BBA 63378

Effect of sulfhydryl groups on 5-aminolaevulinate dehydratase activity

In a previous paper[1] a substrate inhibition of the 5-aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase (adding 5-aminolaevulinate and cyclizing), EC 4.2.1.24) activity, caused by the reaction of –SH groups with the ketonic group of 5-aminolaevulinate, was assumed. This enzyme would represent an interesting case in which a simple metabolite (cysteine, GSH or another with free –SH groups) could act both by activating an enzyme, and at the same time, by inhibiting its activity through combination with the substrate. A similar phenomenon was reported in the case of betaine aldehyde dehydrogenase[2] as well as in the competitive action of –SH groups with α-glycerophosphate and lactate dehydrogenase by their substrates[3].

Sulfhydryl groups easily add to carbonyls giving thiohemiketals. The chemical interaction between 5-aminolaevulinate and cysteine has been measured as described previously[1]. Spectrophotometric measurements (Fig. 1) also suggest a combination between those metabolites. The absorbances of cysteine (Curve A) and 5-amino-

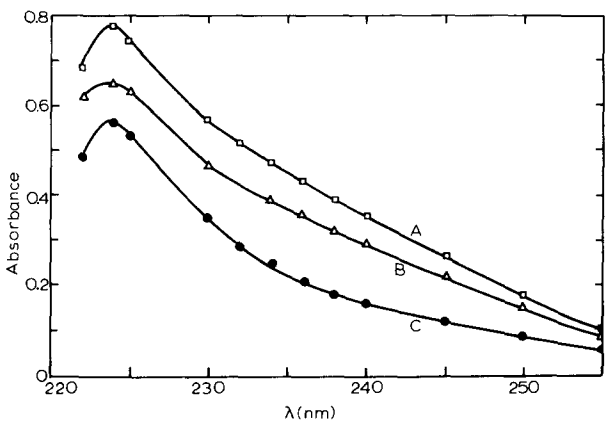

Fig. 1. Combination of 5-aminolaevulinate with cysteine. Curve A, spectra of a 2.5 mM cysteine–HCl solution (glycine buffer, pH 9.7); Curve B, spectra for a 15 mM 5-aminolaevulinate solution (in the same buffer); Curve C, spectra for a solution of 2.5 mM of cysteine and 15 mM of 5-aminolaevulinate. Similar assays were performed with the same concentration of cysteine and 5-aminolaevulinate in 0.1 M HCl and 0.1 M acetate buffer (pH 4.7). In each case, readings were made against the solvent used in preparing the solutions.

laevulinate solutions (Curve B) between 220 and 255 mμ diminished when these solutions were mixed (Curve C). The same occurred at pH 7.4, while at pH 1 the mixture of both solutions yielded the sum of the individual absorbances, thus suggesting that –SH groups did not combine with ketonic groups at that pH.

A technique similar to that given by GRANICK AND MAUZERALL[4] for eliminating the interference of –SH groups with the Ehrlich's reaction[5], was found to be unsatisfactory under the conditions in which the experiments described here were performed. A careful quantitative study with Cu^{2+} (used for the same purpose) at nonoptimal concentrations, showed the interference of this ion in the reaction,

especially when working with enzymic solutions of low protein content; the quantity of Cu^{2+} to be used depended fundamentally on two variables: –SH molarity and protein present in the assay.

In this paper *N*-ethylmaleimide is proposed to eliminate the excess of cysteine added to the incubation systems. Deproteinized porphobilinogen solutions, enzymatically obtained as described below (b), with and without addition of cysteine, were used to assay the effect of *N*-ethylmaleimide on free –SH groups. Appropriate fractions were prepared by heating them 10 min at 55° with *N*-ethylmaleimide solution. After cooling, Ehrlich's reaction was performed[5]. From the values obtained (Table I) it is possible to conclude that the molarity of *N*-ethylmaleimide to be used only depended on the molarity of –SH groups present in the assay. Comparison between values corresponding to Sample 1 (with no *N*-ethylmaleimide treatment) and to Samples 7, 8, 11 and 12 allowed us to assume that heating at 55° in acid medium does not affect the porphobilinogen content.

The effect of –SH groups on 5-aminolaevulinate dehydratase activity was studied both on yeast and rabbit liver. In the second case, the animals were killed

TABLE I

OVERCOMING THE CYSTEINE EFFECT ON EHRLICH'S REACTION BY *N*-ETHYLMALEIMIDE

Each tube contained: 0.1 ml deproteinized solution (b), 100 µl 30% trichloroacetic acid, cysteine and *N*-ethylmaleimide as indicated in the table, and water to 0.3 ml.

Sample	*Cysteine (µmoles)*	*N-Ethyl-maleimide (µmoles)*	$A_{555\ nm}$
1	—	—	0.25
2	—	25	0.26
3	—	50	0.28
4	—	100	0.28
5	5	—	0.05
6	5	25	0.15
7	5	50	0.27
8	5	95	0.28
9	10	—	0.06
10	10	50	0.16
11	10	100	0.25
12	10	190	0.26

and their livers immediately frozen in liquid air to avoid oxidation of the free –SH groups containing metabolites.

(a) Yeast enzymic solutions were prepared by extracting 10 g of a ketonic powder[1] with 50 ml of 20 mM glycine buffer (pH 9.7), 20 mM β-mercaptoethanol, and centrifuged at 16 500 rev./min (18 000 × *g*) for 10 min. The supernatant fraction was centrifuged at 30 000 rev./min (78 480 × *g*) for 1 h in a Spinco model L centrifuge. The solution obtained was dialyzed for 1 h against the same mixture in which the extraction was carried out, and used immediately.

(b) In the case of liver, 1 g of tissue was homogenized in a Potter–Elvehjem homogenizer in 3 ml of 0.067 M phosphate buffer (pH 6.9) according to Gibson *et al.*[6]. After centrifugation at 16 500 rev./min for 10 min, the supernatant fraction was used as the enzymic solution.

For estimating 5-aminolaevulinate dehydratase activity in (a) and (b), anaerobic incubations were performed using Thunberg tubes. Substrate curves obtained previously allowed the choice of the nonsaturating substrate concentrations where the action of an excess of –SH groups could be observed.

For the yeast enzyme assay, 0.2 ml of enzyme solution, 20 μmoles of glycine buffer (pH 8.8), 5-aminolaevulinate and cysteine as indicated in Tables I and II (final vol., 0.5 ml) were incubated at 55° for 1 h. Since the substrate is pyrrolyzed by heating at 55° and at that pH, a blank was run for each enzyme activity measurement. Cysteine was dissolved in satd. Na_2SO_4 solution and its pH adjusted to 8.8 with 5 M KOH.

The enzymic liver system consisted of 0.3 ml of extract representing 100 mg of tissue, 0.5 ml of buffer (34 μmoles), 5-aminolaevulinate solution at pH 7 and cysteine at the same pH (in the molarities indicated in Tables I and II) and distilled water up to 1 ml. The substrate was in the lateral arm during preincubations with cysteine (15 min). Incubation lasted 1 h at 37.5°.

Incubation systems, both from yeast and from liver, were deproteinized with 30% trichloroacetic acid. Aliquots of the filtrates were added with warm *N*-ethylmaleimide solution (50 or 100 μmoles for yeast or liver enzyme, respectively) and heated in a water bath at 55–60° (15 min). After cooling, Ehrlich's reaction[5] was performed. A very stable color was obtained and read at 555 nm.

The effect of incubation with several cysteine concentrations on 5-aminolaevulinate dehydratase activity both from yeast and rabbit liver can be seen in Tables IIa and IIb, respectively.

Table IIa shows the values corresponding to experiments performed with two different concentrations of cysteine. Thus it is possible to observe a decrease in the rate of porphobilinogen formation when the cysteine molarities are increased; moreover, higher amounts of porphobilinogen are produced for the same cysteine concentration when the substrate molarity is doubled. For these experiments the free –SH groups would be from cysteine (tabulated) and from the β-mercaptoethanol used in the enzyme preparation.

In the case of the liver enzyme (Table IIb), activities obtained by incubation with 15 mM cysteine (which is approximately the molarity used to measure 5-amino-

TABLE II

EFFECT OF CYSTEINE ON 5-AMINOLAEVULINATE DEHYDRATASE FROM YEAST AND RABBIT LIVER
Details are given in the text.

5-Aminolaevulinate (mM)	*Cysteine (mM)*	*Activity (%)*	*5-Aminolaevulinate (mM)*	*Cysteine (mM)*	*Activity (%)*
(a) Yeast			*(b) Rabbit liver*		
2.9	0	100	0.075	15	100
2.9	3.9	41	0.075	100	61
2.9	9.8	0	0.075	150	53
5.8	0	100	1.25	15	100
5.8	3.9	71	1.25	100	90
5.8	9.8	0	1.25	150	59

laevulinate dehydratase from mammal tissues) are taken as 100% of activity. Tables IIa and IIb summarize the percentages of activity obtained with higher cysteine concentrations for the two molarities of the substrate.

From this study it is possible to consider that 5-aminolaevulinate may react with free –SH groups, especially when added in relatively high concentrations, and thus influence the rate of porphobilinogen formation. Although cysteine molarities needed to reduce enzymic porphobilinogen production seem too high with respect to physiological levels of tissues, the molarity of 5-aminolaevulinate needed to measure the activity *in vitro* must also be high. 5-Aminolaevulinate and free –SH groups (expressed as cysteine) were measured in yeast and liver according to the methods of URATA AND GRANICK[5] and GRUNERT AND PHILLIPS[7], respectively. Values of 90 nmoles of 5-aminolaevulinate and 12 μmoles of cysteine per g of fresh yeast were obtained, while fresh rabbit liver gave contants of 19 nmoles and 2 μmoles per g, respectively; the ratio of the values obtained for cysteine and 5-aminolaevulinate was roughly higher than 100 for both biological sources, so that a chemical interaction would be possible at physiological pH's. Also it would be necessary to consider such interactions in kinetic studies, especially to ascertain the real 5-aminolaevulinate concentration in the incubation mixtures.

The author wishes to express her gratitude to Dr. Luis F. Leloir and Dr. Carlos E. Cardini for allowing her to use the laboratories of the Instituto de Investigaciones Bioquímicas for the performance of this work. She is also indebted to Dr. Israel D. Algranati for helpful advice and to the other members of the Institute for their kind collaboration.

Instituto de Investigaciones Bioquímicas "Fundación Campomar", Obligado 2490, Buenos Aires (28) (R. Argentina)

OFELIA L. CLARA DE BARREIRO

1 O. L. C. DE BARREIRO, *Biochim. Biophys. Acta*, 139 (1967) 479.
2 H. A. ROTSCHILD AND E. S. GUZMÁN BARRON, *J. Biol. Chem.*, 209 (1954) 511.
3 A. DUGAICZIK, M. MALECKI AND J. J. EILER, *J. Biol. Chem.*, 243 (1968) 2236.
4 S. GRANICK AND D. MAUZERALL, *J. Biol. Chem.*, 232 (1958) 1119.
5 G. URATA AND S. GRANICK, *J. Biol. Chem.*, 238 (1963) 811.
6 K. D. GIBSON, A. NEUBERGER AND J. J. SCOTT, *Biochem. J.*, 61 (1955) 618.
7 R. GRUNERT AND P. H. PHILLIPS, *Arch. Biochem.*, 30 (1951) 217.

Received December 12th, 1968

BBA 63376

UDP-D-glucuronate 4-epimerase in blue-green algae

Pectin, a polymer of D-galacturonic acid is a major fraction of higher plant polysaccharides. In algae, D-galacturonic acid-containing polysaccharides are quite rare and appear to be restricted to Cyanophyceae[1] and Rhodophyceae[2,3]. Even in