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PORPHYRIN BIOSYNTHESIS IN *RHODOPSEUDOMONAS PALUSTRIS*—V. PURIFICATION OF PORPHYRINOGEN DECARBOXYLASE AND SOME UNUSUAL PROPERTIES*

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Abstract—Uroporphyrinogen decarboxylase (EC 4.1.1.37) has been purified 16-fold from *Rp. palustris* to a specific activity of 210 nmol of total decarboxylated porphyrinogens III formed/hr per mg of protein and about 50% yield. The *Rp. palustris* enzyme exhibits some unusual properties as compared with URO-D from other sources.

2. The purified enzyme is a monomer with a molecular weight of ~46,000, an isoelectric point of 4.6 and an optimum pH of 6.9 and 6.8 with urogen III and I substrate. Neither GSH nor EDTA seem to be necessary for activity, and the decarboxylation rate and the distribution of the reaction products was not affected either by the presence or absence of oxygen.

3. The *Rp. palustris* enzyme is a thermo-stable protein, heating at 60°C for 15 min enhanced several times activity. This is the first time that heat treatment is included as one of the steps to purify URO-D.

4. Thermal activation followed an identical profile using either substrate. The ratios of specific activity for the type III and I isomer of urogen remained constant throughout the purification. These findings are indicating that a single enzyme catalyzes the four decarboxylations occurring from urogen to coprogen.

5. Kinetic data employing urogen III and I as substrate showed that the pattern of accumulated intermediates was rather different depending on whether type III or I isomer was used.

6. While decarboxylation of urogen III responds to the usual scheme:

$$\text{octagen III} \xrightarrow{v_1} \text{heptagen III} \xrightarrow{v_2} \text{coprogen III},$$

where $v_1 \gg v_2$ and decarboxylation of heptagen III is the rate-controlling step.

7. Decarboxylation of urogen I revealed a completely different and characteristic picture fitting the scheme:

$$\text{octagen I} \xrightarrow{v_1'} \text{pentagen I} \xrightarrow{v_2'} \text{coprogen I},$$

where again $v_1' \gg v_2'$ and the removal of the final carboxyl group from pentagen I becomes the rate-limiting step.

INTRODUCTION

Accumulation of porphyrins with 8, 7, 6, 5 and 4-COOH had been reported in a variety of biological materials, coming from both porphyric patients or animals and *in vitro* porphyrin biosynthetic systems; indicating that decarboxylation of uroporphyrinogen (urogen) was a stepwise process.

That porphyrinogens with 7 to 5 —COOH were the true intermediates in this reaction was demonstrated in our laboratory (Batlle and Grinstein, 1962a,b, 1964a,b; San Martin de Viale and Grinstein, 1968) and early studies on the enzymic decarboxylation of urogen were carried out using crude preparations from *Chlorella* (Bogorad, 1958), rabbit reticulocytes (Mauzerall and Granick, 1958), avian erythrocytes (Batlle and Grinstein, 1962b, 1964b) and *Rhodopseudomonas spheroides* (Hoare and Heath, 1958, 1959).

During the biosynthesis of protoporphyrin (proto), Uroporphyrinogen decarboxylase (URO-D) (EC 4.1.1.37), catalyzes the sequential removal of the four carboxyl groups of the acetate side chains of the octacarboxylic porphyrinogen (octagen) to finally yield coproporphyrinogen (coprogen); with the formation of intermediate hepta, hexa and penta-carboxy-porphyrinogens (hepta, hexa and pentagens). The same enzyme also acts decarboxylating a large number of both natural and synthetic acetate-substituted porphyrinogens (Jackson *et al.*, 1976a; Smith *et al.*, 1976).

Partially and highly purified enzyme preparations have been obtained from bacterial cells, avian and

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human erythrocytes, mouse spleen, rat liver, tobacco leaves and reticulocytes (for references see Batlle and Koopmann, 1986).

In all cases so far investigated, a single protein, either monomeric or dimeric, appears to catalyze the decarboxylation of all acetic acid residues; however it is still unclear whether these reactions occur at the same active center or not, although there is a report suggesting that there are four different active sites and there is also reciprocal inhibition of decarboxylation by isomers I and III for each porphyrinogen substrate (De Verneuil *et al.*, 1980).

From kinetic properties (Mauzerall and Granick, 1958; Batlle and Grinstein, 1962b, 1964b; Tomio *et al.*, 1970; García *et al.*, 1973; Smith and Francis, 1979; Kardish and Woods, 1980; De Verneuil *et al.*, 1980) it has been postulated that the reaction could occur in a two-stage process, the initial step involving a rapid removal of the first carboxyl group in ring D of urogen III, followed by a slower elimination of the further carboxyl groups from rings A, B and C. The same scheme was proposed to work in case of urogen I being the substrate.

The decarboxylation of urogen I appears to proceed by a non-specific route, in contrast to the preferred clockwise decarboxylation of urogen III (Jackson *et al.*, 1976b, 1977). However, except for the proposal of Barnard and Akhtar (1975), suggesting that removal of the carboxyl group might occur via an enzyme-catalized protonation and deprotonation reaction, no other mechanism has yet been postulated to explain the action of URO-D on its substrates.

URO-D is also of interest with regard to several pathological conditions in humans (Kushner *et al.*, 1976) and various animal species (De Matteis *et al.*, 1961; Taljaard *et al.*, 1971) and also in certain mutants of *S. cerevisiae* (Urban Grimal and Labbe-Bois, 1981; Rytka *et al.*, 1984), in which decreased enzymic activity leads to massive accumulation and/or increase elimination of highly carboxylated porphyrins.

As already stated one of the early studies on URO-D was carried out in *Rp. spheroides* (Hoare and Heath, 1958, 1959) but there had not been yet any other report on this enzyme in other photosynthetic bacteria. Due to the close relationship between *Rp. spheroides* and *Rp. palustris*, and the very limited information about the enzymes involved in porphyrin synthesis in this organism, which seems to be a convenient source for many of these enzymes, we have undertaken a systematic study of porphyrin biosynthesis in this non-sulfur purple bacterium (Viale *et al.*, 1980a,b, 1983, 1985; Juknat *et al.*, 1986; Kotler *et al.*, 1986; Koopmann *et al.*, 1986; Lombardo *et al.*, 1986; Schoua *et al.*, 1986; Vázquez *et al.*, 1986) and we will report here some of our results on the URO-D.

The purpose of our study was to purify and characterize the enzyme in order to obtain some experimental evidence to enable us to clarify the mechanism of action of this enzyme not yet established (Batlle and Koopmann, 1986) and to explain the molecular basis for the diminished URO-D activity found under some abnormal conditions.

We describe a method for the purification of URO-D and various properties of the enzyme. It was found that a single monomeric protein catalizes the decarboxylation of both urogen III and I to the corresponding coprogen. However, with the *Rp. palustris* URO-D, the pattern of accumulated intermediates was rather different depending on whether type I or III substrate was used; showing that, while decarboxylation of the 7-COOH porphyrinogen, is the rate-limiting step when urogen III is the substrate, decarboxylation of urogen I proceeds rapidly until formation of pentagen I; so that removal of the final carboxyl group is the rate-limiting step when the type I isomer of urogen is the substrate.

MATERIALS AND METHODS

Porphyrins

Uroporphyrin I octamethyl ester was a gift from Dr Torben K. With, central County Hospital, Svendborg, Denmark. Uroporphyrin III methyl ester was obtained from turacin, the uroporphyrin copper complex in the flight feathers of Musophagidae (Turaco), following the procedure developed by Nicholas & Rimington (1951) and simplified by With (1957). Synthetic uroporphyrin II and IV were a gift from Professor Claude Rimington FRS. Free porphyrins were obtained from their methyl esters as already described (Batlle and Grinstein, 1964a) and porphyrinogens were prepared by reduction of free porphyrins according to Mauzerall and Granick (1958).

Other materials

Sephadex gels were purchased from Pharmacia Fine Chemicals. All other chemicals were obtained from Sigma Chem. Co, BDH, Oxoid, Fluka AG or E. Merck-Darmstadt and were of the highest grade available. All solutions were made up in ion and metals-free glass distilled water.

Source material of enzyme

Rp. palustris was originally obtained from the collection of the Microbiology and Inmunology Unit-Fac. Cs. Exactas y Naturales, University of Buenos Aires. The growth and harvesting of cells were carried out as already described by Viale *et al.* (1980b). The cells were harvested after two days of growing, washed with 0.05 M Tris–HCl buffer, pH 7.0, unless otherwise indicated and the pellet thus obtained was either immediately worked or could be kept at −20°C until being used. All manipulations from harvesting onwards were carried out at 4°C, except specifically pointed out.

The cells were suspended in the same buffer employed for washing (60 mg wet wt: ml buffer), the cell suspension was disrupted by ultrasonic treatment in an ultrasonic power unit (Soniprep 150, MSE) for 60 sec at 8 μ, in volumes of about 10 ml each and the resulting homogenate (H) was further diluted (1:3; v/v) with the incubation buffer and then centrifuged at 25,000*g* for 30 min. URO-D activity was confined to the supernatant (S) which was therefore employed as starting source for purification of the enzyme.

Determination of URO-D activity

Enzymic activity was assayed using either urogen III or I as indicated and measuring the amounts of hepta-, hexa-, penta- and coprogens formed. Unless otherwise stated the standard incubation conditions were as follows: the reaction mixture contained 0.5 ml of the enzyme preparation in 0.05 M Tris–HCl buffer pH 7.0 (between 0.2 to 1 mg depending on the degree of purification), 10–15 μM urogen, (mM GSH, 0.1 mM EDTA and buffer to make a final volume of 2 ml. Other conditions or any added or omitted component are indicated in the text or in the legends to tables and figures. Incubation was carried out in the dark, for 60 min at 37°C with constant mechanical shaking and under free-oxygen nitrogen. The pH was controlled before and after incubation with a combination-electrode pH

meter. Blanks were always run containing only substrate or enzyme in buffer solution. The reaction was stopped by adding 12 N HCl to a final concentration of 5% (W/v); after mixing, porphyrinogens were oxidized to porphyrins by illumination under air with white light for 30 min; then the precipitated protein was filtered off, washed three times with 1 ml each of 5% HCl and all filtrates were collected together. Porphyrins were dried, in a desiccator under vacuum and converted to their methyl esters by incubation overnight with the esterification mixture 5% (v/v) H_2SO_4 in methanol. Porphyrin methyl esters were extracted into chloroform and dried. To identify and quantify the porphyrins, the dried methyl esters were redissolved in chloroform and analyzed by high performance thin layer chromatography (HPTLC) Seubert and Seubert, 1979), or by HPLC (Polo *et al.*, 1983) on a Spherisor S 5 W silica column (250 × 4.6 mm) (Spectra-Physics) using a Varian 5000 HPLC instrument, equipped with a loop injector; a UV Varian 50 detector, or a Shimadzu RF-510 spectrofluorophotometer (Excitation at 400 nm, emission at 625 nm) and a Varian CDS 111L integrator giving directly the relationship between the area of each chromatographic peak and the concentration of the porphyrin. Porphyrins were eluted, within 5 min, by a mixture of ethyl acetate and *n*-hexane (55:45; v/v) at a pressure of 42–50 atm and a flow rate of 2 ml/min.

Enzyme unit

One unit of URO-D activity was defined as the amount of enzyme that catalyzes the formation of 1 nmol of porphyrinogen(s)/hr at 37°C under the standard incubation conditions. The specific activity being the number of units per mg of protein. Urogen decarboxylation can be expressed either as the sum of nmol of hepta-, hexa-, penta- and copro-gens, that is total porphyrinogens (TP) formed or as the nmol of coprogen formed (C); in some experiments, results were represented as the nmol of each of the decarboxylation intermediates formed individually.

Protein concentration was determined by the method of Bradford (1976).

Molecular weight measurements were performed by gel filtration, Sephadex columns were prepared and calibrated as already described (Batlle *et al.*, 1965; Batlle, 1967; Locascio *et al.*, 1969). The same columns were used throughout this study. Protein content and enzyme activity were determined in the column eluates.

Starch gel electrophoresis was based on the methods described by Bodman (1961).

RESULTS

Purification of the enzyme (Table 1)

Heat treatment. Portions (about 15 ml) of the supernatant fraction obtained as detailed in Materials and Methods were heated with constant stirring in a water bath at 60°C during 15 min, after this time the supernatant was immediately cooled in an ice bath. The system thus treated was centrifuged at 12,000 ***g*** for 10 min and the sedimented protein precipitate was discarded.

Ammonium sulphate fractionation. To the supernatant obtained from the heat treatment solid ammonium sulphate was added to 45% saturation. After 1 hr the material was centrifuged at 12,000 ***g*** for 10 min; the sediment had low activity and only capacity for partially decarboxylating urogen III to heptagen (20%) and hexagen (5%); therefore it was discarded. The supernatant solution was then brought to 75% saturation with ammonium sulphate and after 1 hr was centrifuged at 12,000 ***g*** for 10 min. Ammonium ions were always removed from enzyme

Table 1. Purification of *Rp. palustris* URO-D

Step	Substrate (urogen)	Total protein (mg)	Total units		Specific activity		Yield (%)		Purification (fold)	
			C	TP	C	TP	C	TP	C	TP
1. Homogenate	I	689	4150	6357	6.05	9.26	100	100	1	1
2. Supernatant	I	303	3475	5392	11.48	17.82	84	85	2	2
3. Heat treatment	I	96	2891	4656	30.14	48.55	70	73	5	5
4. 45–75% std $(NH_4)_2SO_4$	I	37	2254	4000	62.60	111.00	54	63	10	12
5. Sephadex G-100	I	23	2125	3867	93.03	169.24	51	61	15	18
5. Sephadex G-100	II	—	—	—	121.87	195.00	—	—	—	—
1. Homogenate	III	689	6035	8965	8.79 (1.45)	13.06 (1.41)	100	100	1	1
2. Supernatant	III	303	5280	7842	17.45 (1.52)	25.92 (1.45)	87	87	2	2
3. Heat treatment	III	96	4718	7007	49.18 (1.63)	73.04 (1.50)	78	78	6	6
4. 45–75% std $(NH_4)_2SO_4$	III	37	3542	5454	98.36 (1.57)	151.50 (1.36)	59	61	11	12
5. Sephadex G-100	III	23	2987	4812	130.81 (1.40)	210.61 (1.24)	49	54	15	16
5. Sephadex G-100	IV	—	—	—	115.62	185.00	—	—	—	—

Experimental conditions are described in the text. Activity was measured using the four isomers of urogen as indicated and expressed either as the amount of coprogen formed (C) or that of total decarboxylated porphyrinogen products formed (TP). Numbers in parenthesis indicate the ratio between the activity found with urogen III and that found with urogen I.

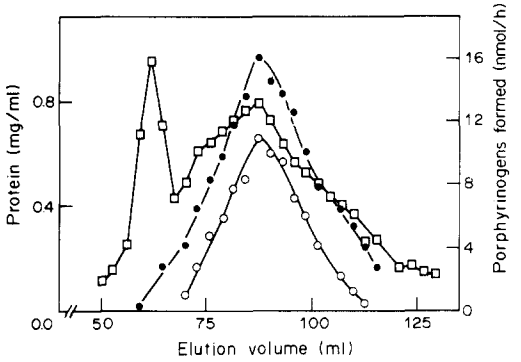

Fig. 1. Gel filtration of *Rp. palustris* URO-D. Experimental conditions are described in the text. Activity is expressed either as the amount of coprogen formed (○) or that of total decarboxylated porphyrinogen products found (●). Protein content (mg/ml) (□). Urogen I was used as substrate.

preparations before measurements of activity by molecular sieving with Sephadex G-25.

Gel filtration on Sephadex G-100. The 45–75% saturated ammonium sulphate fraction (about 45 mg protein) was suspended in a small volume (4–4.5 ml) of 0.05 M Tris–HCl buffer pH 7.0 and applied to a Sephadex G-100 column (2.4 × 45 cm), equilibrated and eluted with the same buffer at a flow rate of 20 ml/hr. Fractions of 3 ml were collected and those containing activity were pooled. Figure 1 shows a typical elution diagram. Sephadex G-100 filtration resolved two main protein peaks, URO-D activity was associated with the latter, yielding a single and rather symmetrical band. Elution profiles employing urogen I or III as substrate were identical. Inclusion of 1 mM dithiothreitol in the buffer, which was previously deairated, for equilibrium and elution of Sephadex G-100 column did not improved the degree of purification. Active fractions were concentrated by ammonium sulphate precipitation up to 75% saturation and stored at −15°C or immediately used for further studies. Under these conditions the enzyme is very stable, negligible loss of activity was detected after 3 months of storage.

The enzyme was purified between 15 and 18-fold with a high overall yield (about 50%). The isoelectric point determined electrophoretically was about 4.6. The apparent molecular weight (MW), using either urogen I or III as substrates was estimated to be 46,000 ± 4600 by Sephadex G-100 gel chromatography (Fig. 1). Using this value the turnover of *Rp. palustris* URO-D was calculated to be 0.11, 0.14, 0.15 and 0.16 mol products/mol of enzyme/min at the optimum pH with urogen I, II, III and IV as substrate respectively.

Enzyme activity towards urogen I and III copurified at each step of the purification procedure. At the optimum pH the rate of utilization of urogen III was about 1.5 times that of urogen I confirming results reported for purified human erythrocyte URO-D (de Verneuil *et al.*, 1983). Urogen II was nearly as good substrate as urogen III, then urogen IV followed and urogen I was the octagen decarboxylated at the lowest rate.

The absorption spectrum of URO-D showed a single absorbance maximum at 278 nm and no other

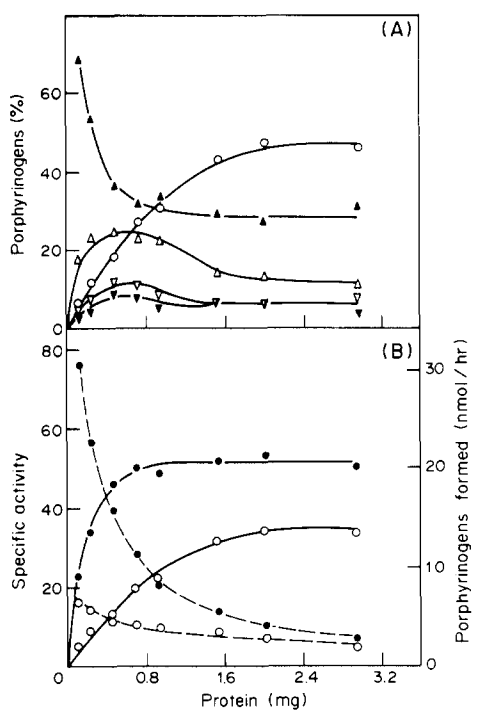

Fig. 2. Effect of protein concentration on URO-D activity at a fixed concentration of urogen. The enzyme source was the supernatant, Urogen III (12 μM) was used as substrate. Experimental conditions are described in the text. (A) Pattern of urogen consumption (▲) and products formed, coprogen (○), heptagen (△), hexagen (▽) and pentagen (▼); (B) total coprogen formed (○—○), total products formed (●—●) and specific activity expressed either on the basis of coprogen formed (○---○) or total porphyrinogens formed (●---●).

peak indicating the presence of any expected prosthetic group such as for instance pyridoxal phosphate was detected.

Enzyme concentration (Fig. 2)

Among the variables always tested to determine the optimum conditions for measuring enzyme activity, is protein concentration; when its influence on the reaction velocity was investigated with the *Rp. palustris* URO-D, it was found that (Fig. 2A) appearance of heptagen increased up to nearly 0.2 mg of protein, there remained constant in the range 0.2–1.0 mg and at greater concentrations a decrease was observed. The formation of coprogen augmented linearly up to 0.7 mg of enzyme and then seemed to reach a plateau, while concentrations of hexa and pentagen were nearly constant over the entire range assayed. If the formation of total decarboxylation products is analysed, a picture like that of coprogen is seen, except that the constant level is attained at lower concentrations of protein (*c.* 0.8 mg). However, if we calculate specific activity (Fig. 2B), expressed in either way, we observed that, when excess protein is present in the incubation mixture, specific activity fell; the less purified the enzyme the more noticeable this effect was. Therefore, in agreement with earlier observation (Straka and Kushner, 1983), to obtain both reproducible and accurate results with this enzyme, it

Table 2. Effect of heating on URO-D activity

Preparation	Total protein (mg)	Total units		Specific activity		Yield (%)		Purification (fold)	
		C	TP	C	TP	C	TP	C	TP
S	109.7	1490	2870	13.58	26.16	100	100	1.0	1.0
S 50°10′	63.6	1788	3319	28.11	52.17	120	115	2.0	2.0
S 50°15′	62.7	1702	3295	27.14	52.46	114	114	2.0	2.0
S 50°20′	58.2	1589	3210	27.29	53.57	107	108	2.0	2.0
S 55°5′	55.7	1646	3320	29.57	59.63	110	115	2.0	2.0
S 55°15′	45.7	1702	3120	37.25	68.27	114	108	3.0	2.6
S 55°20′	51.1	1475	3062	28.87	59.96	99	106	2.0	2.0
S 60°5′	35.4	1702	3062	48.00	86.36	114	106	4.0	3.3
S 60°10′	33.4	1710	3040	51.20	90.10	114	105	3.8	3.4
S 60°15′	31.4	1760	3009	56.11	95.94	118	104	4.0	4.0
S 65°5′	25.5	411	1368	16.14	54.62	28	47	1.2	2.1

Experimental conditions are described in the text. Urogen I was used as substrate. Activity is expressed either as the amount of coprogen I (C) formed or the amount of total decarboxylation products (pentagen I plus coprogen I) formed (TP). The supernatant (S) fraction was used as enzyme source.

was important to keep the amount of protein used in the standard assay mixture between 0.6–1 mg.

Heat stability (Table 2)

In most sources investigated so far, URO-D behaves as a thermo-labil enzyme. Activity was completely destroyed by heating the avian erythrocyte URO-D for 5 min at 60°C (Tomio *et al.*, 1970) or the mouse spleen enzyme for 5 min at 50°C (Romeo and Levin, 1971), or the bovine liver enzyme for 10 min at 60°C (Straka and Kushner, 1983). However, when purifying Deaminase for *Rp. palustris*, by heating the supernatant, we observed that the resulting fractions retained most of their decarboxylating activity (Kotler *et al.*, 1986). Therefore the effect of heating on the activity of *Rp. palustris* URO-D was investigated. Aliquots of the enzyme were preincubated at different temperatures, at various time intervals samples were removed and activity was then measured under the standard conditions, using urogen I or III as substrate. Table 2 shows the effect of heat on protein content and specific URO-D activity with urogen I, similar data were obtained with urogen III. As can be seen in Fig. 3, thermal activation followed virtually the same rate for both substrates.

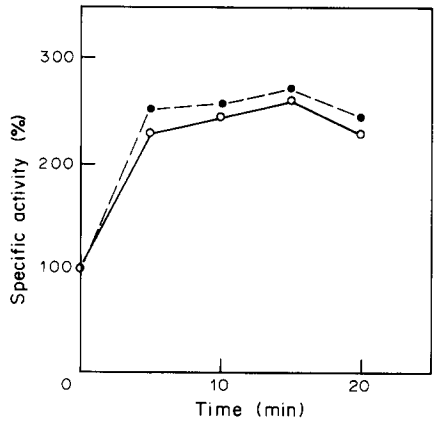

Fig. 3. Thermal activation of *Rp. palustris* URO-D. Experimental conditions are given in the text. Results obtained by heating different time intervals at 55°C are shown and using urogen I (○) or III (●) as substrates. The activity of the controls expressed on the basis of total porphyrinogens formed was taken as 100%.

It is obvious that *Rp. palustris* URO-D is a heat stable protein, pre-heating for 15 min at temperatures as high as 60°C increases specific activity up to 4-times with optimal yield. Heat treatment was then included as one of the steps followed for purifying the enzyme from this source.

Effect of pH (Fig. 4)

It was indeed very interesting that the rates of reaction of human erythrocyte URO-D using type I and III substrates were greatly affected by pH, thus an optimum pH of 6.8 was found for isomer III of porphyrinogens while optimum pH values for isomer I were significantly lower (De Verneuil *et al.*, 1983). Consequently the effect of pH on the decarboxylation of urogen III (Fig. 4A) and I (Fig. 4B) was here investigated. With the former, the enzyme had an optimum pH of 6.9, and 6.8 when using the latter substrate.

Precipitation with acetic acid to pH 5.0–5.5 is a procedure sometimes used to purify enzymes (Sancovich *et al.*, 1969), including URO-D from mouse spleen (Romeo and Levin, 1971) and bovine liver (Straka and Kushner, 1983). The effect of lowering the pH with acetic acid on URO-D activity was tested. The supernatant kept at 0°C was adjusted to pHs 4.3, 5.0 and 5.5 by the dropwise addition of glacial acetic; equilibrated for 20 min with constant stirring, then the mixture was centrifuged for 10 min at 12,000 *g* and the precipitate discarded. The supernatant was taken to pH 7.0 with 0.5 N OHK and URO-D measured under the standard conditions (Table 3). Recovery of activity from the pH 5.0 supernatant was nearly 100% and purification over 2-fold.

Taking into account these results the combination of both acid precipitation and heating was tested to find out whether it might improve purification. A portion of the supernatant was first treated to pH 5.0 and the resulting supernatant heated at 60°C for 15 min, while another portion was first heated and then adjusted to pH 5.0. As it can be seen in Table 4, most of the proteins eliminated by acid precipitation should have apparently being removed during heat treatment, which is the procedure giving the best purification by itself in only one step, making the tested combination unnecessary.

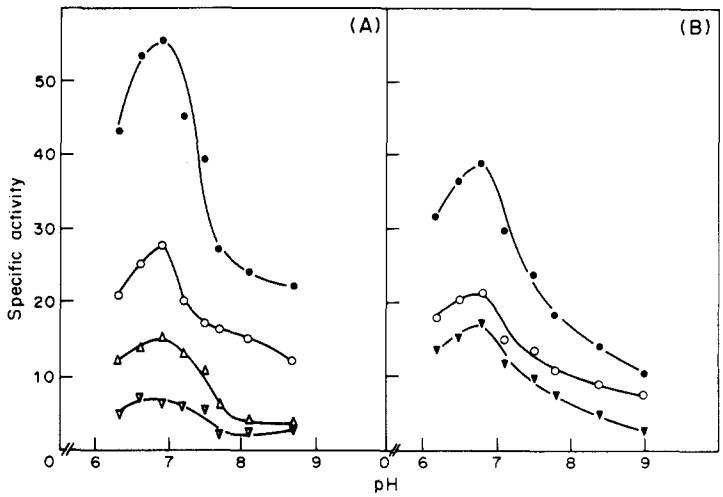

Fig. 4. Effect of pH on *Rp. palustris* URO-D activity. Experimental conditions are described in the text. 45–75% std ammonium sulphate fraction was used as enzyme source. 0.05 ,M Tris–HCl, Na-phosphate and glycine-OH were employed to cover de range of pH studied. Specific activity is expressed on the basis of the amount of total porphyrinogens formed (●); or that of each of the decarboxylation products, coprogen (○), heptagen (△), hexagen (▽) and pentagen (▼), using urogen III (A) or I (B) as substrate. GSH and EDTA were omitted.

Table 3. Effect of acid precipitation on URO-D activity

	Total units		Specific activity		Yield (%)		Purification (fold)	
Preparation	C	TP	C	TP	C	TP	C	TP
S	2036	4435	12.12	26.40	100	100	1	1
S pH 4.3	1350	1590	12.17	56.43	17	36	1	2
S pH 5.0	2009	3946	30.43	59.78	99	89	2.5	2.3
S pH 5.5	1764	3465	17.28	33.95	87	78	1.4	1.3

Experimental conditions are described in the text. Urogen I was used as substrate. C (coprogen formed) TP (total products formed). The supernatant (S) fraction was used as enzyme source.

Effect of atmosphere, GSH and EDTA (Table 5)

URO-D activity is usually measured under anaerobic conditions, in some cases decarboxylation is also dependent on the presence of GSH in the incubation mixture and EDTA is often added along with (Tomio *et al.*, 1970). However, in one of the earliest papers on URO-D, Mauzerall and Granick (1958) found that the enzyme was not affected by changing from aerobic to anaerobic conditions, and that the activity of URO-D was neither greatly influenced by added GSH nor largely modified by EDTA.

Activity of *Rp. palustris* URO-D was assayed in the presence and absence of these compounds and under anaerobiosis, in Thunberg tubes and in oxygen-free nitrogen and also under aerobiosis. Neither GSH nor EDTA seem to be necessary for activity, and interestingly enough, the decarboxylation rate and the distribution of the reaction products was not affected either by the presence of oxygen. Nevertheless, to protect the enzyme from both possible trace heavy metals inhibition and auto-oxidation as it increases

Table 4. Effect of combining heat treatment and acid precipitation

	Specific activity		Purification (fold)	
Preparation	C	TP	C	TP
S	18.94	32.47	1	1
S pH 5.0	41.40	75.25	2.2	2.3
S ϕ 60°	71.97	123.38	3.8	3.8
S pH 5.0; ϕ 60°	73.86	133.12	3.9	4.1
S ϕ 60°; pH 5.0	70.70	129.57	3.7	4.0

Experimental conditions are described in the text. Urogen I was used as substrate. C (coprogen formed), TP (total products formed). The supernatant (S) fraction was used as enzyme source.

Table 5. Effect of atmosphere, GSH and EDTA on URO-D activity

		Specific activity	
Atmosphere	Additions	C	TP
Thunberg tubes	1. None	8.0	12.1
	2. GSH 1 mM	9.0	13.0
	3. EDTA 0.1 mM	8.5	12.0
	4. GSH 1 mM EDTA 0.1 mM	8.5	11.5
N_2	1. None	8.0	11.5
	2. GSH 1 mM	8.5	12.5
	3. EDTA 0.1 mM	7.5	12.5
	4. GSH 1 mM EDTA 0.1 mM	8.0	12.4
Air	1. None	7.5	12.0
	2. GSH 1 mM	8.3	11.5
	3. EDTA 0.1 mM	8.0	12.2
	4. GSH 1 mM EDTA 0.1 mM	8.5	11.5

Experimental conditions are described in the text. The supernatant fraction was used for activity with urogen I as substrate. Final concentrations in the incubation mixture are indicated. GSH and EDTA were omitted in the controls. Anaerobiosis was achieved by using either Thunberg tubes or oxygen free nitrogen atmosphere in a Dubnoff bath.

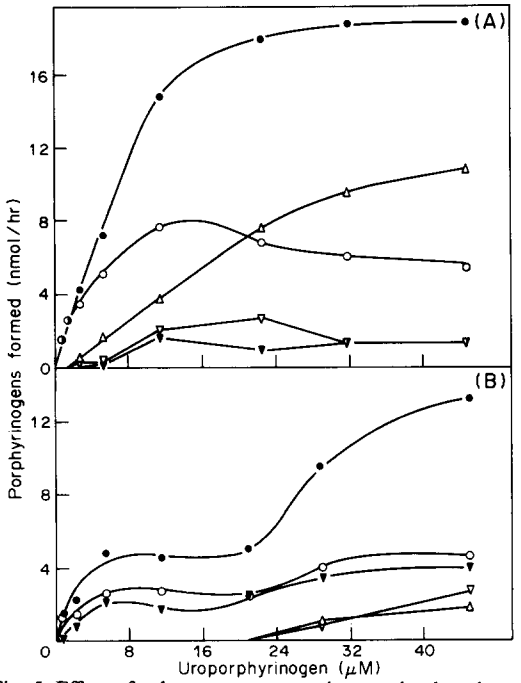

Fig. 5. Effect of substrate concentration on the decarboxylation of urogen III (A) and I (B). Experimental conditions are given in the text. The supernatant fraction was used as enzyme source. Total decarboxylated products (●), coprogen (○), heptagen (△), hexagen (▽) and pentagen (▼). GSH EDTA were omitted.

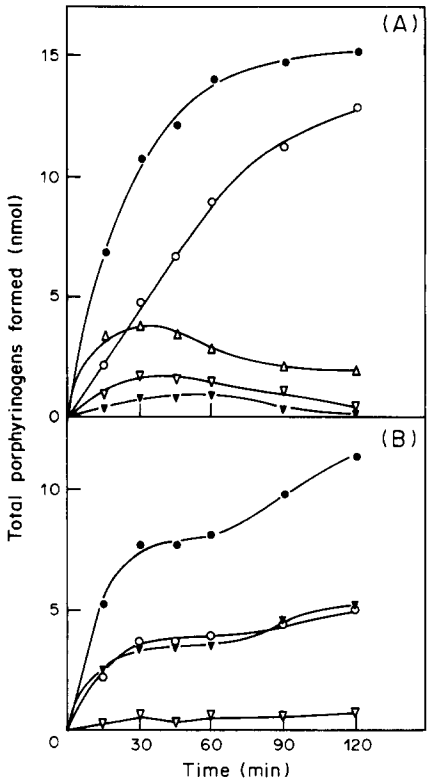

Fig. 6. Time course of the reaction. Experimental conditions are given in the text. Urogen III (A) and I (B) were used as substrate. The supernatant fraction was used as enzyme source. Total decarboxylated products (●), coprogen (○), heptagen (△), hexagen (▽) and pentagen (▼). GSH and EDTA were omitted.

its purity, GSH and EDTA were usually added to the incubation mixture; which was very convenient carried out in open tubes in a Dubnoff bath under N_2.

Effect of substrate concentration, isomer type (Fig. 5) and time-course of the reaction (Fig. 6)

A series of experiments were carried out in which the concentration of either urogen I or III was varied between 0.8 and 45 μM.

The effect of substrate concentration on the formation of coprogen III and hepta, hexa and pentagen III intermediates is depicted in Fig. 5A. At low substrate concentration (<3 μM) the predominant product was coprogen III and formation of the other intermediates was almost negligible. At about 12 μM of urogen III maximum amount of coprogen III was found and at higher concentrations of substrate coprogen III formation diminished, whereas heptagen III greatly accumulated; obviously the amount of total decarboxylated porphyrinogens III increased.

When urogen I was used as substrate (Fig. 5B) the rate of decarboxylation was less than that of urogen III. Again at low urogen I concentration the main product was coprogen I but above 2 μM, pentagen I began to accumulate and from about 5–20 μM, nearly equal amounts of coprogen I and pentagen I were the predominant porphyrinogens formed. Only at very high concentrations of urogen I (>20 μM) intermediates with 7 and 6 carboxyls began to accumulate in significant quantities. Straka and Kushner (1983) have observed that at 30–50 μM of urogen I the amount of pentagen intermediate exceeded that of coprogen I; but as far as we know, this is the first source of URO-D where there is such a striking difference in the composition of the reaction products depending whether urogen III or I were used as substrate. This picture was also influenced by the ratio between the concentration of the substrates and the enzyme in the incubation media (Fig. 2), as well as the reaction time as we can see in Fig. 6, where the time course of urogen III and I decarboxylation was compared at a fixed substrate concentration.

The rates of formation of coprogen III were linear up to 1 hr, whereas heptagen III accumulated rapidly within 30 min and then decreased. The accumulation of hexa and pentagen III was low. These results are in good agreement with the view that the first decarboxylation of urogen III yielding heptagen III is not the rate limiting step (García *et al.*, 1973).

With urogen I as substrate, only copro and pentagen accumulated until they reached apparent steady-state levels. Again a unique picture of reaction products emerged from urogen I, indicating that in this case, the rate limiting step is the decarboxylation of pentagen I.

Furthermore, the stoichiometry of the reaction was investigated by comparing the total amount of decarboxylated porphyrinogens formed and that of urogen consumed. Balance studies indicated that all the urogen disappearing was accounted for by the intermediate porphyrinogen(s) and the coprogen formed.

DISCUSSION

As already stated *Rp. palustris* has provided a good source for studying the enzymes involved in tet-

rapyrrole biosynthesis in photosynthetic organisms. Succinyl CoA-Synthetase (Schoua *et al.*, 1986), δ-Aminolevulinic acid Synthetase (Viale *et al.*, 1980a,b, 1983, 1985), Porphobilinogenase (Juknat *et al.*, 1986), Deaminase (Kotler *et al.*, 1986), Coproporphyrinogenase (Koopmann *et al.*, 1986) and now URO-D has been identified from this bacterium. Interestingly, activity of the initial homogenate prepared from *Rp. palustris* (0.217 units/mg protein/min) was the highest value so far reported; so although the overall purification was only 16–18 fold, the final fraction obtained with a yield of about 50% had a very high specific activity. The turnover number of the purified URO-D (between 0.11 and 0.16 mol/mol of enzyme/min, depending on the isomer of urogen used as substrate) was of the same order of magnitude as those calculated for other enzymes in the porphyrin pathway from the same organism [0.40 for Porphobilinogenase, Juknat *et al.* (1986); and 0.80 for Deaminase, Kotler *et al.* (1986)] and also within the range of values calculated for URO-D from other sources; 2–12 mol/mol enzyme/min from human erythrocytes (De Verneuil *et al.*, 1983; Elder *et al.*, 1983), 0.17–1.8 from avian erythrocytes (Tomio *et al.*, 1970; Kawanishi *et al.*, 1983); 0.71 from bovine liver (Straka and Kushner, 1983).

Purified *Rp. palustris* URO-D has a molecular weight of $46{,}000 \pm 4600$, in good agreement with values reported for the human erythrocyte enzyme [39,000–46,000 De Verneuil *et al.*, 1983; Elder *et al.*, 1983]. A molecular weight of about 57,000 was found for the bovine liver URO-D (Straka and Kushner, 1983) but both De Verneuil *et al.* (1983) and Elder *et al.* (1983) had observed that the molecular weight of the native URO-D purified from fresh blood was about 1.5 times that obtained from either out dated blood or from SDS-polyacrylamide electrophoresis; suggesting that, either a conformational change or a proteolytic cleavage of a fragment of the protein might have occurred during storage or during purification.

A monomeric enzyme is expected to have the same structure in different tissues whereas if the enzyme is a dimer, at least one of the units should be common to any each tissue and the presence of tissue-specific subunits would be possible. Recent findings (Elder and Urquhart, 1984), provided evidence against the existence of tissue-specific isoenzymes of human URO-D specified by independent genomic sequences, supporting the view that the human URO-D is a monomer of mol. wt 40,000. The avian erythrocyte enzyme is dimeric (Kawanishi *et al.*, 1983). The URO-D from *Rp. palustris* appears to be a monomer; still, both the alternative of being a protomer of two subunits, identical or not, very tightly associated (Batlle *et al.*, 1978; Batlle and Stella, 1978) and the possibility of aggregation of these subunits under certain experimental conditions, such as varying protein or salt concentration (Llambías and Batlle, 1971a,b; Rossetti *et al.*, 1980; Stafforini *et al.*, 1980) are presently being investigated.

The obtainment of purified *Rp. palustris* URO-D with high specific activity and in good yield, enables us to establish the nature of this enzyme and to perform structural and mechanistic studies on the reaction mechanism (Batlle and Koopmann, 1986).

The enzyme has optimal activity at pH 6.8–6.9 for urogen I and III as substrate as reported for URO-D from most sources and it also appears to be an acidic protein (isoelectric point 4.6).

This is the first time that a heat treatment is included in the procedure developed for purification of URO-D, because the *Rp. palustris* enzyme is a thermo-stable protein and heating at 60°C for 15 min increased 4-fold the activity of the supernatant. It was also observed that at all temperatures tested, using urogen I or III as substrate, the ratio of URO-D activities remained virtually the same as the enzyme became activated, indicating that a single protein would be involved in the decarboxylation of all porphyrinogens, as already suggested (Romeo and Levin, 1971; Elder and Tovey, 1977; De Verneuil *et al.*, 1983). Purification of the enzyme with a constant ratio of decarboxylation rates among the two isomers of urogen as substrates further supports that a single enzyme catalyzes the four decarboxylations from urogen to coprogen.

The kinetic properties of the purified *Rp. palustris* URO-D were in good agreement with those previously reported (Tomio *et al.*, 1970; Smith and Francis, 1979; De Verneuil *et al.*, 1980, 1983; Kawanishi *et al.*, 1983; Straka and Kushner, 1983; Cantoni *et al.*, 1984), so at low substrate concentration or under conditions in which the rates of enzyme to substrate is high, the data here presented showed that the predominant reaction product was coprogen, while nil or very small amounts of intermediate porphyrinogens were formed; but, when an excessive amount of urogen III was used, heptagen III accumulated resulting in the inhibition of coprogen III formation and indicating that heptagen III is also inhibiting its further decarboxylation as already observed (García *et al.*, 1973). In a normal cell, urogen III concentration remains far below saturating levels, so under physiological conditions the ratio of enzyme to substrate would be high enough to predict that the only reaction product would be coprogen III; moreover, very small amounts of intermediates accumulate or are excreted by normal organisms.

It has been proposed that urogen decarboxylation occurred in two stages (Tomio *et al.*, 1970)

$$\text{8-COOH} \xrightarrow{v_1} \text{7-COOH} \xrightarrow{v_2} \text{4-COOH}$$

where v_1 is greater than v_2; therefore the decarboxylation of the heptagen would be the rate limiting step, and v_2 is often most susceptible to various chemical and physical agents than v_1 (San Martín de Viale *et al.*, 1969; Tomio *et al.*, 1970; Straka and Kushner, 1983).

In all sources so far examined, kinetic results using either urogen III or I as substrate or both seem to be in agreement with the two stage process (Straka and Kushner, 1983; Kawanishi *et al.*, 1983; Cantoni *et al.*, 1984), where the first decarboxylation of urogen is not the rate-limiting step. In this paper, we have obtained evidence indicating that, when urogen III is the substrate, decarboxylation of heptagen III is the step proceeding at lower rate, in keeping with the usual scheme:

$$\text{Octagen III} \xrightarrow{v_1} \text{Heptagen III} \xrightarrow{v_2} \text{Coprogen III}$$

where $v_1 \gg v_2$ and decarboxylation of the heptagen is the rate-controlling step.

But, for the first time, a different picture emerged when urogen I was used, where decarboxylation of the substrate proceeds rapidly up to the formation of pentagen I and removal of the final carboxyl group becomes the rate-limiting step; as it is proposed in the following scheme:

$$\text{Octagen I} \xrightarrow{v_1'} \text{Pentagen I} \xrightarrow{v_2'} \text{Coprogen I}$$

where $v_1' \gg v_2'$

The differential behaviour of *Rp. palustris* URO-D towards urogen III and I, still indicating the existence of two distinct decarboxylation rates, suggests that the sequential decarboxylation of urogen III and I probably occurs at two or possibly more different catalytic sites, which might or might not be interacting active sites; but, that could allow the enzyme recognize either isomer. It was proposed that the position of the side chains on ring D or urogen III favours the more rapid formation of a specific enzyme–substrate complex and, rotation of the substrate for subsequent decarboxylation (Cantoni *et al.*, 1984).

Having an enzyme which seems to be able to distinguish somehow, between urogen III and I, it can be possible to comparatively study the kind of interaction existing between this enzyme and both substrates and to obtain more experimental information as to the probable mechanism of this most unusual enzymic decarboxylation, where a single protein, apparently not requiring any cofactor, catalyzes four different reactions in the conversion of the octagen to the coprogen.

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