mg protein)	13.3	4.3
Kinetics	hyperbolic	hyperbolic
K_m (μM)	10	167
V_{max} (nmol/h)	1.78	12.50
Inhibitors	Pb^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+} ammonia PCMB, NEMI, DTNB	Pb^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+} ammonia PCMB, NEMI, DTNB

flected in inhibition of both substrate consumption and porphyrinogen synthesis, as it was observed with heavy metals.

Moreover, if thiol groups other than cysteine-242 are involved in the conformation of the active center of the enzyme, their modification could interfere with the correct spacial orientation of the growing linear polypyrrole, so that the synthesis of the hydroxymethylbilane and the ring closure could be prevented and thus explain diminished or ceased porphyrinogen synthesis.

Since these effects of thiol inactivating reagents were similar on both, PBG-D and the complex, and as isomer ratio was unchanged in their presence, we propose that they act essentially on the PBG-D moiety of the complex. However, we cannot exclude some additional effects of these compounds on pig liver isomerase itself or on the formation of the active complex and its structural arrangement.

Results presented here support the idea that both enzymes, PBG-D and isomerase, cannot function independently, but are interacting with each other in a complex, as already postulated by other authors [13, 16, 37–39]. Moreover, specific activity of pig liver PBG-D is 3 times higher than that of the complex suggesting that the isomerase could exert some control on PBG-D activity, as previously suggested [3, 16]. This kind of regulatory function of the isomerase was also observed when the effect of ammonia on PBG consumption was analyzed (Fig. 5).

Since HMB was recently found to be the substrate for the isomerase, studies were stimulated to test separatly both, isolated PBG-D and isolated uroporphyrinogen III synthase. However, it has to be kept in mind that the natural enzyme system forming the physiological precursor of hemoproteins *in vivo*, uroporphyrinogen III, is the PBG-D-I system. Its properties seem to be quite different from those of the isolated enzymes. It is therefore of great interest to continue studies on the pig liver PBG-D-I system to obtain further experimental evidence on the possible regulatory role of the isomerase in uroporphyrinogen synthesis.

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