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# PORPHYRIN BIOSYNTHESIS IN *RHODOPSEUDOMONAS PALUSTRIS*—II. EVIDENCE ON THE EXISTENCE OF A FACTOR REGULATING AMINOLEVULINATE SYNTHETASE ACTIVITY

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**Abstract**—1. No changes in ALA-S activity were observed when different preparations of *R. palustris* were stored at 4°C for various periods of time.

2. Mixing supernatants from pigmented and decoloured *R. palustris* cells, showed that the activity of ALA-S was several times higher than expected, suggesting the presence of an activator.

3. Supernatants from photosynthetically and aerobically grown cells were heated and the effect of the protein-free supernatant was tested on both red and white supernatants. The heated supernatant from aerobic cells increased ALA-S when added to red and white preparations, but the heated red supernatant only activated red supernatant and had no action on the white cells enzyme.

4. By gel filtration on Sephadex G-25 of cell free extracts from *R. palustris* either aerobically or anaerobically grown, a low molecular weight compound was separated, which added back to the homologeous enzyme enhanced its activity confirming the existence of one or two low-molecular weight and heat-stable factors which would act stimulating ALA-S activity.

5. A scheme is proposed to explain the role of these factors on the control of ALA-S in *R. palustris*.

## INTRODUCTION

It is widely accepted that levels of Aminolevulinic Acid (ALA) play a central role in the control of porphyrin biosynthesis in living systems. The enzyme responsible for its formation, Aminolevulinate Synthetase (ALA-S) (EC 2.3.1.37) has been detected in various organisms; one of the most active sources are the photosynthetic bacteria of the family *Rhodospirillaceae*. From these, ALA-S has been extensively studied in *Rhodopseudomonas sphaeroides* (Jordan & Shemin, 1972) and also investigated in *Rhodospirillum rubrum* (Kikuchi *et al.*, 1958a) but to the best of our knowledge, there had not been yet any report on ALA-S in *Rhodopseudomonas palustris*, the most common non sulfur purple bacterium.

The activity of *R. sphaeroides* ALA-S has been shown to vary significantly in response to environmental changes, particularly oxygen tension (Lascelles, 1959; Marriot *et al.*, 1969). The enzyme activity in cells grown anaerobically in the light is several times higher than in cells grown aerobically in the dark which have no photosynthetic pigments.

During early attempts to purify the enzyme from *R. sphaeroides*, variations in its activity were observed (Kikuchi *et al.*, 1958b) and finally ALA-S was resolved in two different iso-functional enzymes, a low activity and a high activity form (Tuboi & Hayasaka, 1972). Interconversion of these forms has been shown to be dependent on the presence of certain thio-compounds and endogenous enzymes, suggesting that the activity of *R. sphaeroides* ALA-S might be controlled at a molecular level (Neuberger *et al.*, 1973; Wider de Xifra *et al.*, 1976).

Aerobically grown *R. palustris* does not contain bacteriochlorophyll and ALA-S is also much lower than in cells photosynthetically grown. When the pigmented cells are transferred to conditions of high oxygen tension, ALA-S activity immediately diminishes, resembling some how the behaviour of *R. sphaeroides* ALA-S. However, on some other respects *R. palustris* ALA-S exhibits different properties (Viale *et al.*, 1980).

Studies reported here were designed to elucidate the mechanism controlling ALA-S activity in this non sulfur purple bacterium, which seems to involve one or two low molecular weight cofactors.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., BDH, Oxoid or Fluka AG, unless otherwise stated.

Sephadex was from Pharmacia Fine Chem. Uppsala.

*R. palustris* was obtained from the collection of the Microbiology & Immunology Unit-Facultad de Ciencias Exactas y Naturales, University of Buenos Aires.

The growth and harvesting of cells, preparation of cell-free extracts, determination of protein, dry weight and ALA-S activity were carried out as already described by Viale *et al.* (1980). One unit of ALA-S activity is defined as the amount of enzyme which catalyses the formation of 1 nmol of ALA in 60 min under the standard incubation conditions. Specific activity is expressed as units per mg of protein.

Preparation of Sephadex column and gel-chromatography were as described by Batlle *et al.* (1965).

The cells were grown anaerobically in the light, and aerobically in the dark, we will refer to them as photosynthetically (Ph) and aerobically (A) grown respectively. Crude cell-free extracts of both type of cultures were centrifuged either at 15,000 *g* for 15 min, or at 140,000 *g* for 2 hr. Pellets had negligible activity and were discarded. The resulting supernatants from Ph and A cells will be designated as 15 Ph, 140 Ph, 15 A and 140 A.

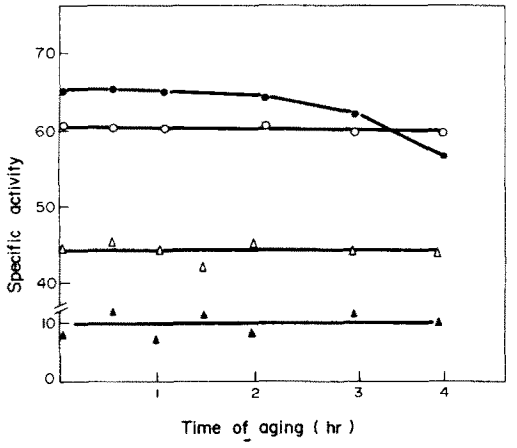


Fig. 1. Effect of aging on ALA-S activity in: A mixture of equal amounts of homogenates obtained from *R. palustris* grown photosynthetically and aerobically (H.Ph + H.A) (○). and 15,000 *g* supernatants from the same cells (15Ph + 15A) (●) were used; 15Ph supernatant (△) and 15A supernatant (▲).

## RESULTS AND DISCUSSION

### *Effect of aging and mixing experiments*

Spontaneous activation of *R. sphaeroides* ALA-S was observed, when crude extracts were stored at 0–4°C for various periods of time (Marriot *et al.*, 1969; Tuboi *et al.*, 1969). Similar changes in this enzyme activity were also found when crude extracts or supernatants of soybean callus were aged at 4°C. (Wider de Xifra *et al.*, 1971) A mechanism explaining the activation of ALA-S and its control involving sulphur compounds of low molecular mass and endogenous enzymes has been proposed by Wider de Xifra *et al.* (1976).

The effect of aging on ALA-S activity was also investigated in *R. palustris* (Fig. 1). When crude extracts of either Ph or A cells were stored at 4°C for different times no changes in activity occurred. Similar results were observed aging the 15,000 *g* supernatants, or mixing extracts and supernatants from anaerobic and aerobic cells. No evidence on the presence of an inhibitor or activator was therefore obtained from these assays.

On the other hand, as already stated, the activity of ALA-S in cells photosynthetically grown is higher than in those aerobically grown. Therefore, to investigate if these changes are due to the presence of an inhibitor in the latter preparation or an activator in the former or both, mixing experiments with extracts and supernatants from Ph and A cells were carried out (Table 1). When crude extracts of both cells were mixed, the activity of the mixture was the sum of its components. Very little or no change was also observed when mixing supernatants from the same type of cells; however it was interesting to find out that, when mixing 15 Ph or 140 Ph supernatants with 15 A or 140 A supernatants, increase in activity occurred; we therefore assumed, that such increases were due to the Ph supernatants, these might contain an activator which was acting on the enzyme in the A preparations.

Table 1. ALA-S activity in *R. palustris* preparations from photosynthetically and aerobically grown cells, and mixing experiments

| Fraction | Observed activity (Units) | Theoric activity (Units) | % Increased activity |
|---|---|---|---|
| H.Ph | 32.94 | | |
| H.A | 23.36 | | |
| 15Ph | 43.06 | | |
| 15A | 25.53 | | |
| 140Ph | 43.76 | | |
| 140A | 30.23 | | |
| Pellet Ph | 0.82 | | |
| Pellet A | 0.59 | | |
| H.Ph + H.A | 56.74 | 56.20 | 0 |
| 15Ph + 140Ph | 98.47 | 86.82 | 13 |
| 140Ph + 140Ph | 103.60 | 87.52 | 18 |
| 15Ph + 15A | 125.88 | 68.59 | 84 |
| 15Ph + 140A | 137.64 | 73.79 | 88 |
| 140Ph + 15A | 124.70 | 69.29 | 80 |
| 140Ph + 140A | 135.29 | 73.99 | 83 |
| 140A + 15A | 56.47 | 55.76 | 1 |
| 140A + 140A | 62.00 | 60.46 | 2 |

ALA-S activity was measured in 0.1 ml of crude extracts, 15,000 *g* (15) and 140,000 *g* (140) supernatants and pellets from photosynthetically (Ph) and aerobically (A) *R. palustris* grown cells, either alone or in 0.2 ml by mixing equal volumes of the different preparations as indicated.

### *Heat stability of the endogenous activator*

Fractions of Ph and A supernatants were heated at 100°C for 10 min, and the denatured protein separated by centrifugation. The clear resulting superna-

Table 2. Heat stability of the endogenous factor present in photosynthetically and aerobically *R. palustris* grown cells

| Fraction | Observed activity (Units) | Theoric activity (Units) | % Increased activity |
|---|---|---|---|
| 15Ph | 38.47 | | |
| 140Ph | 39.06 | | |
| 15A | 9.41 | | |
| 140A | 8.35 | | |
| 15Ph + 15A | 65.11 | 47.88 | 36 |
| 15Ph + 140A | 64.48 | 46.82 | 38 |
| 140Ph + 15A | 66.84 | 48.47 | 38 |
| 140Ph + 140A | 68.85 | 47.41 | 45 |
| 15A + 15Ph$\phi$ | 9.64 | 9.41 | 2 |
| 140A + 15Ph$\phi$ | 8.33 | 8.35 | 0 |
| 15A + 140Ph$\phi$ | 10.00 | 9.41 | 6 |
| 140A + 140Ph$\phi$ | 9.06 | 8.35 | 8 |
| 15Ph + 15A$\phi$ | 54.14 | 38.47 | 41 |
| 15Ph + 140A$\phi$ | 53.48 | 38.47 | 39 |
| 140Ph + 15A$\phi$ | 57.08 | 39.06 | 46 |
| 140Ph + 140A$\phi$ | 56.15 | 39.06 | 44 |
| 15Ph + 15Ph$\phi$ | 50.22 | 38.47 | 31 |
| 140Ph + 140Ph$\phi$ | 51.95 | 39.06 | 33 |
| 15A + 15A$\phi$ | 12.70 | 9.41 | 35 |
| 140A + 140A$\phi$ | 11.19 | 8.35 | 34 |

ALA-S activity was measured in 15,000 *g* (15) and 140,000 *g* (140) supernatants from photosynthetically (Ph) and aerobically (A) grown cells. The effect of mixing the 15 and 140 supernatants and the addition of the heated supernatant ($\phi$) was tested as indicated. Experimental conditions are given in the text.

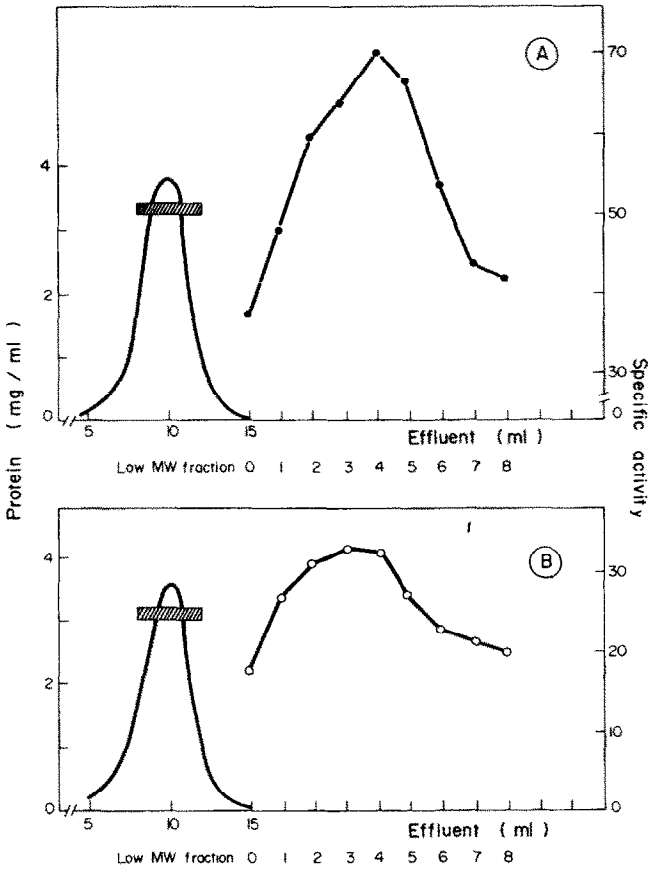


Fig. 2. Separation of a low molecular weight factor modifying ALA-S activity from photosynthetic and aerobic *R. palustris*. 140,000 *g* supernatants from Ph (Part A) and A cells (Part B) were passed through a Sephadex G-25 column (1 × 20 cm) at 4–6°C, equilibrated and eluted with phosphate buffer pH 7.5. Protein was determined (——). The control activity of Ph and A cells was 66.01 and 33.91 units/mg protein respectively. One volume of the most active fraction of excluded enzyme (■) was added to one volume of the retarded column fractions and the activity was measured (●——●) (Ph) and (○——○) (A).

tants were then tested for its activating capacity (Table 2).

The effect of mixing Ph and A supernatants was again observed; but somehow unexpectedly, the addition of heated Ph supernatants to aerobic supernatants did not produce any activation; if only this set of mixtures were tested, it could have been assumed that the activator, if any, was not heat-stable. However, when adding heated A supernatants to anaerobic cell preparations, activation of the same order to that produced by the corresponding non-heated supernatants occurred.

Furthermore, addition of the heated Ph or A supernatants to the Ph and A supernatants, showed that they all increased the activity of ALA-S. These findings were indicating that in aerobic cells a heat-stable compound was present with activating capacity on both the ALA-S from photosynthetically grown *R. palustris* and the ALA-S from aerobic cells. It was also evident that in Ph cells it was present a heat-stable compound which can stimulate the ALA-S activity from Ph cells, but has no action on the enzyme from aerobic cells. Therefore, the activity changes produced by mixing supernatants from Ph and A cells with each other or with deproteinized fractions, seem to be due to two heat-stable compounds, which would participate in the control of ALA-S activity.

*Effect of gel filtration*

Preliminary experiments had shown that ALA-S activity from Ph or A cells diminished after dialysis. To determine if such decrease in activity was due to the loss of an activating component of low molecular mass, which might be identified with the heat-stable compound here detected, 140,000 *g* supernatants of anaerobically and aerobically grown *R. palustris* were passed through a Sephadex G-25 column (Fig. 2).

Protein was excluded with the void volume, and the activity of ALA-S in the protein eluates was 50% reduced, suggesting that a low molecular weight activating component had been separated. As expected by adding back the eluted enzyme with lower activity to the retarded columns fractions, it was obtained an increase in the activity of the excluded ALA-S up to initial level, confirming the existance of an activator in both Ph and A cells.

Were the low molecular weight eluates obtained from A cells able to act on the ALA-S from Ph cells, and which was the action of the retarded fractions prepared from Ph cells on the activity of the enzyme

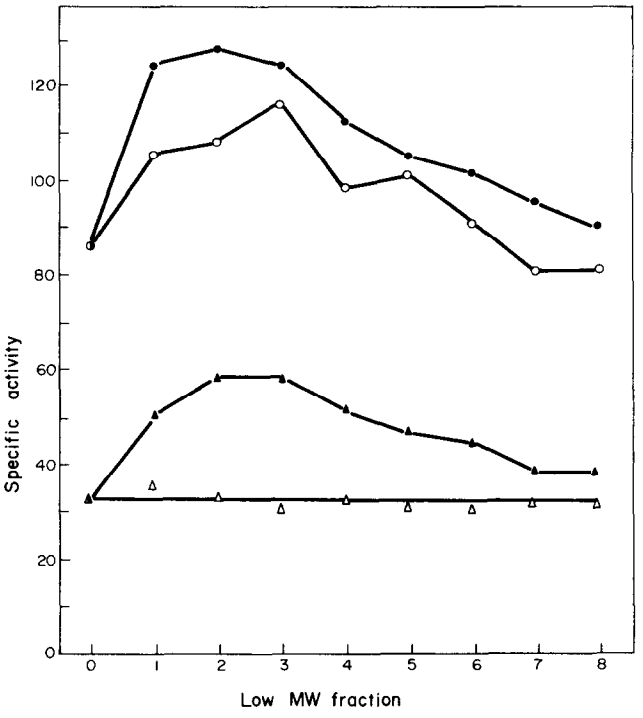


Fig. 3. Effect on ALA-S activity in photosynthetically and aerobically grown cells, of the factor prepared from Ph and A *R. palustris*. 0.1 ml of 140,000 *g* supernatant were added to 0.2 ml of the low molecular weight fraction (F) separated by Sephadex G-25 gel filtration as shown in Fig. 2. ALA-S activity was measured in mixtures of: 140Ph + PhF (○); 140Ph + AF (●); 140A + PhF (△) and 140A + AF (▲).

from A cells? To answer these questions, fractions eluted from Sephadex G-25, containing the low molecular weight compound obtained from Ph and A cells were added to the Ph and A 140,000 *g* supernatants as shown in Fig. 3. Confirming previous results (Table 2), the compound present in aerobically grown *R. palustris*, can stimulate ALA-S activity from both Ph and A cells. However the compound present in photosynthetically grown bacteria can only activate ALA-S from the same source but has no action whatsoever on the enzyme from aerobic organisms.

*Postulated scheme*

Undoubtedly more work is necessary to clarify further the structure of these compounds and the nature of their interaction with the enzyme; however it is evident that they should play an important role in the control of ALA-S activity and although rather speculative we propose the following scheme to explain the present findings (Fig. 4).

We assume that in *R. palustris* ALA-S could exist in two different forms depending on the growing conditions, a low and a high activity form in aerobically and photosynthetically grown cells respectively. Under aerobic conditions a cofactor is formed which binds the enzyme increasing the activity.

Under anaerobic conditions another compound or probably the same cofactor but with its structure changed is formed, which also binds ALA-S although at a different site, resulting in a high activity form. The structure of the protein synthesized by anaerobic cells can be slightly different from that of the enzyme

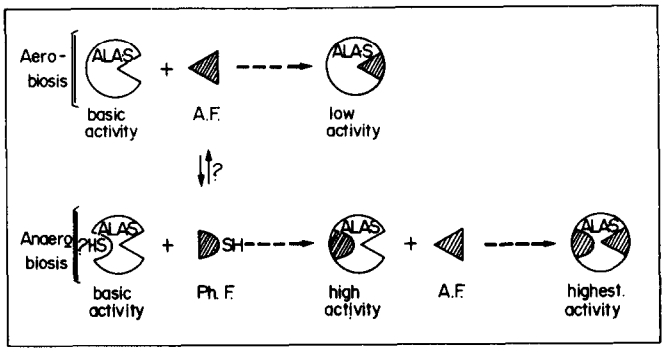


Fig. 4. Postulated scheme for explaining the existance of low molecular weight factors, formed under aerobically (AF) and anaerobically (PhF) grown conditions, controlling the activity of ALA-S in *R. palustris*. See the text for explanation.

from aerobic cells, and addition of the aerobic factor might still further increase its activity. On the other hand, the anaerobic factor finds no site on the aerobic ALA-S to interact with. The possibility of the involvement of sulphydryl groups in the binding of the anaerobic factor to the anaerobic enzyme is also contemplated. Oxygenation might inactivate or prevent the formation of the anaerobic factor; alternatively if sulphydryl groups on the enzyme are essential for its interaction, oxygen could perhaps block the site of binding, modifying the protein structure to that corresponding to the low activity form.

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