THE EFFECTS OF SULFHYDRYL REAGENTS ON THE ACTIVITY OF WHEAT GERM UROPORPHYRINOGEN I SYNTHASE

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1. Introduction

Uroporphyrinogen I (urogen I) synthase [UPGI-S; porphobilinogen ammonia lyase (polymerizating), EC 4.3.1.8] catalyzes the condensation of 4 mol porphobilinogen (PBG). The synthase and uroporphyrinogen III (urogen III) cosynthase convert 4 mol PBG to urogen III, the precursor of the biologically active tetrapyrroles; e.g., hemes, chlorophylls and corrins. The synthase from wheat germ is a single protein with mol. wt ~ 40 000 [1]. It is inhibited by monopyrroles which have at least one free α position, a propionic acid side-chain in a β position and only an aminomethyl or methyl group in the other α position [2,3]. The enzyme is inhibited by ammonium ion and hydroxylamine which presumably dislodge putative precursors of the final product from the active site [4,5]. Studies to identify the amino acid residues at the active site have obtained evidence for one arginine [6] and one lysine residue [7] whose reactions with butanedione and phenylglyoxal and with pyridoxal phosphate, respectively, are inhibited by the substrate, PBG. Inhibition by photooxidation, by *N*-bromosuccinimide and by pyrrolooxygenase suggest that a tryptophan residue(s) is required for activity [8].

Urogen I synthase is a sylfhydryl enzyme. Its activity is inhibited by *N*-ethylmaleimide (NEM), mercuric ion, *p*-chloromercuribenzoate, silver ion [9] and lead [10] but not by iodoacetate [9] or iodoacetamide [8]. Affinity chromatography on mercuri-phenylagarose has been used as a step in the purification of the enzyme [11]. Reaction with mercurials is reversed by thiols such as mercaptoethanol [2], dimercaptopropanol [10], dithioerythritol (DTE) (see below) and cysteine [9].

This paper describes the results of studies to ascertain whether the essential sulfhydryl group(s) resides at the active site. To this end the effects of mercurial reagents and NEM on the enzyme in the presence and absence of PBG or the competitive inhibitor, opsopyrrole dicarboxylic acid (OPD) [12] were observed. Mercury derivatives of PBG (PBG-Hg) and OPD (OPD-Hg) were prepared and their effects on enzyme activity studied.

2. Experimental

PBG was purchased from Porphyrin Products (Logan, UT) and Sigma Chemical Co. (St Louis, MO). *N*-Ethylmaleimide (NEM) was purchased from Aldrich (Milwaukee, WI). *p*-Hydroxymercuribenzoate (PHMB) and wheat germ were obtained from Sigma. All other chemicals were reagent grade or better obtained from standard suppliers.

OPD was prepared by the method in [13] from 2-carboxy-3-(2-carbethoxyethyl-)4-carbethoxymethyl-5-carbethoxypyrrole (CCP) which was generously provided by Dr S. F. MacDonald. CCP (100 mg) was dissolved in 0.6 ml 10% sodium hydroxide in a 20 mm tube. The tube was flushed with argon, frozen in dry ice and sealed under vacuum before heating for 2 h in an oven at 175–178°C. The tube was refrozen and opened and the contents were taken up in 6 ml water, adjusted to pH 3.1 with 2 N HCl and lyophilized. The powder was extracted with ether. The extract was concentrated and the residue was crystallized from hexane to yield 83 mg OPD, m.p. 129–130°C.

Urogen I synthase was prepared from wheat germ according to [9] and used as heat-treated 'fraction B'.

The mercurial derivatives, 'PBG-Hg' and 'OPD-Hg' were obtained by precipitation from reaction of PBG

or OPD with 15% mercuric acetate in 1 M acetate buffer (pH 4.5).

Activities of $HgCl_2$ solutions varied so much that reactions to be compared were run on the same day using dilutions of $HgCl_2$ stock solutions which gave ~80–60% activity remaining without additions.

2.1. *Inhibition studies*

Inhibitor solution (25 μl) in 0.075 M Tris buffer (pH 8.1) was mixed with 25 μl buffer and 75 μl or 100 μl enzyme in a 400 μl tube and incubated at 37°C for 5 min when mercurials were used, up to 1 h when NEM was used and various intervals shown in the figures for time studies. For protection studies with PBG or OPD, 25 μl PBG (2 mg/10 ml buffer) or OPD 1 mg/10 ml buffer) were added instead of buffer alone and preincubated with the enzyme for 5 min before addition of inhibitor. When reversal of inhibition by mercurials was studied, the reaction mixture was made 1 mM in DTE and incubated for an additional 30 min. When reversal of inhibition by NEM was studied, the mixture was dialyzed overnight against buffer before adding DTE.

For high concentrations of inhibitors or PBG or OPD, enzymic mixtures were dialyzed against Tris buffer overnight before assay. All other incubation mixtures were applied to small columns of Sephadex G-25 (Pharmacia, Piscataway, NJ) and centrifuged immediately at half-speed in an International table-top clinical centrifuge for 2 min. The columns had been prepared by adding Sephadex G-25 (1 ml gel by gravity-packing) to 1 ml plastic syringes (Pharmaseal) equipped with polyethylene porous disks and centrifuging for 2 min in the centrifuge at half-speed as above.

2.2. *Enzyme assays*

Eluate or dialyzate (50 μl or 75 μl) was mixed with 25 μl 0.18 mM PBG in a 1.5 ml tube and incubated at 37°C until porphyrin fluorescence was observed under ultraviolet light (1–2 h). Uroporphyrinogen was oxidized completely by adding 0.5 ml iodine reagent (0.01% in 1 M HCl) [14]. After 10 min 0.5 ml water was added to each tube and just before reading a small crystal of sodium thiosulfate was added to discharge the iodine color. Uroporphyrin was measured as A_{405}.

3. Results

Table 1 shows the results of treating the synthase with NEM and with mercurial compounds. PBG at several concentrations (0.14–10 mM) had little or no effect on inhibition by NEM. OPD, on the other hand afforded substantial protection from inactivation by NEM.

Inhibition by mercuric ion, PHMB, PBG-Hg and OPD-Hg was enhanced by PBG. At longer incubation time (30 min) PBG-Hg inhibition was not enhanced by PBG.

OPD enhanced the inhibition by $HgCl_2$. OPD provided the enzyme with some protection from inhibition by PBG-Hg. It had little or no effect on inhibition by OPD-Hg. At similar concentrations the latter two inhibitors were far more effective than mercuric ion or PHMB.

Table 2 shows the results of repeating these experiments with DTE. Inhibition was lifted in most cases in which mercurials were used. PBG retarded reversal of PHMB inhibition. Reversal was partial for PBG-Hg and for PBG-Hg with PBG.

Table 1
Effects of sulfhydryl reagents on urogen I synthase

Inhibitor	Conc. (mM)	Incubation time (min)	% Activity remaining: pretreatment (5 min)		
			None	PBG (mM)	OPD (mM)
NEM	40	60	50	48 (0.18)	78 (0.14)
	50	60	28	33 (10)	62 (10)
$HgCl_2$	0.040	5	60	7 (0.18)	27 (0.14)
	0.066	5	0	0 (0.18)	0 (0.14)
PHMB	0.10	5	70	9 (0.18)	64 (0.14)
	0.20	5	28	0 (0.18)	17 (0.14)
PBG-Hg	0.018	5	62	39 (0.18)	83 (0.14)
	0.030	5	16	8 (0.18)	48 (0.14)
OPD-Hg	0.022	5	95	54 (0.18)	90 (0.14)
	0.043	5	35	0 (0.18)	35 (0.14)

Table 2
Effect of dithioerythritol (DTE) on the inhibition of urogen I synthase by sulfhydryl reagents

Inhibitor	Conc. (mM)	DTE[a] (1 mM)	% Activity remaining[b]: pretreatment (5 min)		
			None	PBG (0.14 mM)	OPD (0.20 mM)
NEM	32	–	57		
		+	50		
$HgCl_2$	0.053	–	91	65	
		+	104	90	
	0.071	–	88		88
		+	94		104
PHMB	0.081	–	50	0	
		+	102	15	
	0.071	–	55		48
		+	105		102
PBG-Hg	0.024	–	14	13	
		+	60	64	
	0.015	–	16		75 (0.18 mM)
		+	54		75
OPD-Hg	0.018	–	91	47	
		+	100	89	
	0.016	–	55		68
		+	94		98

[a] NEM inhibitions were for 1 h at 37°C. Inhibitions by mercurials were for 5 min at 37°C
[b] For NEM inhibition, reaction mixtures were dialyzed before DTE was added. After 30 min at 37°C, the mixtures were put through Sephadex G-25 before assay. For inhibition by mercurials, DTE was added to the reaction mixture, incubated at 37°C for 30 min, and put through Sephadex G-25 before assay

4. Discussion

The protection of urogen I synthase activity by OPD, a competitive inhibitor, from inhibition by NEM and PBG-Hg suggests that one or more essential sulfhydryl groups may reside at the active site of the enzyme. The active site must accommodate two pyrrole units for condensation to take place and it is possible that OPD binds to only one of these sites.

The enhancement effect of PBG on inhibition of the synthase by mercurial compounds may be the result of one or more of the following:

(i) PBG causes conformational changes which expose thiol groups. PBG would be effective because it can bind to both sites postulated above. OPD may not be able to bind exactly as PBG does and thus exhibits a different response.

Other investigators have observed that substrate and competitive inhibitors can sometimes stimulate inhibition of enzymes by thiol reagents. Levulinic acid, a competitive inhibitor of δ-aminolevulinic acid dehydratase potentiated thiol-inactivation by iodoacetamide and protected from inactivation by iodoacetate [15]. Competitive inhibitors of histidine decarboxylase, histamine and imidazole stimulated reaction of essential thiol groups [16]. That substrates induce conformational changes causing enhanced reactivity of thiol groups has been shown with several enzymes [17–21].

(ii) PBG may be activating the mercurial reagent by forming a more reactive reagent. This possibility is being studied more thoroughly.

These results suggest that there is a thiol group at the active site of urogen I synthase. The potentiating effects of PBG on inhibition of synthase by mercurials may be due to some combination of conformational changes, activation of the mercurial, and conversion of the thiol group to its anion.

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References

[1] Higuchi, M. and Bogorad, L. (1975) Ann. NY Acad. Sci. 244, 401–418.
[2] Frydman, R. B. and Frydman, B. (1970) Arch. Biochem. Biophys. 136, 193–202.
[3] Frydman, R. B. and Feinstein, G. (1974) Biochim. Biophys. Acta (1974) 350, 358–373.
[4] Pluscec, J. and Bogorad, L. (1970) Biochemistry 9, 4736–4743.
[5] Radner, R. and Bogorad, L. (1972) Biochemistry 11, 904–910.
[6] Pollack, S. E. and Russell, C. S. (1978) FEBS Lett. 90, 47–50.
[7] Pollack, S. E. and Russell, C. S. (1978) Fed. Proc. FASEB 37, 1339.
[8] Frydman, R. B. and Frydman, B. (1973) Biochim. Biophys. Acta 293, 506–513.
[9] Bogorad, L. (1958) J. Biol. Chem. 233, 501–509.
[10] Lockwood, W. H. and Benson, A. (1960) Biochem. J. 75, 372–381.
[11] Russell, C. S. and Pollack, S. E. (1978) J. Chrom. 166, 632–636.
[12] Carpenter, A. T. and Scott, J. J. (1961) Biochim. Biophys. Acta 52, 195–198.
[13] MacDonald, D. M. and MacDonald, S. F. (1955) Can. J. Chem. 33, 573–579.
[14] Jordan, P. M. and Shemin, D. (1973) J. Biol. Chem. 248, 1019–1024.
[15] Barnard, G. F., Itoh, R., Hohberger, L. H. and Shemin, D. (1977) J. Biol. Chem. 252, 8965–8974.
[16] Lane, R. S. and Small, E. E. (1976) Biochemistry 15, 4175–4179.
[17] Yankeelov, J. A. jr and Koshland, D. E. jr (1965) J. Biol. Chem. 240, 1593–1602.
[18] Nowak, T. and Himes, R. H. (1971) J. Biol. Chem. 245, 1285–1293.
[19] Wolf, D. H. and Ebner, E. (1972) J. Biol. Chem. 247, 4208–4212.
[20] Rippa, M., Grazi, E. and Pontremoli, S. (1966) J. Biol. Chem. 241, 1632–1635.
[21] Karni-Katsadimas, I., Dimitropoulos, C. and Evangelopoulos, A. E. (1969) Eur. J. Biochem. 8, 50–54.