Int. J. Biochem. Vol. 19, No. 4, pp. 373–377, 1987
Printed in Great Britain.

0020-711X/87 $3.00 + 0.00

BIOSYNTHESIS OF PORPHYRINS IN *RHODOPSEUDOMONAS PALUSTRIS*—VI. THE EFFECT OF METALS, THIOLS AND OTHER REAGENTS ON THE ACTIVITY OF UROPORPHYRINOGEN DECARBOXYLASE

GERARDO ENRIQUE KOOPMANN and ALCIRA MARIA DEL C. BATLLE*
Centro de Investigaciones sobre Porfirinas y Porfirias-CIPYP(CONICET and FCEyN, University of Buenos Aires), Pabellón II, Ciudad Universitaria, 1428 Buenos Aires, Argentina

(*Received* 21 *July* 1986)

Abstract—1. The effect of several metals and reagents on the decarboxylation rate of uroporphyrinogen I by using a 16-fold purified preparation of Uroporphyrinogen Decarboxylase from *Rhodopseudomonas palustris*, was studied.

2. 1 mM Hg^{2+} and Cu^{2+} were strong inhibitors, 1 mM Zn^{2+} and Fe^{2+} under certain conditions and 1 mM Fe^{3+} and Cr^{3+} also inactivated the enzyme, but Pb^{2+}, Cd^{2+} and Al^{3+} did not. Metals inhibition was reversed by 1 mM GSH or CySH.

3. 0.1 mM DTNB and PCMB, 1 mM pyridoxal phosphate and 100 mM chloral hydrate, as well as 1 mM 2-methoxy-5-nitrotropone and 0.2 mM diethylpyrocarbonate inhibited Uroporphyrinogen Decarboxylase; while GSH, CySH, *N*-ethylmaleimide, sodium thioglycolate, 1,4-dithioerythritol, EDTA and *O*-phenantroline did not modify activity.

4. Data obtained would indicate that one cysteine, one or two histidine residues and probably a lysine group are required for enzyme activity.

INTRODUCTION

Uroporphyrinogen decarboxylase (Uro-D) (EC 4.1.1.37) catalyzes the sequential removal of four carboxyl groups of uroporphyrinogen (urogen) to yield coproporphyrinogen (coprogen). Numerous reports describing the properties of this enzyme from various sources have appeared (Mauzerall and Granick, 1958; Tomio *et al.*, 1970; Romeo and Levin, 1971; De Verneuil *et al.*, 1983; Elder *et al.*, 1983; Kawanishi *et al.*, 1983; Straka and Kushner, 1983; Koopmann *et al.*, 1986). Studies to identify the amino acid residues at the active site have visualized the presence of cysteine and possible histidine residues (Mauzerall and Granick, 1958; De Verneuil *et al.*, 1983; Elder *et al.*, 1983; Kawanishi *et al.*, 1983; Straka and Kushner, 1983).

The effect of metals, particularly iron, on Uro-D activity *in vitro*, have produced conflicted data. Iron has been found to have no action (Woods *et al.*, 1981, 1984; De Verneuil *et al.*, 1983), to inhibit (Straka and Kushner, 1983; Smith and Francis, 1983; Mukerji *et al.*, 1984) or even to stimulate (Blekkenhorst *et al.*, 1979) urogen decarboxylation. Due to the important role that hepatic iron plays in both the biochemical and clinical expression of the diminished Uro-D activity in porphyria cutanea tarda (PCT) (Kappas *et al.*, 1983), research in this area has attracted great interest.

Uro-D from *Rhodopseudomonas palustris* (Koopmann *et al.*, 1986) exhibits a number of properties similar to the enzyme from other sources; but it also shows some characteristics which are rather unique, making this organism very convenient to carry out mechanistic studies. As it was found in all sources so far examined, decarboxylation of urogen is a two stage process; however, with the *Rp. palustris* enzyme, a differential behaviour towards isomers III and I of the urogen was observed. Decarboxylation of heptaporphyrinogen is the rate-limiting step when urogen III is the substrate, while removal of the final carboxyl group of pentaporphyrinogen becomes the rate controlling step when urogen I is the substrate, with practically no accumulation of other intermediates. For this reaction it was proposed the scheme:

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

where $v_1 \gg v_2$ (Koopmann *et al.*, 1986).

This paper describes the results of studies undertaken to obtain evidence as to the type of essential residues at the active site of *Rp. palustris* Uro-D. To this end, the effect of several metals and various reagents on the enzymic decarboxylation rate of urogen I, was investigated.

MATERIALS AND METHODS

Enzyme preparation

A fraction purified 16-fold from *Rp. palustris*, following the procedure already described (Koopmann *et al.*, 1986) was used throughout this study.

*All correspondence should be addressed to: Professor Alcira Batlle, Viamonte 1881 10°A, 1056 Buenos Aires, Argentina.

Abbreviations: urogen—uroporphyrinogen; pentagen—pentaporphyrinogen; coprogen—coproporphyrinogen; PCMB—*p*-chloromercuribenzoate; DTNB—5,5′-dithiobis(2-nitrobenzoate); GSH—glutathione; CySH—cysteine.

Uro-D assay

Uro-D activity was assayed as previously detailed (Koopmann *et al.*, 1986). Unless otherwise stated, the standard incubation conditions were as follows: the reaction mixture contained 0.1 ml of the enzyme preparation (0.20 mg protein) in 0.05 M Tris–HCl buffer pH 6.8; 15 μM urogen I, and buffer to make a final volume of 2 ml. Studies to ascertain the effect of metals, thiols and other reagents were performed by adding appropriate concentration of assay buffer (up to 0.2 ml) of the test substance. Solutions of the chemicals were either preincubated for 20 min at 37°C or without preincubations; the reaction was allowed to proceed for 60 min at 37°C after the addition of the substrate, with constant mechanical shaking, in the dark and under free-oxygen nitrogen. Controls were run containing no reagents. When the effect of 2-methoxy-5-nitrotropone and diethylpyrocarbonate was tested, the experimental conditions were different, following the procedures described by Tamaoki *et al.* (1967) and Setlow and Mansour (1970) respectively. The rest of experimental conditions were already described (Koopmann *et al.*, 1986).

Enzyme activity was quantitated and expressed on the basis of nmoles of coprogen I (C) and total decarboxylated products (TP) formed per hour. Activity of all controls was taken as 100%.

Other conditions or any added or omitted component are indicated in the text or in the legends to tables. Metals were chloride salts, except lead, copper and zinc acetates and ferrous ammonium sulphate. In all instances a wider concentration of the different compounds was tested, however only data obtained at the maximum are here reported.

Other materials or methods not specified here were those previously reported (Koopmann *et al.*, 1986).

RESULTS

Table 1 shows the inhibitory effect of some metals on the activity of Uro-D. Hg^{2+} and Cu^{2+} are the most potent inhibitors of the enzyme, as already demonstrated (Woods *et al.*, 1981; De Verneuil *et al.*, 1983; Straka and Kushner, 1983). Zn^{2+} and Fe^{2+}, under certain conditions, are also inhibitors. Hg^{2+}, Zn^{2+} and Fe^{2+} inhibitions can be reversed by GSH or CySH, suggesting that these metals do not denature the enzyme but probably bind reversibly to sulphydryl groups at the active site. Confirming previous findings (Woods *et al.*, 1981) Pb^{2+}, a well known inhibitor of δ-aminolevulinate dehydratase (ALA-D)

Table 1. Effect of metals on *Rp. palustris* Uro-D activity

Incubation conditions			Activity			
			nmol porphyrinogens/hr		%	
Reagents added	Conc. (mM)	Preinc.	C	TP	C	TP
Control		+	11.81	19.12	100	100
		–	11.95	19.18	100	100
GSH	1.0	+	11.42	18.11	97	95
		–	12.55	19.13	105	100
CySH	1.0	+	10.84	16.66	92	87
		–	12.04	19.22	101	100
Cu^{2+}	1.0	+	0	0.42	0	2
		–	0.50	1.12	4	6
Cd^{2+}	1.0	+	11.56	17.37	98	91
		–	12.52	20.27	105	106
Pb^{2+}	0.5	+	11.79	19.10	100	100
		–	11.97	19.20	100	100
	1.0	+	10.82	16.62	92	87
		–	12.20	19.95	102	104
Hg^{2+}	1.0	+	0	0	0	0
		–	3.24	10.62	27	55
Hg^{2+}	1.0	+	7.88	12.59	67	66
GSH^{+}	1.0[b]	–	15.64	23.90	131	125
Zn^{2+}	0.5	+	10.17	17.23	86	90
	1.0	+	0.46	0.97	4	5
		–	9.44	15.82	79	82
Zn^{2+}	1.0					
GSH^{+}	1.0[b]	+	12.77	21.55	108	113
Fe^{2+}	0.1	+	10.36	17.37	88	91
		–	10.19	17.51	85	91
	0.5[a]	+	10.12	17.18	86	90
		–	9.69	16.61	81	87
	1.0[a]	+	5.63	14.16	48	74
		–	9.10	18.19	76	95
	1.0	+	3.92	8.95	33	47
		–	10.09	17.89	84	94
Fe^{2+}	1.0[a]					
GSH^{+}	1.0[b]	+	12.50	19.16	106	100
Fe^{2+}	1.0[a]					
$CySH^{+}$	1.0[b]	+	13.18	18.56	112	97
Fe^{3+}	1.0	+	4.35	8.80	37	46
Cr^{3+}	1.0	+	4.97	10.65	42	56
Al^{3+}	1.0	+	10.87	18.76	92	98

Experimental conditions are indicated in the text, except that incubations were carried out under standard conditions in Tris–HCl buffer or in 0.05 M sodium phosphate buffer pH 6.8 where indicated ([a]). (+) With preincubation, (–) without preincubation. ([b]) In these cases, when the enzyme was preincubated with the metal ion, GSH (or CySH) was added just before the substrate immediately prior to assay. The final concentration of each reagent in the reaction mixture is indicated.

and various other thiol enzymes, did not affect Uro-D activity; Cd^{2+} did not inhibit the enzyme either.

In most cases greater inhibition was attained when the enzyme was previously incubated with the metal; this is particularly noticeable with 1 mM Hg^{2+}, Zn^{2+} and Fe^{2+}.

Hg^{2+} and Fe^{2+} also affected more strongly decarboxylation of pentagen I, that is v_2, the rate-limiting step of the reaction (Koopmann *et al.*, 1986), was more sensible to inhibition than v_1. Here the pattern of porphyrinogen decarboxylated products showed a greater proportion of pentagen at the expenses of coprogen; in the presence of GSH however, not only inhibition was prevented but the original distribution of copro and pentagen of the controls was again obtained.

It is interesting that the inhibitory action of 1 mM Fe^{2+} was observed using either Tris–HCl or phosphate buffer, although it was somehow greater when preincubation was done with the former.

Trivalent ions Fe^{3+} and Cr^{3+} also diminished Uro-D activity; Al^{3+} instead, did not.

Table 2 describes the effects of several compounds on Uro-D activity. As already noted (Koopmann *et al.*, 1986), the presence of different thiols with or without preincubation, did not affect the enzyme. As expected, DTNB strongly inhibited, and it must be noted that here too the second step in the decarboxylation of urogen I was more sensible than the first. On titration with this reagent (data not shown), approximately five free SH groups per 46,000 of molecular weight were found; however, enzymic activity was rapidly lost after disappearance of only one thiol group.

Sulphydryl-inactivating chemicals such as PCMB and chloral hydrate, clearly inhibited Uro-D activity, while *N*-ethylmaleimide only produced about 15% reduction; anyhow these findings were confirming previous results (Kardish and Woods, 1980; De Verneuil *et al.*, 1983; Elder *et al.*, 1983; Kawanishi *et al.*, 1983; Straka and Kushner, 1983) and supporting the view that a thiol group would play an important role in the enzyme. Neither EDTA nor *o*-phenantroline had any effect (Mauzerall and Granick, 1958; Tomio *et al.*, 1970; Romeo and Levin, 1971; Woods *et al.*, 1981; De Verneuil *et al.*, 1983; Elder *et al.*, 1983; Kawanishi *et al.*, 1983; Koopmann *et al.*, 1986), indicating that the purified Uro-D contains no metal (Kawanishi *et al.*, 1983).

Interestingly, inactivation with diethylpyrocarbonate is suggesting that histidine residues may reside at the active site of the enzyme (Kawanishi *et al.*, 1983). Moreover, the presence of one or more essential amino groups was also indicated by the inactivating effect of 2-methoxy-5-nitrotropone and pyridoxal phosphate.

Additional support for the existence of cysteine and histidine rests as part of the active center was found analyzing the pH profile of *Rp. palustris* Uro-D (Koopmann *et al.*, 1986), that showed a maximum of 6.9 and 6.8 for urogens III and I as substrate, and inflection points at 6.40 and 8.45, which are known

Table 2. Effect of various reagents on *Rp. palustris* Uro-D activity

Incubation conditions			Activity			
			nmol porphyrinogens/hr		%	
Reagents added	Conc. (mM)	Preinc.	C	TP	C	TP
Control		+	9.71	17.03	100	100
		−	9.86	17.11	100	100
GSH	0.5	+	9.49	16.98	98	100
	1.0	+	9.67	17.31	100	102
	2.0	+	9.53	16.48	98	97
	5.0	+	9.74	17.00	100	100
	7.0	+	10.11	18.66	104	110
	10.0	+	9.68	18.35	100	108
CySH	1.0	+	9.39	16.20	97	95
	5.0	+	8.27	15.46	85	91
Na thioglycolate	1.0	+	9.09	14.96	94	88
		−	9.92	17.15	101	100
1,4-Dithioerythritol	1.0	+	9.28	15.64	96	92
		−	9.98	17.08	101	100
DTNB	0.1	+	1.68	5.64	17	33
		−	1.65	5.44	17	32
Chloral hydrate	100.0	+	5.9	12.15	61	71
PCMB	0.1	+	0.41	6.02	4	35
N-ethyl maleimide	1.0	+	8.22	14.68	85	86
EDTA	0.1	+	10.08	18.03	104	106
		−	9.82	17.44	100	102
o-Phenantroline	0.1	+	9.63	17.58	99	103
		−	10.05	18.01	102	105
Pyridoxal-5-phosphate	1.0	+	3.86	9.38	40	55
	25.0	+	3.21	7.81	33	46
Control (MNT)	[a]		5.08	10.44	100	100
MNT	1.0[a]	[b]	2.32	5.94	46	57
Control (DEP)	[a]		4.20	10.14	100	100
DEP	0.2[a]	[b]	2.91	7.48	69	74
	1.0[a]	[b]	2.03	6.81	48	67

Experimental conditions are the same as those indicated in Table 1 or in the text. Where indicated ([a]) 0.05 M sodium phosphate buffer was used. (+) With preincubation, (−) without preincubation. ([b]) Pretreatment times for 2-metoxi-5-nitrotropone (MNT) and diethylpyrocarbonate (DEP) were 180 and 60 minutes respectively, at room temperature.

to correspond with pK values observed for histidine (6.00) and cysteine (8.33) (Lehninger, 1975). The rabbit (Mauzerall and Granick, 1958) and the avian (Kawanishi *et al.*, 1983) erythrocyte enzymes also had inflection points at pHs 8.2 and 6.0 respectively.

DISCUSSION

The inhibition pattern of the metal ions studied appears to support the presence of both cysteine and histidine at the active site of *Rp. palustris* Uro-D. Depending on the oxidation state, metals are preferentially bound to different types of ligands, involving atoms which can extend from highly electronegative or hard donor atoms such as oxygen, to the more polarizable or soft atoms such as sulfur. Ions binding hard donors are classified as hard cations while those binding soft donors are called soft cations, and there seem to be a good correlation between the biochemical activity of a metal ion and some parameters measuring their hardness or softness (Jones and Vaughn, 1978).

From the softest metal ions tested, Pb^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Fe^{2+}; Hg^{2+} and Cu^{2+} were strongly inhibitory and to a lesser extent Zn^{2+} and Fe^{2+}; however, Pb^{2+} and Cd^{2+} were not. These metals are usually found bound to soft ligands, in this case most likely to sulfur donor portion of Uro-D. This observation is in agreement with the involvement of a cysteine residue, from the inflection point at pH 8.45 in the pH profile of enzyme activity, the reversal of metal inhibition by thiols and the inactivation by DTNB and SH reagents.

Confirming previous findings (Mukerji *et al.*, 1984; Mukerji and Pimstone, 1986), Fe^{2+} and Zn^{2+} were found to inactivate Uro-D to different degree depending on the experimental conditions; GSH reversed inactivation, supporting further the view that these metals should be blocking the active site by reversibly binding essential SH groups.

In spite of its well known affinity for sulfhydryl residues, neither Pb^{2+} nor Cd^{2+} inhibited Uro-D. These results can be explained if a monothiol rather than a dithiol group is at the active site, as already suggested (Woods *et al.*, 1981), because these metals are strong inhibitors of enzymes possessing more than one SH residue or vicinal dithiols at the active center (Seehra *et al.*, 1981); additional support for this view came from results of inhibition by arsenate (Batlle *et al.*, 1965; Woods *et al.*, 1981), and titration with DTNB (De Verneuil *et al.*, 1983); indicating that at least one SH in the Uro-D is involved in the decarboxylation reaction mechanism.

According to Klopman (1968) metal hardness classification, Fe^{3+} and Cr^{3+} are intermediate ions, capable of accepting electrons from tertiary aromatic nitrogen as found in histidine; these results, together with diethylpyrocarbonate inactivation and the inflection point at pH 6.4 in the pH profile of enzyme activity, are strongly indicative of histidine at or near the active site. Furthermore, amino acid composition of human (De Verneuil *et al.*, 1983) and avian (Kawanishi *et al.*, 1983) erythrocytes showed between 11 and 12 histidine residues per mol of the native enzyme, and results on photoinactivation of human red blood cell Uro-D by porphyrins (Batlle *et al.*, 1986) would also imply a mechanism involving blocking or a conformational change of the active site through binding of a histidine functional residue. Specific studies on the role of histidine in the reaction mechanism of urogen decarboxylase are in progress.

Some indication of essential amino groups for catalysis was also obtained from the action of 2-methoxy-5-nitrotropone and pyridoxal phosphate.

In conclusion, present data would indicate that one cysteine, one or two histidine residues and possibly one lysine group are required for enzyme activity and therefore are involved in the reaction mechanism of urogen decarboxylation by *Rp. palustris* Uro-D.

Acknowledgements—Alcira M. del C. Batlle holds the post of Principal Scientific Researcher in the Argentine National Research Council (CONICET) and Gerardo E. Koopmann is a Research Fellow at the CONICET. This work forms part of the Doctoral Thesis of Gerardo E. Koopmann to be submitted to the University of Buenos Aires for his Ph.D. degree. We are grateful to Dr Torben K. With for generously giving us his samples of uroporphyrin I. This work was supported by grants from CONICET, the SECYT, the University of Buenos Aires, the Secretaría de Salud Pública de la Nación and Banco de la Nación Argentina.

REFERENCES

Batlle A. M. del C., Benson A. and Rimington C. (1965) Purification and properties of coproporphyrinogenase. *Biochem. J.* **97,** 731–740.

Batlle A. M. del C., Salamanca R. E. de, Chinarro S., Afonso S. G. and Stella A. M. (1986) Photodynamic inactivation of red cell uroporphyrinogen decarboxylase by porphyrins *Int. J. Biochem.* **18,** 143–147.

Blekkenhorst G. H., Eales L. and Pimstone N. R. (1979) Iron and porphyria cutanea tarda: activation of uroporphyrinogen decarboxylase by ferrous iron in porphyria cutanea tarda. *S. Afr. med. J.* **56,** 918–920.

De Verneuil H., Sassa S. and Kappas A. (1983) Purification and properties of uroporphyrinogen decarboxylase from human erythrocytes. A single enzyme catalyzing the four sequential decarboxylations of uroporphyrinogens I and III. *J. biol. Chem.* **258,** 2454–2460.

Elder G. H., Tovey J. A. and Sheppard D. M. (1983) Purification of uroporphyrinogen decarboxylase from human erythrocytes. Inmunochemical evidence for a single protein with decarboxylase activity in human erythrocytes and liver. *Biochem. J.* **215,** 45–55.

Jones M. M. and Vaughn W. K. (1978) HSAB theory and acute metal ion toxicity band detoxification processes. *J. Inorg. Nucl. Chem.* **40,** 2081–2088.

Kappas A., Sassa S. and Anderson K. E. (1983) The porphyrias. In *The Metabolic Basis of Inherited Disease* (Edited by Stanbury J. B., Wyngaarden J. B., Fredrickson D. S., Goldstein J. L. and Brown M. S.), pp. 1301–1384. MacGraw–Hill, New York.

Kardish R. M. and Woods J. S. (1980) Measurement of heme biosynthetic enzymes in mammalian tissues. I. Uroporphyrinogen decarboxylase. *J. appl. Biochem.* **2,** 159–167.

Kawanishi S., Seki Y. and Sano S. (1983) Uroporphyrinogen decarboxylase. Purification, properties and inhibition by polychlorinated biphenyl isomers. *J. biol. Chem.* **258,** 4285–4292.

Klopman J. (1968) Metal hardness classification. *J. Am. Chem. Sci.* **90,** 223–230.

Koopmann G. E., Juknat de Geralnik A. A. and Batlle A. M. del C. (1986) Porphyrin biosynthesis in *Rhodopseudomonas palustris* V: Purification of porphyrinogen

decarboxylase and some unusual properties. *Int. J. Biochem.* **18,** 935–944.

Lehninger A. L. (1975) In *Biochemistry*, 2nd end, 78 pp. Worth, New York.

Mauzerall D. and Granick S. (1958) Porphyrin biosynthesis in erythrocytes III. Uroporphyrinogen and its decarboxylase. *J. biol. Chem.* **232,** 1141–1162.

Mukerji S. K., Pimstone N. R. and Burns M. (1984) Dual mechanism of inhibition of rat liver uroporphyrinogen decarboxylase activity by ferrous iron: Its potential role in the genesis of porphyria cutanea tarda. *Gastroenterology* **87,** 1248–1254.

Mukerji S. K. and Pimstone N. R. (1986) *In vitro* studies of the mechanism of inhibition of rat liver uroporphyrinogen decarboxylase activity by ferrous iron under anaerobic conditions. *Archs Biochem. Biophys.* **244,** 619–629.

Romeo G. and Levin E. Y. (1971) Uroporphyrinogen decarboxylase from mouse spleen. *Biochim. biophys. Acta* **230,** 330–341.

Seehra J. S., Gore M. G., Chaudhry, A. G. and Jordan P. M. (1981) 5-Aminolevulinic acid dehydratase: The role of sulfhydryl groups in 5-aminolevulinic acid dehydratase from bovine liver. *Eur. J. Biochem.* **114,** 263–269.

Setlow B. and Mansour T. E. (1970) Studies on heart phosphofructokinase. Nature of the enzyme desensitized to allosteric control by photo-oxidation and by acilation with ethoxyformic anhydride. *J. biol. Chem.* **245,** 5524–5533.

Smith A. G. and Francis J. E. (1983) Synergism of iron and hexachlorobenzene inhibits hepatic uroporphyrinogen decarboxylase in inbred mice. *Biochem. J.* **214,** 909–913.

Straka J. G. and Kushner J. P. (1983) Purification and characterization of bovine hepatic uroporphyrinogen decarboxylase. *Biochemistry* **22,** 4664–4672.

Tamaoki H., Murase Y., Minato S. and Nakanishi K. (1967) Reversible chemical modification of protein by 2-methoxy-5-nitrotropone. *J. Biochem., Tokyo* **62,** 7–14.

Tomio J. M., García R. C., San Martín de Viale L. C. and Grinstein M. (1970) Porphyrin biosynthesis VII. Porphyrinogen carboxy-lyase from avian erythrocytes. Purification and properties. *Biochim. biophys. Acta* **198,** 353–363.

Woods J. S., Kardish R. M. and Fowler B. A. (1981) Studies on the action of porphyrinogenic trace metals on the activity of hepatic uroporphyrinogen decarboxylase. *Biochem. biophys. Res. Commun.* **103,** 264–271.

Woods J. S., Eaton D. L. and Lukens C. B. (1984) Studies on porphyrin metabolism in the kidney. Effects of trace metals and glutathione on renal uroporphyrinogen decarboxylase. *Molec. Pharmacol.* **26,** 336–341.