SULPHYDRYL GROUPS AND THE *IN VITRO* ENZYMATIC SYNTHESIS OF 5-AMINO-LAEVULINIC ACID AND PORPHOBILINOGEN IN *RHODOPSEUDOMONAS SPHEROIDES**

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1. Introduction

Reactions of -SH groups have been implicated in control mechanisms of the tetrapyrrol pathway [1, 2]. In previous papers the author has studied the effect of Cy on δALA dehydratase in liver and yeast [3, 4]. There are several reports concerning the properties of these enzymes in microorganisms grown under different oxidation-reduction conditions [5–7].

Lascelles [5] reported that "the addition of glutathione improved the synthesis of δALA provided that the final concentration did not exceed 1 mM". Others [7, 8] found that neither glutathione nor Cy was necessary in the enzymatic assay for δALA synthetase. This paper reports amperometric and colorimetric SH determinations performed in *Rh. spheroides* extracts. The effect of Py P and Cy in the *in vitro* assay of δALA synthetase was studied using pigmented cells subjected to different treatments. δALA dehydratase stimulation by addition of Cy was also investigated.

Abbreviations:

δALA : 5-amino-laevulinic acid
PBG : porphobilinogen
Cy : cysteine
Py P : pyridoxal phosphate
DTNB : 5-5'-dithio-bis(2NO_2 benzoic acid)
CoASH: coenzyme A
TCA : trichloroacetic acid
NADH : nicotinamide-adenine dinucleotide

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2. Experimental and results

Rh. spheroides ATH 2-4-1 was grown anaerobically in the light according to Lascelles [9] and harvested in the late exponential phase (after 76 hr of incubation). The pigmented cell suspensions were subjected to one of three different treatments: (a) Centrifuged and washed 3 times with 0.9% saline. (b) The cell suspension (in medium "S" described by Lascelles [9] was aereated in the dark for 4 hr at 37° while filtered humid air was bubbled into the cell suspension. Cells were then treated as under (a). (c) The pigmented pellet obtained in (a) was resuspended in a basal incubation medium (2 mg dry weight of cells/ml) containing glycine and fumarate as major components and treated according to Goto et al. [10] to establish the effect of modifying aerobic conditions.

Pellets of packed cells were resuspended in 0.05 M Tris-HCl buffer (pH 7.4) for amperometric titrations of -SH groups and enzymatic assays both for δALA and PBG. Sonication was carried out at 0° under N_2 for 3 min using a Sonifier Cell Disruptor (Heat System Co., Melville, Long Island, N.Y.). A clear supernatant fraction obtained from the extract by centrifuging for 2 hr at 105,000 *g* (0°–4°) was used for colorimetric measurements of -SH groups and for enzymatic assays. The whole extract was used for amperometric titrations of -SH groups.

2.1. *-SH content in extracts of Rh. spheroides*

Amperometric titrations were performed by the method of Kolthoff and Harry [11]. Bacterial extracts obtained after treatment (a), (b) or (c) above were

Table 1
-SH group contents in extracts of *Rh. spheroides*. Results in μmoles of Cy. Experimental conditions are described in the text.

Amperometric titrations		Colorimetric measurements	
Sample	Total -SH groups ($\frac{\mu moles}{dry\ wt.}$)	Total -SH groups (μmoles/g of protein)	Non-protein -SH groups (μmoles/g dry wt.)
(a)	15	35	3
(b)	55	77	7.5
(c)	23	29	–

Note the higher values for sample (b) with respect to those of sample (a) with both methods.

used. Mixtures contained 14 mM Tris buffer pH 7.4, 10 mM KCl, 8 M urea and extracts representing 20 to 30 mg dry weight of bacteria. Preincubation was carried out anaerobically for 1 hr at 0° in Thunberg tubes before titration.

Standards run with Cy under the same conditions included gelatin (10 mg%) as indicated by Benesh et al. [12]. The dry weight of bacterial extracts was measured colorimetrically at 680 nm (table 1).

Colorimetric measurements: Determination of total -SH (including those of proteins and metabolites) were made by Ellman's method [13]. Supernatant fractions in 8 M urea obtained by ultracentrifugation were treated with DTNB; after 60 min, readings were made at 412 nm using a control sample with the same components but without DTNB. Blanks were run for DTNB at the same pH. Results expressed as μM of -SH groups/g of protein are presented in table 1; protein contents were determined according to Lowry et al. [14]. Values for non protein -SH groups performed by Ellman's method in whole extracts followed the same pattern observed for total -SH groups determined by amperometric titration.

2.2. *Enzymatic formation for δALA*

Interactions between aminothiols and Py P [15] may account for the low values of enzymatic synthesis reported by other authors [10] using vigorously aereated cell preparation. Assay of Py P concentrations showed that 4 mM of this cofactor promotes increased enzymatic activity in (b) with respect to (a) extracts

Table 2
Enzymatic formation of δALA. Experimental details are given in the text. Activity is expressed in nmoles of δALA/mg protein/hr.

Effect of Py P			Effect of Cy preincubation (Py P 6 mM)		
Sample	0.1 mM	4 mM	with no Cy	with Cy 0.5 mM	% activation
(a)	152	168	180	193	7
(b)	35	193	201	222	11

The assays did not include more than 0.6 mg of protein for sample (b).

(table 2). In other assays not included in this paper, a higher content of Py P was necessary.

Enzymatic formation of δALA was measured as described by Kikuchi et al. [8] except that Py P concentrations were 0.1 mM, 4 mM or 6 mM when Cy was included. The action of Cy was assessed by preincubating the whole mixture (excluding ATP, Py P and CoASH) for 15 min at 34°. After adding the excluded components tubes were incubated for 1 hr. Half saturated $CuSO_4$ solution (5λ) was added and after 5 min, 30% TCA was used to precipitate proteins. δALA was estimated colorimetrically with acetylacetone by the method of Mauzerall and Granick [16] with some modifications and together with δALA standards.

Table 2 shows the effect of Py P concentration and the effect of Cy on the δALA formation. Cy (1 mM) gave lower values than those obtained for the unactivated mixture although 6 mM Py P was used. The use of mercaptoethanol [17] which probably pro-

Table 3
Effect of Cy on δALA dehydratase. The experimental conditions are described in the text. Activities are expressed as nmoles of PBG/mg/hr.

Sample	Addition	Activity
(a)	None	7.3
	Cy 1 mM	47.3
	Cy 2 mM	59.7
(b)	None	< 1
	Cy 1 mM	1.6
	Cy 2 mM	4.3

duces a dissociable thiohemiketal with Py P does not seem to interfere with δ ALA synthetase assay.

The small effect of Cy on δ ALA formation shown in table 2 does not suggest a sulphydryl activation of the enzymes involved.

2.3. *δALA dehydratase activity*

The conditions used in this assay were similar to those of Gibson [18] with slight modification. Activation with 1 mM and 2 mM of Cy were performed during 15 min. Incubations lasted 1 hr at 37°. Treatment with 5λ of half saturated $CuSO_4$ solution was followed by deproteinisation using TCA. Estimation of PBG was made by the method of Urata and Granick [19]. Results (table 3) show the striking effect of Cy preincubation on δ ALA dehydratase and the remarkably different activities of anaerobic and aereated cells.

3. Discussion

Heyl et al. [20] showed that LCy and L penicillamine (which contains a free -SH group) reacted with Py P to give 4 thiazolidine carboxylic acid derivatives. Buell and Hansen [15] demonstrated that Py P reacted with aminothiols to give stable complexes the necessary condition being proximity between $-NH_2$ and -SH groups:

L penicillamine [21] caused a reduced activity of alanine-glutamic and aspartic-glutamic transaminase. Inhibition was also acomplished by the simultaneous administration of piridoxine or by addition in vitro of Py P. L penicillamine and L Cy are thought to inhibit δ ALA synthetase by forming thiazolidine rings with the Py P [1]. The increased δ ALA formation observed when using relatively high Py P concentrations resemble results quoted by Kuchinskas et al. [21]. The *in vivo* interaction between aminothiols and Py P in *Rh. spheroides* is still to be investigated. Results obtained in the present work show that the relative amount of Py P and -SH influence δ ALA formation. The values for -SH groups or δ ALA formation varied with the conditions of treatments of bacterial cells. For instance vigorous aereation of (b) or (c) suspensions resulted in increased values of -SH. It would be interesting to investigate if the activation of δ ALA synthetase from semianaerobic cells reported by Marriot et al. [22] in the presence of oxygen is related to the level of -SH groups. According to these authors activation of δ ALA synthetase does not begin until all the added NADH has being oxidised. This phenomenon could be dependent, for instance, upon enzymatic systems such as NAD (PH_2) glutathione reductase which would oxidise -SH groups. If this were so, the *in vitro* assay of δ ALA synthetase would work even at low Py P concentrations. No explanation can be offered for the observation that δ ALA dehydratase activity exhibits low values if Cy is not added even though the aereated extract contains a high -SH level.

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