5-AMINOLAEVULINATE HYDRO-LYASE FROM YEAST
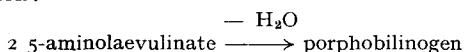## ISOLATION AND PURIFICATION

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SUMMARY

An enzyme, aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase (adding 5-aminolaevulinate and cyclizing), EC 4.2.1.24), which catalyzes the following reaction:

$$2 \text{ 5-aminolaevulinate} \xrightarrow{- H_2O} \text{porphobilinogen}$$

has been isolated from yeast. Its purification and some of its properties have been studied. 5-Aminolaevulinate synthetase, another enzyme involved in porphyrin biosynthesis, has been detected in the mitochondrial particles.

INTRODUCTION

The biosynthesis of porphyrins in respiration-deficient mutants of *Saccharomyces cerevisiae*, grown under special conditions, was described by SUGIMURA et al.[1]. This fact and the presence of cytochrome in normal yeast would indicate that all the enzymes related to porphyrin biosynthesis should be present. Nevertheless, GIBSON, NEUBERGER AND SCOTT[2] could not find activity of aminolaevulinate dehydratase (5-aminolaevulinate hydro-lyase (adding 5-aminolaevulinate and cyclizing), EC 4.2.1.24) in this microorganism. This paper describes the isolation, purification and a kinetic study of this enzyme in yeast.

A method for the disruption of the yeast cell membrane, which allowed the detection of aminolaevulinate dehydratase in the supernatant fraction and of aminolaevulinate synthetase in the mitochondria, is also reported.

MATERIALS AND METHODS

1. The study was made on *S. cerevisiae* in steady state. Several methods for disrupting the membrane were tried: manual treatment of frozen cells in a mortar and a French pressure cell. The use of a Raytheon 10K sonic oscillator for 15 min with glass beads was less effective but allowed the assay of aminolaevulinate synthetase

in the mitochondria. The most convenient and practical method was lyophilization of a thick suspension of cells in water, followed by grinding of the dry powder, suspended in acetone, with a Virtis homogenizer, at 40 000 rev./min for 20 min. For this operation the glass container was placed in a solid CO_2–alcohol bath. After filtration and washing with cold acetone and ether, a very stable powder was obtained which maintained its activity for several months.

2. 5-Aminolaevulinate was purchased from Sigma Chemical Company (St. Louis, Mo.).

3. $Ca_3(PO_4)_2$-gel was prepared according to COLOWICK[3].

4. DEAE-cellulose from Serva (0.84 mg/g) was used as described by PETERSON AND SOBER[4].

5. Estimation of 5-aminolaevulinate and porphobilinogen was made by te methods of SHUSTER[5] and URATA AND GRANICK[6], respectively. Porphobilinogen was prepared in our laboratory according to the method of COOKSON AND RIMINGTON[7].

6. Protein estimations were made (mg of protein per ml of enzyme solution) using the method of LOWRY et al.[8].

7. The estimation of aminolaevulinate dehydratase activity was made in a similar way to Gibson's experiments[2]. Thunberg tubes were used to measure the enzymic activity. In general, the system contained the enzyme fraction (dissolved in 20 mM glycine buffer at the desired pH), a buffer whose molarity ranged from 60 mM to 100 mM, and the substrate dissolved in the same buffer or adjusted to the same pH. The final volume was 1 ml.

Deproteinization was effected using 0.4 ml of 0.3 M trichloroacetic acid.

The adsorption of porphobilinogen to the protein fraction used, ranged between 12 and 20%, according to the porphobilinogen concentration. In the presence of 9.5 mg of protein per ml, recoveries of 80 and 88% were obtained for concentrations of porphobilinogen of 1 μM and 10 μM, respectively. The same concentrations were used for the K_m determinations; the corresponding corrections for each point of $1/v$ were made.

Since the activities of the enzyme preparations were low, deproteinization with colloidal Fe_2O_3 and $CuSO_4$ did not give satisfactory results.

8. The extraction and estimation of aminolaevulinate synthetase was effected as follows: 15 g of yeast was suspended in a mixture of 0.25 M sucrose, 20 mM Tris buffer (pH 7.4) and 5 mM EDTA, and then submitted to sonication. Mitochondria were obtained by differential centrifugation at 70 000 $\times$ g, following a preliminary separation of "débris cellulaires" at 7000 $\times$ g. The pellet was treated with 20 mM thioglycollate, 0.25 M sucrose, 10 mM Tris buffer (pH 7.4) and 5 mM EDTA for 1 h. The aerobic system of incubation consisted of 50 mM [2-^{14}C]glycine (2.7 μC), 0.5 mM ATP, 0.25 mM succinyl-CoA (prepared according to SIMON AND SHEMIN[9]), 30 mM KCl, 6 mM $MgCl_2$, 0.2 ml of mitochondria suspension, representing 5 g of fresh yeast, 50 mM Tris buffer, pH 7.4, and 125 mM pyridoxal phosphate, in a final vol. of 2.0 ml. After incubation at 37.2° for 1 h, the system was deproteinized with 0.2 ml of 0.3 M trichloroacetic acid. The pyrrols formed according to MAUZERALL AND GRANICK[10], were extracted with ether at pH 1.0 and submitted to chromatography on Whatman No. 1 paper. The chromatograms were scanned in a Packard radiochromatogram scanner, Model 7200.

RESULTS

Purification of aminolaevulinate dehydratase

A summary of the data on purification is presented in Table I. All operations were carried out between 0–4°.

TABLE I

PURIFICATION OF AMINOLAEVULINATE DEHYDRATASE
Experimental details are given in the text.

Fraction	Protein (mg/ml)	Specific activity (mμmoles porphobilinogen per mg protein per h)
Step 1: supernatant fraction obtained from centrifuging at 78 000 × g	37.0	5.1
Step 2: redissolved precipitate obtained from treatment with $(NH_4)_2SO_4$ (30–50% satn.)	28.5	22.7
Step 3: supernatant fraction after treatment with $Ca_3(PO_4)_2$ gel	7.1	49
Step 4: eluted from DEAE-cellulose column	0.02	4700

Extraction of the enzyme. The dry acetone powder was treated with 20 mM buffer (20 g/100 ml of buffer) using a magnetic stirrer, in a two-step operation. The buffer was selected according to the study to be made with the solubilized proteins contain-

Fig. 1. Stages of purification of aminolaevulinate dehydratase as monitored by polyacrylamide-gel electrophoresis using barbital buffer (pH 8.6; $I = 0.1$).
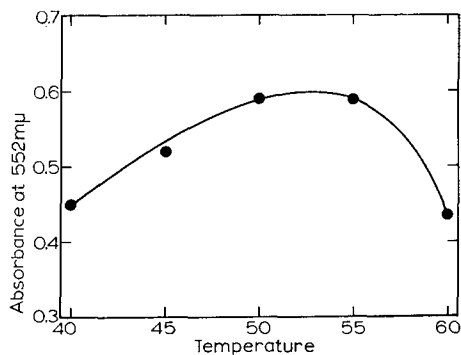
Fig. 2. Optimal temperature. The incubation mixture contained 0.1 M glycine–NaOH buffer, 5 mM 5-aminolaevulinate and 0.2 ml of the enzyme fraction from Step 2. The final volume was 1 ml. Incubations were performed for 30 min.

ing the enzyme. The buffered solution extracted from yeast was spun at 18 000 × g for 15 min and then at 78 000 × g for 1 h (Step 1). A non-viscous solution was thus obtained with which reproducible fractionation steps using $(NH_4)_2SO_4$ may be carried out. After testing different salt concentrations, most of the enzyme activity was found in the fraction obtained at 30–50% satn. (Step 2). This fraction was dialyzed against 20 mM phosphate buffer (pH 7.6) to allow further purification. For inhibition assays, 20 mM sodium phosphate buffer (pH 6.8) was used, but the enzyme flocculated within a few hours in this buffer and became inactive.

Purification on $Ca_3(PO_4)_2$ *gel.* The dialyzed fraction obtained from Step 2 was treated with 4 portions of 1 ml of $Ca_3(PO_4)_2$ gel (32 mg/ml). The latter was eluted with 0.1 M glycine buffer (pH 9.6) containing 5% $(NH_4)_2SO_4$. Most of the enzyme remained in the supernatant fraction (Step 3).

Purification on DEAE-cellulose. The supernatant fraction obtained from Step 3 was dialyzed against 10 mM glycine buffer (pH 7.7). It was then passed through a DEAE-cellulose column, equilibrated with 10 mM glycine buffer (pH 7.7). Protein was eluted from the column by increasing the molarity of the glycine buffer from 10 to 200 mM in a stepwise fashion (Step 4). Four peaks were obtained of increasing enzymic activity at glycine molarities of approx. 20, 50, 100 and 200 mM, the most active being that eluted with the greatest ionic strength buffer. The results obtained and the increases in specific activity at each step are shown in Table I.

Purification of the enzyme was followed by electrophoresis on polyacrylamide, using the technique of RAYMOND AND WEINTRAUB[11] (Fig. 1).

Optimal Temperature

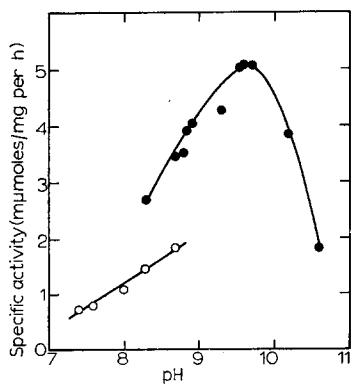As shown in Fig. 2, the optimal temperature range was 50–55°.

Fig. 3. Optimal pH. The system contained: 5 mM 5-aminolaevulinate, 100 mM Tris buffer or 70 mM glycine buffer and 0.3 ml or 0.2 ml of the enzyme fraction from Step 2 for each buffer, respectively. Incubations were performed at 38° for 60 min when Tris buffer (○) was used and at 55° during 30 min when glycine buffer (●) was present.
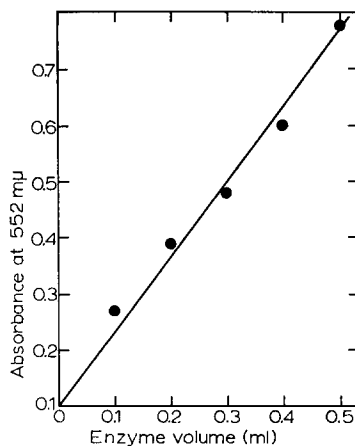
Fig. 4. Relation between activity and enzyme concentration. The system contained: 60 mM glycine–NaOH buffer (pH 9.6), 5 mM 5-aminolaevulinate, and the supernatant fraction treated with 30% $(NH_4)_2SO_4$ and containing 25 mg protein per ml. The final volume was 1 ml and the temperature of incubation 55°.

Optimal pH

Buffer systems ranging from pH 5.95 to 10.6 were assayed. In each case the 30–50% fraction from Step 2 was dissolved in the appropriate buffer and dialyzed against it. The pH of the incubation mixture was measured before and after incubation. Determinations made in a carbonate–bicarbonate system failed to give concordant values, perhaps due to the dissociation of the bicarbonate anion when it was incubated *in vacuo*; it was found that the pH did not maintain its value. The optimal pH was obtained in glycine–NaOH buffer between pH 9.5 and 9.7 (Fig. 3.).

Relation between activity and enzyme concentration

A linear relation between these factors was maintained under the conditions of the experiment (Fig. 4).

Determination of K_m

Since the optimal pH was about 9.5–9.7, the K_m measurements were made in an alkaline medium. It was observed that the substrate solution turned violet-red in this medium. According to NEUBERGER AND SCOTT[12], 5-aminolaevulinate forms a 2,5-disubstituted pyrazinyl derivative at these pH values. It was necessary therefore to
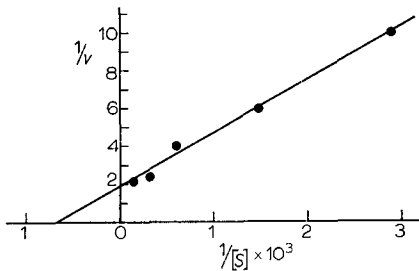

Fig. 5. Determination of K_m. The system contained: 80 mM glycine–NaOH buffer (pH 9.6), 5-aminolaevulinate ranging from 0.345 μM to 6.45 μM and 0.1 ml of the enzyme fraction (Step 2) representing 9.5 mg of protein. Incubations were performed at 55° for 30 min. Results are plotted as 1/absorbance and 1/[S]: absorbance represents initial reaction rate; [S] is expressed as molar concentrations of 5-aminolaevulinate.

evaluate the residual 5-aminolaevulinate at each point of the K_m determination. At pH 9.5, a 30% conversion was observed with all 5-aminolaevulinate concentrations used. The K_m value obtained, under optimal conditions, was 1.5 mM (Fig. 5).

Effect of inhibitors

As the aminolaevulinate dehydratases studied in different organisms and tissues are sulfhydryl enzymes, tests with classic inhibitors were made. To avoid decomposition of the reagent SH groups in the alkaline medium, sodium phosphate buffer (pH 6.8) was used, in spite of the low enzyme activity at that pH. The results obtained indicate that it is also a sulfhydryl enzyme (Table II).

Glutathione and cysteine, under special conditions, were found to be inhibitors of the enzyme, although random values were obtained. It may be assumed that the substrate inhibition by the SH groups takes place on the highly reactive ketonic group of 5-aminolaevulinate.

TABLE II

The percentage of inhibition is calculated with respect to the activity of the following mixture: 60 mM sodium phosphate buffer (pH 6.8), 5 mM 5-aminolaevulinate and 0.1 ml of the enzyme from Step 2. The final volume is 1 ml. Porphobilinogen was estimated as described under MATE-RIALS AND METHODS.

Inhibitor (1 mM)	Inhibition (%)
N-Ethylmaleimide	30
Iodoacetamide	55
5,5'-Dithio-bis(2-nitrobenzoic acid)	85
p-Chloromercuribenzoate	85
Hg^{2+}	90
Cu^{2+}	90

To obtain inhibition curves, according to WEBB[13], it is necessary to know K_s (the substrate constant). Since K_s could not be determined up to now, estimation of the SH group of cysteine in the presence of 5-aminolaevulinate was measured according to GRUNERT AND PHILLIPS[14]. The results are shown in Table III. Mg^{2+}, assayed in Tris buffer, had no effect on porphobilinogen biosynthesis. As is the case for other dehydratases, Tris buffer acted by partially inhibiting the enzyme, with respect to sodium phosphate buffer. The addition of 1 mM EDTA to the medium containing sodium phosphate buffer (pH 6.8) leads to approx. 35% inhibition of the enzymic activity. α,α'5-dipyridyl at 1 mM did not inhibit the enzyme.

TABLE III

Cysteine (1 mM) was incubated with 0.1 M phosphate buffer (pH 7.6) and the indicated amounts of 5-aminolaevulinate. The incubation was made in vacuo, at 38° during 15 min. A fraction of the solution was assayed for cysteine as indicated by GRUNERT AND PHILLIPS[14].

5-Amino-laevulinate (μmoles)	Absorbance at 520 mμ
0	0.290
0.5	0.241
1.0	0.229
2.5	0.200

DISCUSSION

Aminolaevulinate dehydratase has been isolated from several biological sources: mammalian tissues[2], Rhodopseudomonas spheroides[15], propionic acid bacteria[16], etc. In all cases, the enzyme acts in the second step in the pathway of tetrapyrrole biosynthesis, thus leading to the formation of heme, chlorophyll, etc., depending on the biological source.

The normal yeast, studied in the present paper, shows a low activity of amino-laevulinate dehydratase, at least under biological pH and temperature. For instance

the extract obtained from the acetone powder (the latter representing 33% in dry weight) only yielded 5 mμmoles/mg per h, a much lower activity than those reported for aminolaevulinate dehydratase from other sources. The same was true for aminolaevulinate synthetase for which, in a radioactivity uptake experiment, an incorporation of 540 counts/min, representing 0.08% of added counts, was obtained. The final products can vary quantitatively, depending on the culture medium in which the yeast is grown.

According to WALLACE AND LINNANE[17], using an anaerobic medium with 5% glucose, an enhancement of porphyrin biosynthesis is obtained, relative to that of cytochromes. Therefore, a high activity of the related enzymes can be expected to be found in that medium.

This enzyme exhibited a pH optimum of 9.5–9.7, very different from that reported by GIBSON, NEUBERGER AND SCOTT[2] for the enzyme from ox liver (pH 6.8), by WALERYCH[16] for that from propionic acid bacteria (pH 7), by M. TOMIO (personal communication) for Harderian rat gland (pH 6.8) and by BURNHAM AND LASCELLES[15] for *R. spheroides* (pH 7.8–8.0). The optimal temperature (50–55°) was found to be similar to that for the enzymes from propionic acid bacteria (60°) and Harderian gland (55°).

From the inhibition reactions performed, it was deduced that it is also a sulfhydryl enzyme. The studies of NANDI AND SHEMIN[18] with aminolaevulinate dehydratase from *R. spheroides* demonstrated that there is the formation of a Schiff base between the substrate 5-aminolaevulinate and a basic amino acid of the enzyme. This allows us to explain the inhibition by SH groups (Table III) as being due to the interaction of cysteine SH groups with the keto group of the substrate, thus diminishing the substrate concentration.

In general, on the basis of several activity determinations in the presence of cysteine, it is possible to conclude that at high substrate concentrations, the activation of the enzyme by SH groups prevails with respect to the interaction between SH groups and 5-aminolaevulinate; this problem needs further investigation and kinetic studies are in progress.

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