Occurrence and Some Properties of Two Types of δ -Aminolevulinic Acid Synthase in a Facultative Methylotroph, *Protaminobacter ruber*

Kazuyoshi SATO,* Koichi ISHIDA,** Makoto SHIRAI***
and Shoichi SHIMIZU

Department of Food Science and Technology, Faculty of Agriculture,
Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464, Japan

Received March 29, 1985

In addition to the α -ALA synthase already reported (Fraction I: molecular weight, 100,000; optimal pH, *ca.* 8.0), an isozyme (Fraction II: molecular weight, 64,000; optimal pH, *ca.* 6.4) was found in *Protaminobacter ruber* grown in the dark after an initial light period (20~30 hr). The fraction I- and II-enzymes were separable by gel-filtration through Sepharose 6B. While the former was formed constitutively, the latter was formed inducibly under the conditions for bacteriochlorophyll formation. Therefore, the fraction II-isozyme seems to be responsible for the biosynthesis of bacteriochlorophyll.

In the preceding paper,¹⁾ we reported the purification and properties of the δ -aminolevulinic acid (δ -ALA) synthases from a facultative methylotroph, *Protaminobacter ruber*, and a photosynthetic bacterium, *Rhodospseudomonas spheroides*. Although the specific activity of the δ -ALA synthases was highest in cells collected at the early logarithmic phase of growth, bacteriochlorophyll was not sufficiently formed to be detected in such cells, which were used in the previous study.¹⁾ It was of interest to examine the δ -ALA synthase from *P. ruber* cultured under conditions in which bacteriochlorophyll is formed, and to investigate the regulation mechanism for tetrapyrrole biosynthesis. Therefore, we examined the δ -ALA synthase in *P. ruber* cultured under three different cultivation conditions; *i.e.* in continuous darkness (dark conditions), in the dark after an initial light period (light-dark conditions) and in continuous light (light conditions), and we found the presence of an

isozyme of the δ -ALA synthase in *P. ruber*, which was especially abundant in cells cultured under the light-dark conditions, with which bacteriochlorophyll was inducibly formed. The isozyme was not detected in cells which were collected at the early logarithmic phase of growth, which were used in the previous study.¹⁾ The present paper describes the presence and some properties of the isozyme in *P. ruber*.

MATERIALS AND METHODS

Cultivation conditions. *P. ruber* NR-1 was cultivated by a two-step cultivation method, essentially as described in the preceding paper on an NK type shaker (Nippon Ikakikai Seisakusho Co.) fitted with illumination equipment.^{1,2)} The light intensity was about 2,000 lux on the surface of Sakaguchi flasks. For the dark cultivation, the flasks were covered with aluminum foil. The reciprocating shaker was operated at 96 strokes per min at 30°C. After cultivation for 37 hr, the cells were collected by centrifugation at 7,000 $\times g$ for 30 min at 4°C, and stored at -80°C until use. Where indicated, L-type test tubes were

Present addresses:

* Department of Curriculum Studies, Faculty of Education, Hiroshima University, Fukuyama 720, Japan.

** Takasaki Research Laboratory, Nippon Kayaku Co., Ltd., Iwahana-cho, Takasaki 370-12, Japan.

*** Toray Industries Inc., Ohe-cho, Minato-ku, Nagoya 457, Japan.

used instead of Sakaguchi flasks for the cultivation.

Cell-free extracts and column chromatography. Preparation of cell-free extracts and column chromatography were performed as described in the preceding paper.¹⁾ δ -ALA synthase activity, protein content and molecular weight were also determined according to the methods used in the preceding study.¹⁾

Extraction and determination of bacteriochlorophyll. Bacteriochlorophyll was extracted and determined as previously described.^{3,4)}

RESULTS

Time courses of bacterial growth, bacteriochlorophyll formation and specific activity of δ -ALA synthase under three different cultivation conditions

P. ruber was cultivated in L-type test tubes under the following three cultivation conditions: (i) 48-hr darkness, (ii) 20-hr light and 28-hr darkness, and (iii) 48-hr light. The time course of bacterial growth was similar under all the conditions (Fig. 1(a)). However, bacteriochlorophyll formation was considerably affected by the cultivation conditions. Under the dark conditions an appreciable amount of bacteriochlorophyll was formed, and its formation was markedly stimulated by the cultivation of *P. ruber* under the 20-hr light and 28-hr dark conditions (Fig. 1(b)). δ -ALA synthase in the cells showed the highest activity at 7 to 8 hr cultivation in all cases. In the cells cultivated under the conditions of continuous darkness or 20-hr light followed by 28-hr darkness, another peak of δ -ALA synthase activity appeared concomitantly with the bacteriochlorophyll formation, while this activity decreased steadily in cells cultured in the light (Fig. 1(c)).

Sephacrose 6B column chromatography of δ -ALA synthase

δ -ALA synthase from *P. ruber* cultivated under 32-hr light and 5-hr dark conditions was partially purified by ammonium sulfate fractionation (0~40%) and Sepharose 6B column chromatography. Almost all the δ -ALA synthase was recovered in the 0~40% ammonium

FIG. 1. Time Courses of Bacterial Growth (A), Bacteriochlorophyll Formation (B) and δ -ALA Synthase Activity (C) under the Following Cultivation Conditions: Light ($\circ$); 20-hr Light and 28-hr Darkness ($\blacktriangle$); Darkness ($\bullet$).

Bacteriochlorophyll formed was determined by subtracting the value at zero time cultivation, *i.e.* that attributable to the inoculated cells, from that at each cultivation time.

TABLE I. PARTIAL PURIFICATION OF δ -ALA SYNTHASE (Light 32 hr $\rightarrow$ Dark 5 hr)

Fraction	Total activity (nmol/hr)	Relative activity (%)
Crude extract	3400	
Ammonium sulfate fraction (0~40% sat.)	4030	100
Sephacrose 6B filtration		
Fraction I	1170	29
Fraction II	> 2400	60

FIG. 2. Purification of the Ammonium Sulfate Fraction (0~40% Saturation) by Sepharose 6B Column Chromatography.

δ -ALA synthase activity (○), protein (●).

FIG. 3. Purification of the Ammonium Sulfate Fraction (0~40% Saturation) by Sephacryl S-200 Column Chromatography.

δ -ALA synthase activity (○), protein (●).

sulfate fraction, and it was separated into two fractions by Sepharose 6B column chromatography (Table I, Fig. 2).

*Isozyme of δ -ALA synthase in *P. ruber**

Two fractions having δ -ALA synthase activity obtained on Sepharose 6B column chromatography (Fig. 2) were supposed to contain isozymes. On Sephacryl S-200 column chromatography of the 0~40% ammonium sulfate fraction, however, only one activity peak appeared with a shoulder on the elution side of high molecular substances, as shown in Fig. 3. Then, the peak fractions were divided into 3 parts as shown in Fig. 3, which were called fractions A, B and C. These fractions were applied on a column of Sepharose 6B. The results are shown in Fig. 4. δ -ALA synthase activity was detected largely in the main elution peak of protein in the case of fraction A

from Sephacryl S-200 column chromatography. When fraction B was applied on the Sepharose 6B column, δ -ALA synthase activity was detected in two fractions, I and II. However, fraction C was composed of only one fraction showing δ -ALA synthase activity; *i.e.* fraction II. Each fraction was further purified on DEAE-Sephacel, and the molecular weights of the two fractions were determined by Sephadex G-200 column (1.2 $\times$ 100 cm) chromatography. Chymotrypsinogen A (25,000), human serum albumin (45,000), bovine serum albumin (68,000) and aldolase (158,000) were used as reference standards. As shown in Fig. 5, the molecular weights of fractions I and II were estimated to be about 100,000 and 64,000, respectively. Figure 6 shows the effect of pH on the δ -ALA synthase activities of the two fractions. The enzyme in fraction I had an optimal pH in the vicinity of 8.0 and that in

FIG. 4. Sepharose 6B Column Chromatography of Sephacryl S-200 Fractions (A, B, C). δ -ALA synthase activity (○), protein (●).

FIG. 5. Molecular Weight Determination of Fractions I and II.

fraction II at *c.a.*6.0.

Bacteriochlorophyll formation and δ -ALA synthase activity in cells cultivated under several cultivation conditions

Table II summarizes the effects of light on bacteriochlorophyll formation and δ -ALA synthase activity in *P. ruber*. Bacteriochlorophyll was formed in the dark (0.23 nmol/

FIG. 6. Effect of pH on the Activities of Fractions I (A) and II (B).

Potassium phosphate buffer (●), Tris-HCl buffer (△) and glycine-NaOH buffer (○).

TABLE II. EFFECTS OF LIGHT ON BACTERIOCHLOROPHYLL FORMATION AND δ -ALA SYNTHASE ACTIVITY

Conditions	Bchl. formed (37 hr) (nmol/ml)	δ -ALA synthase activity (nmol/hr/mg)
Light (37 hr)	0	8.3
Light (32 hr) $\rightarrow$ Dark (5 hr)	0.91	20.2
Dark (37 hr)	0.23	13.6

ml), but not under continuous illumination. The changing of the conditions from light to dark markedly increased the bacteriochlorophyll formation. The level of δ -ALA synthase activity changed almost in parallel with that of bacteriochlorophyll formation under the above cultivation conditions. The δ -ALA synthase was present even in cells cultivated in the light, in which no bacteriochlorophyll was formed, and so it seems to be a constitutive enzyme for other tetrapyrrole syntheses.

The bacterial growth was almost the same under these three cultivation conditions. All the cells cultivated under these three different conditions contained the isozyme (fraction II) of δ -ALA synthase at different levels, as shown in Fig. 7. The change in δ -ALA synthase level was attributable to this isozyme level which changed notably according to the cultivation

FIG. 7. Purification of the Ammonium Sulfate Fraction (0~40%) by Sepharose 6B Column Chromatography.

Protein (●); δ -ALA synthase activities in cells cultured in the light (37-hr) (○), in the light (32-hr) followed by darkness (5-hr) (△) and the dark (37-hr) (×).

conditions. Formation of this isozyme was induced by the transfer of the cells from the light to the dark, parallel with the inducible formation of bacteriochlorophyll. Although continuous illumination completely blocked bacteriochlorophyll biosynthesis, the isozyme was formed a little under the same conditions. In contrast, the level of the constitutive enzyme (fraction I) did not change at all, irrespective of the cultivation conditions.

DISCUSSION

Regulation of tetrapyrrole biosynthesis has been examined by many workers, but the true mechanism is still obscure.⁵⁾ The most important control step for tetrapyrrole biosynthesis is considered to be δ -ALA synthase step, which was reported to be regulated by heme through feedback inhibition and also by intracellular trisulfide activators.^{5,6)}

Control of the synthesis of each tetrapyrrole through isozymes of δ -ALA synthase is most attractive, if they really act as in the case of the three forms of aspartate kinase involved in the biosynthesis of threonine, methionine and lysine.⁷⁾ Two fractions showing δ -ALA synthase activity were found during purification of the enzyme from *R. spheroides*.^{8,9)} Each of fractions I and II from *R. spheroides* contains two forms of enzyme, active and less active. However, the physiological roles of these forms of enzyme are unknown so far. Our

finding of two forms of δ -ALA synthase is likely to facilitate the elucidation of the physiological roles of the isozymes.

As shown in Fig. 1(c), the specific activity of the *P. ruber*- δ -ALA synthase decreased steadily with cultivation time under the light conditions, while it increased again after about 12 hr and 20 hr under the dark and the light-dark conditions, respectively. These times were almost coincident with those of bacteriochlorophyll formation. In the case of δ -ALA dehydratase, the pattern of the specific activity was similar to that of δ -ALA synthase from cells cultivated in the light, and it was not affected by the cultural conditions (unpublished data). The increase in δ -ALA synthase activity accompanied by bacteriochlorophyll formation was probably due to the *de novo* synthesis of the δ -ALA synthase isozyme. δ -ALA synthase seems to be present in cells as constitutive and inducible forms (fractions I and II in Fig. 2, respectively). The level of the latter form of enzyme depends on the cultivation conditions (Fig. 7) and corresponds very well to the level of bacteriochlorophyll synthesized in the cells. The differences in molecular weight (fraction I, 100,000; fraction II, 64,000) and optimal pH (fraction I, *ca.*8.0; fraction II, *ca.*6.0) strongly suggest that both forms of the enzyme are isozymes. End-products such as heme, adenosylcobalamin and methylcobalamin did not cause any inhibition of either form of enzyme, but rather stimulated the bacteriochlorophyll

formation at higher concentrations ($5 \sim 50 \mu\text{M}$, unpublished data). These results well coincide with those of an *in vivo* experiment in which bacteriochlorophyll formation was stimulated by the addition of end-products.¹⁰⁾

Although the regulation mechanism for tetrapyrrole synthesis through the δ -ALA synthases in *P. ruber* should be examined in more detail, we assume that the inducible enzyme mainly participates in bacteriochlorophyll formation and its activity changes according to the cultivation conditions, while the constitutive enzyme participates in the synthesis of vitamin B₁₂ and cytochromes.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 56560110) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) K. Sato, K. Ishida, O. Mutsushika and S. Shimizu, *Agric. Biol. Chem.*, **49**, 3415 (1985).
- 2) K. Sato and S. Shimizu, *J. Nutr. Sci. Vitaminol.*, **26**, 557 (1980).
- 3) K. Sato, *FEBS Lett.*, **85**, 207 (1978).
- 4) K. Sato and S. Shimizu, *Agric. Biol. Chem.*, **43**, 1669 (1979).
- 5) J. Lascelles, "The Photosynthetic Bacteria," ed. by R. K. Clayton and W. R. Sistrom, Plenum Press, New York and London, 1978, pp. 795~808.
- 6) J. D. Sandy, R. C. Davies and A. Neuberger, *Biochem. J.*, **150**, 245 (1975).
- 7) J. E. Baily and D. F. Ollis, "Biochemical Engineering Fundamentals," McGraw-Hill Book Company, New York, 1977, p. 325.
- 8) S. Tuboi and S. Hayasaka, *Arch. Biochem. Biophys.*, **150**, 690 (1972).
- 9) M. Fanica-Gaignier and J. D. Clement-Metral, *Eur. J. Biochem.*, **40**, 13 (1973).
- 10) K. Sato, K. Ishida, T. Kuno, A. Mizuno and S. Shimizu, *J. Nutr. Sci. Vitaminol.*, **27**, 439 (1981).