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PORPHYRIN BIOSYNTHESIS

X-PORPHYRINOGEN CARBOXY-LYASE FROM AVIAN ERYTHROCYTES FURTHER PROPERTIES

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SUMMARY

Several properties of porphyrinogen carboxy-lyase from normal chicken erythrocytes were studied.

1. The utilization of the substrate uroporphyrinogen (8-COOH), and the formation of intermediate products (porphyrinogens of 7-, 6- and 5-COOH) and the final product coproporphyrinogen (4-COOH) were investigated as function of time and substrate concentration. The results confirm a two-stage hypothesis involving firstly, the elimination of the first carboxyl group from uroporphyrinogen and secondly the elimination of the further three carboxyl groups to form coproporphyrinogen. The elimination of the first carboxyl group is not the rate-limiting step in this multiple decarboxylation because large amounts of 7-COOH porphyrinogen were accumulated.

The effect of temperature on the stepwise decarboxylation process also suggests an easier elimination of the first carboxyl group.

2. Cysteine and glutathione inhibited the decarboxylation process at low concentrations, but at higher concentrations cysteine continues to inhibit while glutathione allows the recovery of enzyme activity.

3. Studies of the effect of uroporphyrinogen concentration on the first and second stages; and of 7-COOH porphyrinogen concentration on the second stage revealed both substrates as inhibitors of their own decarboxylations. Furthermore, when 7-COOH porphyrinogen was incubated in the presence of uroporphyrinogen, it further inhibited the first decarboxylation of uroporphyrinogen. 7-COOH porphyrinogen was a stronger inhibitor than 8-COOH porphyrinogen.

INTRODUCTION

Decarboxylation of uroporphyrinogen to coproporphyrinogen occurs in four steps[1–3]:

8-COOH → 7-COOH → 6-COOH → 5-COOH → 4-COOH porphyrinogen.

The enzyme system catalysing this transformation has been studied in rabbit reticulocytes[1], *Rhodopseudomonas spheroides*[4] and mouse spleen[5]. We have purified it from chicken erythrocytes and studied some of its properties[6,7]. The influence of several chemical and physical agents: anaerobiosis, NaCl, EDTA, GSH, boiled rat liver extract, preheating, showed a different susceptibility in the removal of the first carboxyl group from uroporphyrinogen and the subsequent three groups.

However, we have obtained a single protein entity responsible for the overall decarboxylation. The present work describes a more detailed study of the behaviour of the decarboxylating system, carried out in order to clarify this process.

MATERIALS AND METHODS

Cysteine, GSH, and DEAE-Cellulose (0.83 meq/g) were purchased from the Sigma Chemical Co. Ion exchange resin used was De Acidite FF/1P (Permutit). All other reagents were of analytical grade.

Uroporphyrin III (8-COOH porphyrin) and 7-COOH porphyrin III were removed from the urine of hexachlorobenzene-intoxicated rats by shaking it with De Acidite FF/1P anion exchange resin (7 g of resin to 100 ml of urine). The resin with the combined porphyrins was recovered by filtration. Extraction was repeated with more resin until no absorption bands of porphyrins were observed in the filtrate. The combined resins were washed 3 times with distilled water and the washings discarded. The washed resin was repeatedly extracted with 25% HCl (w/v) until the acid extracts exhibited no red fluorescence. The HCl solution was titrated with concentrated NaOH to pH: 3.0–3.2. A complete precipitation of the porphyrins was obtained by leaving this solution at 4 °C for 24–48 h. The precipitated porphyrins were collected by filtration on filter paper, dried in a dessicator and then esterified by suspension in a mixture of methanol–sulphuric acid (95:5, v/v).

Porphyrinogen preparations were carried out according to Mauzerall and Granick[1].

Purified porphyrinogen carboxy-lyase was obtained as previously described[7]: enzyme eluted from DEAE-Cellulose columns is referred to as "purified enzyme".

Incubations were performed anaerobically in the dark. Other conditions are described in the figure legends. Further isolation and quantitative determination of porphyrins were as previously described[7].

Initial velocities were calculated by the method of Algranati[8].

RESULTS AND DISCUSSION

Course of reaction

The effect of uroporphyrinogen III concentration on the course of decarboxylation was investigated (Fig. 1). The formation of the final product (4-COOH porphyrinogen) was always linear with time. The amount of 5- and 6-COOH porphyrinogens was in all cases small and constant.

7-COOH porphyrinogen accumulated in greater amounts than the other intermediates (5- and 6-COOH) and the kinetics of its production shows that it is at first

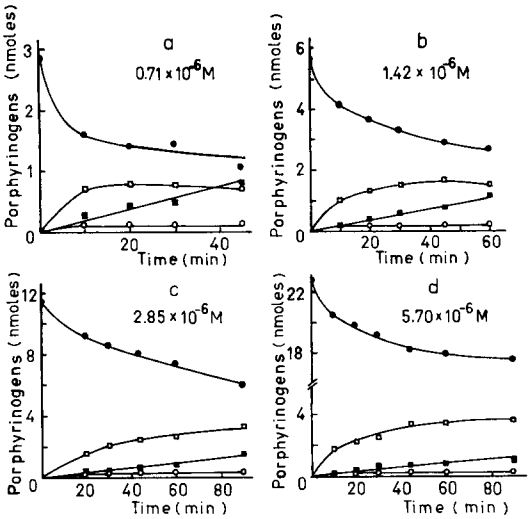


Fig. 1. The decarboxylation process presented as a function of time. The reaction mixture contained 67 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM GSH, 0.14 mg/ml purified enzyme, and uroporphyrinogen concentrations as indicated. Incubation temperature: 37 °C. ●—●, 8-COOH porphyrinogen; □—□, 7-COOH porphyrinogen; ○—○, 6- or 5-COOH porphyrinogen; ■—■, 4-COOH porphyrinogen.

formed at a higher velocity than any other decarboxylation product. This rate decreases as the reaction proceeds and the accumulation reaches a maximum as shown in Fig. 1a and 1b. Maximum accumulation is attained in about 20 min with the lowest uroporphyrinogen concentration (Fig. 1a) and about 50 min with the next higher concentration (Fig. 1b). With the two highest concentrations maximum accumulation was not observed even at 90 min (Fig. 1c and 1d).

When the enzyme concentration was twice that used in experiments of Fig. 1, the highest accumulation of 7-COOH porphyrinogen was obtained earlier. The present results confirm the two-stage hypothesis reported in the previous paper[7].

It is possible to express the results of our experiments in the two following ways:

(1) v_1 = nmoles of 7- + 6- + 5- + 4-COOH porphyrinogens formed per unit time.

(2) v_2 = nmoles of 4-COOH porphyrinogen formed per unit time.

The first way indicates the rate of elimination of the first carboxyl group from uroporphyrinogen (first stage), while the second expresses the rate of the overall process. The observed accumulation of the first intermediate (7-COOH porphyrinogen) shows that the first stage is not the rate-limiting step. Therefore, the second way of expression corresponds to the second stage, *i.e.* the elimination of the last three carboxyl groups.

Additional support for the above statements was obtained by incubation at temperatures ranging from 0 °C to 60 °C. Logarithms of v_1 and v_2 were plotted against reciprocal temperatures (Fig. 2). The energy values E_1 (10.3 kcal/mol) and E_2 (14.8 kcal/mol) were determined from the slopes of the straight lines obtained. E_1 is related to the first decarboxylation stage and E_2 involves the second. E_2 must not be considered as an activation energy but represents an unknown relation involving

mainly the velocity constants of the second stage steps. The fact that $E_1 < E_2$ would imply a greater velocity for the first decarboxylation.

It is noteworthy that enzyme decarboxylation of uroporphyrinogen is carried out even at 0 °C, the 7-COOH porphyrinogen being only accumulated and detecting no other decarboxylation products. Similar results were obtained at 25 °C provided that short incubation times (10–15 min) were used.

Effect of sulphydryl compounds

Preliminary results have shown an opposite influence of glutathione and

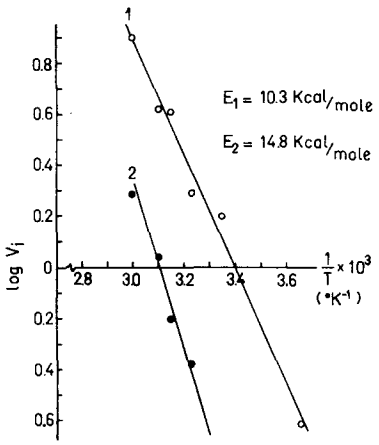


Fig. 2. Arrhenius plots. Incubation conditions were as in Fig. 1c, except the temperatures. ○—○ (1), v_1 (nmoles of 7- + 6- + 5- + 4-COOH porphyrinogen formed/20 min); ●—● (2), v_2 (nmoles of 4-COOH porphyrinogen formed/20 min).

cysteine on enzyme activity[7]. The present work on the influence of sulphydryl compounds shows a rather complex behaviour of the enzyme, depending on the nature of the thiol reagent and its concentration.

As can be seen in Fig. 6a, both Curve 1 and 2 show an exponential inhibition by cysteine throughout the concentration range assayed. This could be due to a breakage of disulfide bridges necessary for full enzyme activity.

The influence of glutathione concentration on reaction velocity is shown in Fig. 6b. At the lowest concentrations, no or little effect was observed. As concentration increased, a sharp decrease can be seen, followed by a further increase. This dual behaviour was observed for both Stage 1 and 2. The inhibition observed at low glutathione concentrations was less sharp than that produced by cysteine and can be ascribed to the same reasons. Recovery of activity at higher concentrations might be related to an activating function of GSH, which would act as a bivalent anion, not as a sulphydryl protecting reagent[9].

The activating effect of GSH would appear once most of the disulfide bridges are broken. This possibility is in agreement with the results shown in Fig. 6c. When cysteine is present in sufficient amount (1 mM) to break disulfide bridges, GSH may express its activating effect. In fact the curves of Fig. 6c can be superimposed on that part of the curves in Fig. 6b beyond 1 mM glutathione.

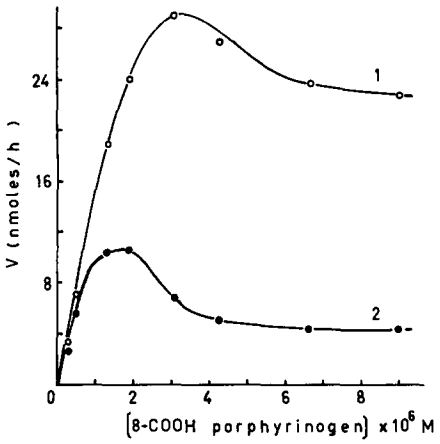


Fig. 3. Effect of uroporphyrinogen III (8-COOH porphyrinogen) concentration on enzyme activity. The reaction mixture contained 67 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM GSH, 0.10 mg/ml purified enzyme, and substrate concentrations as indicated. Incubation temperature, 37 °C. ○—○ (1), v_1; ●—● (2), v_2.

Substrate concentration

The effect of two of the substrates (8-COOH and 7-COOH porphyrinogen III) on reaction velocity was studied.

The plot of initial velocity *vs* uroporphyrinogen concentration is shown in Fig. 3. Curve 1 represents the velocity of disappearance of 8-COOH porphyrinogen (v_1) and Curve 2 the velocity of appearance of the final product, 4-COOH porphyrinogen (v_2). Both curves are hyperbolic at low substrate concentrations, reach a maximum and then decrease. Thus, high substrate concentrations inhibit uroporphyrinogen utilization and coproporphyrinogen formation. Maximum utilization of uroporphyrinogen is obtained at a substrate concentration of about $3 \cdot 10^{-6}$ M and for coproporphyrinogen production at $1.6 \cdot 10^{-6}$ M. Moreover, the velocities appear to approach a constant value at great substrate concentrations. This is more evident in Curve 2. The shape of Curve 2 shows a maximal velocity at a lower substrate concentration than Curve 1, and it begins to decrease when v_1 is still increasing. Such a behaviour was unexpected since Curve 2 represents the removal of three succesive carboxyl groups from 7-COOH porphyrinogen and therefore it should increase while v_1 is increasing.

We believe that this behaviour can be explained in two ways:

(1) 8-COOH porphyrinogen would inhibit the three succesive decarboxylations from 7-COOH porphyrinogen, even before inhibition of its own decarboxylation takes place.

(2) 7-COOH porphyrinogen would inhibit its own decarboxylation as long as v_1 is sufficiently large as compared to v_2. When v_1 begins to decrease, *i.e.* the rate of 7-COOH porphyrinogen formation diminishes, v_2 is stabilized at a constant value, thus indicating that the inhibition is reduced.

An inhibition was actually found when 7-COOH porphyrinogen was used as the substrate (Fig. 4) thus confirming one of the above possibilities. The highest velocity was obtained at about $2 \cdot 10^{-6}$ M substrate. Unlike the results plotted in

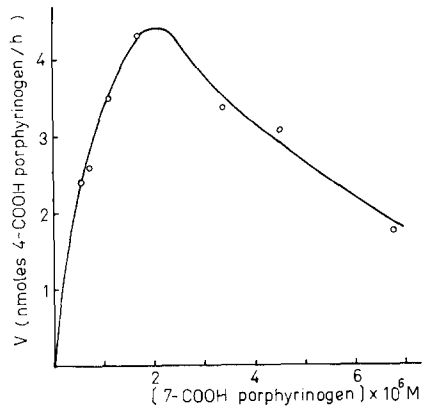


Fig. 4. Effect of 7-COOH porphyrinogen III concentration on enzyme activity. Incubation conditions were as in Fig. 3, except the enzyme concentration which was 0.13 mg/ml. Velocities are expressed as nmoles of 4-COOH porphyrinogen formed per h (v_2).

Fig. 3, even at high substrate concentrations the velocity kept on decreasing.

From the reciprocal plots of substrate concentration curves, apparent K_M values within the range $3 \cdot 10^{-6}$–$5 \cdot 10^{-6}$ M were obtained for uroporphyrinogen and $1.3 \cdot 10^{-6}$ M for 7-COOH porphyrinogen.

When decreasing amounts of 7-COOH porphyrinogen and increasing amounts of 8-COOH porphyrinogen were incubated together, the results plotted in Fig. 5 were obtained. The velocity of removal of the first carboxyl group from 8-COOH porphyrinogen (Curve 1) proceeded slowly for low abscissa values and increased together with 7-COOH porphyrinogen concentration decrease. Thus, v_1 gradually approached the value that could be obtained if no 7-COOH porphyrinogen were added (as is the case in Fig. 3).

Comparing Curves 1 of Figs 3 and 5, a fundamentally different shape can be observed. While in Fig. 3, v_1 initially increases substantially and then levels off, in Fig. 5 a slow initial increase is followed by a fast growth.

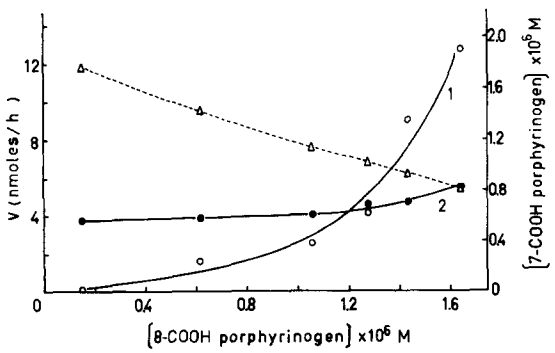


Fig. 5. Effect of 7-COOH porphyrinogen III on 8-COOH porphyrinogen decarboxylation. The reaction mixture contained 67 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM GSH, 0.12 mg/ml purified enzyme, 8-COOH porphyrinogen concentrations as indicated on the abscissa and 7-COOH porphyrinogen as indicated on the right hand ordinate (△---△). Initial velocities correspond to the left hand side ordinate. ○—○ (1), v_1; ●—● (2), v_2.

At least two reasons could explain these results:

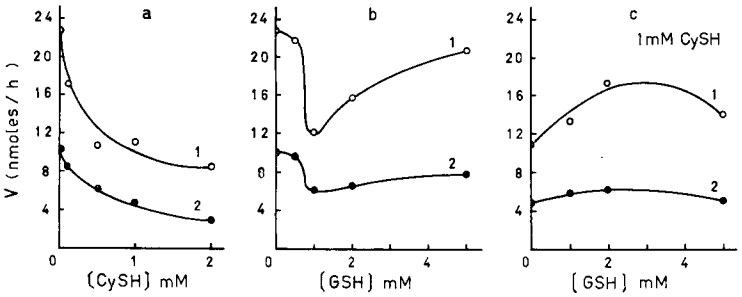


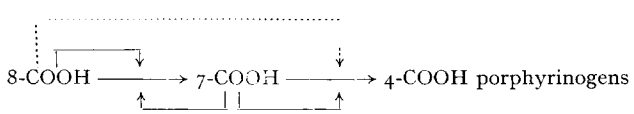
Fig. 6. Effect of thiols. The reaction mixture contianed 67 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 2.9 μM uroporphyrinogen, 40–75% $(NH_4)_2SO_4$ enzyme fraction (0.45 mg/ml), GSH and/or cysteine as indicated. Incubation temperature, 37 °C. ○—○ (1), v_1; ●—● (2), v_2.

(1) 7-COOH prophyrinogen may be preferentially decarboxylated by the enzyme, thus diminishing the extent of 8-COOH porphyrinogen decarboxylation. Such a behaviour should be reflected in decreasing values of v_2 as 7-COOH porphyrinogen concentration decreases. As this was not the case, this first possibility can be discarded. In fact, v_2 is initially fairly constant with increasing 8-COOH porphyrinogen concentrations, tending to increase later (Fig. 5). This behaviour can be due to a inhibition by 7-COOH porphyrinogen of its own decarboxylation and to a velocity increase produced by increasing 8-COOH porphyrinogen concentrations. This implies a stronger inhibitor effect of 7-COOH porphyrinogen than that of 8-COOH porphyrinogen, suggested from the experiment of Fig. 3.

(2) 7-COOH porphyrinogen may inhibit 8-COOH porphyrinogen decarboxylation, perhaps similar to a substrate inhibition. This second possibility seems more probable.

Briefly, the effects of 8-COOH porphyrinogen and 7-COOH porphyrinogen concentrations on enzyme activity indicate that the decarboxylation process is affected as follows: (a) 8-COOH porphyrinogen inhibits its own decarboxylation (Curve 1, Fig. 3). (b) 7-COOH porphyrinogen inhibits its own decarboxylation (Fig. 4). (c) 7-COOH porphyrinogen inhibits 8-COOH porphyrinogen decarboxylation (Fig. 5). (d) 8-COOH porphyrinogen may inhibit 7-COOH porphyrinogen decarboxylation (Curve 2, Fig. 3).

A tentative scheme for the above statements could be:



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