CHAPTER VI

STUDIES ON RHIZOBIAL HAEMOGLOBIN AND δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY

IN

RHIZORIUM JAPONICUM
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RHIZOBIUM JAPONICUM

A unique characteristic of the symbiotic relationship between Rhizobium and leguminous species is the presence of a red pigment in the root nodules. Kubo (1) identified the compound as a haemoglobin which was confirmed by Virtnen (2) and Keilin and Wang (3). A positive relationship between nitrogen fixing effectiveness and leghaemoglobin (LB) content was indicated constantly (4). The probable function of LB is the regulation of intranodule oxygen tension (5), and its presence is correlated with rhizobial nitrogenase (2). Bergersen (6) hypothesized that LB serves a dual function in nitrogen fixation: first as oxygen carrier to mitochondria and bacteroids for ATP production via oxidative phosphorylation, and second as a modulator of oxygen tension to prevent inhibitory levels of oxygen from reaching the nitrogenase system.

Appleby (7) has reported that soybean rhizobia grown in the laboratory culture, contain a pigment with the characteristic of a haemoglobin. This "Rhizobial haemoglobin" as called by Appleby (7), for the
haemoglobin produced in pure culture, has the molecular weight in the range of 20,000 - 25,000, which is higher than that found for LB. It has been speculated that the "Rhizobial haemoglobin" may be a primitive form of the present day LB(8). This chapter discusses some of the conditions which lead to the synthesis of Rhizobial haemoglobin in R.japonicum. It also discusses some of the properties of the enzyme δ-aminolevulinate (δ-ALA) synthetase involved in the synthesis of haemoglobin. The pathway for the haem synthesis has been shown in Fig.1. δ-ALA synthetase is the first enzyme involved in the biosynthesis of haem from glycine and succinyl CoA. This step of formation of aminolevulinic acid (ALA) is known to be the rate limiting step in most organisms including bacteria (9).

The spectral properties of rhizobial haemoglobin, leghaemoglobin and its derivatives, are not appreciably different from those of mammalian haemoglobin. Characteristic absorption spectra of ferroleghaemoglobin, oxyferroleghaemoglobin and ferrileghaemoglobin, have been reported (1,10) and the prosthetic group of the protein has been indentified as protoheme (11). Appleby (7) obtained peaks at 430 nm and 555nm for haemoglobin extracted from R.japonicum. The haemoglobin from
GLYCINE + SUCCINYL-CoA

δ-ALA synthetase

δ-AMINOLEVULINIC ACID

2-ALA → Porphobilinogen synthetase

PORPHOBILINOGEN

4-PBG → (deamination)

UROPORPHYRIN OGEN III → VITAMIN B₁₂ → (decarboxylation)

COPROPORPHYRINOGEN III → (oxidation)

DEUTEROPORPHYRINOGEN-2,4-

BIS,β-HYDROXY PROPIONIC ACID → (decarboxylation)

PROTOPORPHYRINOGEN

{O₂}

PROTOPORPHYRIN

Fe

HEME → HEMOGLOBIN, MYOGLOBIN

CATALASE, PEROXIDASE

CYTOCHROME b, b₅, P₄₅₀

CYTOCHROME c

CYTOCHROME a
R. japonicum strain 211 D used in the present study, was one step purified using ammonium sulphate precipitation. A pyridine haemochrome was prepared using the method of Appleby (12), which showed a peak at 410 nm (Fig. 2). Bovine haemoglobin was used as the standard showing peak at 400 nm. No distinguished peak at 555 nm was observed in R. japonicum 211 D as reported by Appleby in R. japonicum (7). The peak at 410 nm obtained in R. japonicum 211 D was observed only when the culture was grown on succinate as the carbon source. Glucose grown cells did not show presence of haem. This suggested inducible nature of enzyme(s) of haem biosynthesis, as succinyl CoA is one of the precursors of haem synthesis.

The response of R. japonicum to sodium succinate as the carbon source compared to glucose was studied. The concentration of sodium succinate, supplemented in the medium was comparable to 0.5% glucose in terms of carbon atoms. Succinate was found to be a better carbon source compared to glucose (Fig. 3), while glycine (0.05%) when supplemented along with succinate, was found to be promoting the growth of R. japonicum. The data in Fig. 4 and 5 show the optimum concentration of succinate and glycine required for haem synthesis in R. japonicum. With 0.75% succinate in the medium, maximum haem synthesis
Fig. 2. Absorption spectra of haemoglobin from Bovine (O) and H. japonicum (•).
Fig. 3. Growth curves of *R. japonicum* with 0.5% glucose (•), 0.75% succinate (○) and 0.75% succinate + 0.05% glycine (×) in the medium as the carbon source.
Fig. 4. Effect of succinate on haemoglobin content in \textit{H. japonicum}.

Fig. 5. Effect of glycine on haemoglobin content in \textit{H. japonicum}.
occurred (Fig. 4), while increasing concentration of glycine in the medium from 0 to 50 mg%, showed a linear increase in the haem content reaching a plateau beyond this concentration. Richmond and Solomon (13) also observed stimulation of haem synthesis by succinate and glycine in soybean nodules.

As haemoglobin was found to be synthesized by this strain of *R. japonicum*, attempts were made to study the first enzyme, δ-aminolevulinate synthetase (δ-ALA synthetase) of haem biosynthesis under the conditions of haem synthesis. Godfrey and Dilworth (14) observed haem synthesis from $^{14}$C-δ-aminolevulinic acid in laboratory cultured bacteria, as well as in bacteroid forms of *Rhizobium lupini* isolated from serradella root nodules. The activity of the enzyme δ-ALA synthetase has been reported in a non-sulphur bacterium *Rhodopseudomonas spheroides* (15), and some of the non-photosynthetic bacteria like *Protoniobacterium shermanii* (16), *Spirillum itersonii* (17) and *Micrococcus denitrificans* (18).

The enzyme δ-ALA synthetase from *R. japonicum* was found to be oxygen sensitive. During the course of the investigation it was observed that when grinding of cells using mortar and pestle was prolonged, it resulted in the loss of δ-ALA synthetase activity (Table I). Homogenized
Table 1: Effect of grinding on the activity of \( \delta \)-ALA synthetase in \( R. \) \( ianonicum \)

<table>
<thead>
<tr>
<th>Enzyme extraction methods</th>
<th>Sp. activity (Units/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Grinding (2 hours)</td>
<td>6.1</td>
</tr>
<tr>
<td>Grinding (4 hours)</td>
<td>Nil</td>
</tr>
<tr>
<td>Homogenizing</td>
<td>11.9</td>
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preparation using homogenizer (B. Brown and Co., West Germany) restored the activity. The loss in the activity could be due to prolonged exposure of the homogenate to air. Lascelles (19) also demonstrated the loss of activity of \( \delta \)-ALA synthetase by exposure to air. An oxygen-sensitive \( \delta \)-ALA synthetase has also been reported in \( R. \) \( spheroides \) by Tait and his group (20).

The activity of \( \delta \)-ALA synthetase with respect to protein concentration is shown in Fig.6. Maximum activity was obtained at 0.48 mg/ml concentration. Higher concentration than this was found to be inhibitory. Similar type of results were reported by Tait (18) in \( M. \) \( denitrificans \). The reported pH optima for \( \delta \)-ALA synthetase from \( R. \) \( spheroides \) is 7.4 (20). The enzyme from \( R. \) \( japonicum \) showed optimum pH of 7.5 (Fig. 7). Maximum activity was obtained in tris-HCl buffer. The data listed in Table 2 shows specific activity of \( \delta \)-ALA synthetase
Fig. 6 Activity of 6-aminolevulinate synthetase with respect to enzyme protein concentration.
Fig. 7 pH dependent activity of δ-aminolevulinate synthetase of *R. japonicum*. Acetate buffer (○), sodium phosphate buffer (□) and tris-HCl buffer (x).
Table 2: Time incubation dependence of \( \delta \)-ALA synthetase from *R. japonicum*

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>Sp. activity (Units/mg protein)</th>
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<tbody>
<tr>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>20</td>
<td>71</td>
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<tr>
<td>40</td>
<td>61</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
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with respect to increase in time of incubation of the reaction. Increase in time of incubation above 20 min. showed inhibition in the activity of \( \delta \)-ALA synthetase from *R. japonicum*. This could be due to the inhibition by the product of the reaction. As the system used here to measure \( \delta \)-ALA synthetase is a crude one, the \( \delta \)-ALA formed might be further getting channelized for the formation of protoporphyrin and finally to haem. Burnham and Lascelles (21) observed inhibition of \( \delta \)-ALA synthetase by haem in *R. spheroides*. In contrast, the \( \delta \)-ALA synthetase in isolated liver mitochondria was found to be insensitive to hemin even at 2.5 x \( 10^{-5} \)M concentration (22). Bottomley and Smithee (23) reported inhibition of the mitochondrial \( \delta \)-ALA synthetase enzyme from rabbit bone marrow by hemin or protoporphyrin.
As mentioned previously, haem was only found to be present in succinate grown cells, glucose grown cells did not show presence of haem (Fig. 2). So it was of interest to study the activity of δ-ALA synthetase under different cultural conditions. Succinate and succinate + glycine grown cells exhibited higher activity of δ-ALA synthetase compared to the glucose grown cells (Table 3), suggesting inducible nature of the enzyme. Inducible nature of δ-ALA synthetase has also been reported in animal system by Granick and Urata (24) and Hayashi et al (25).

Figures 8 and 9 show substrate dependence and Linewaver Burk plots of δ-ALA synthetase for the substrates succinate and glycine respectively. The Km values for succinate and glycine were found to be 1.54 and 5.9 mM respectively in \textit{R. japonicum}. The apparent Km values of
Fig. 8. Substrate dependence (A) and Lineweaver-Burk Plot (B) of 6-aminolevulinate synthetase for succinate. Saturating concentration of glycine was used for enzyme assays.
Fig. 9  Substrate dependence (A) and Lineweaver-Burk Plot (B) of δ-aminolevulinate synthetase for glycine. Saturating concentration of succinate was used for enzyme assays.
about 12 mM for glycine and 10 mM for succinyl CoA were found with the purified ALA synthetase from *M. denitrificans* (18), while the Km values for succinyl CoA and glycine from *R. spheroides* were found to be $2.2 \times 10^{-3}$ mM and 3.0 mM, respectively (26). In all these systems the Km value for succinyl CoA is low, compared to the Km value for glycine. The higher Km for glycine suggests that glycine may be a limiting substrate in these systems. The Km value of 6-ALA synthetase for succinate from *R. japonicum* cannot be compared with the Km value for succinyl CoA obtained for 6-ALA synthetase from other systems. The probable reason being that, in *R. japonicum* the reaction catalysed by succinyl CoA synthetase, converting free succinate and CoA into succinyl CoA, has been coupled with 6-ALA synthetase reaction. The apparent Km value of 6-ALA synthetase for succinate may thus, not be true. The Km of succinyl CoA synthetase for succinate has been reported to be 5.0 mM in animal system (27).

The haem content and 6-ALA synthetase activity were studied at different growth hours in laboratory grown *R. japonicum*. Maximum haem content was found to be present around 28 hours of growth and then content declines (Fig. 10). The activity of 6-ALA synthetase also follows the same pattern, suggesting a direct correlation between haem synthesis and 6-ALA synthetase activity. Godfrey and Dilworth (14) have tried to study
Fig. 10. Haem content (○) and the activity of \( \delta \)-aminolevulinate synthetase (●) during the growth of \( \textit{R. japonicum} \).
the synergistic effect between cytoplasmic and particulate fractions of serradella nodule to incorporate δ-ALA into haem. From the rate of δ-ALA incorporation into haem, they have suggested that the synthesis of haem in *R.* _lupini_ may be adequate to produce the required amount of LB (leghaemoglobin) haem. Moreover, strains of *R.* _meliloti_ accumulated porphyrins in culture medium under rather specific conditions (28). Laboratory grown rhizobia, under the conditions of plentiful ALA, were found to release the product into medium which could be true for bacteroids also. Jackson and Evans (29) observed that ALA is incorporated at least ten fold more readily into haem than any precursors of ALA. Similar responses to exogenous ALA have been observed in other bacteria (9). The considerable synergistic effect between cytoplasmic and particulate fractions of serradella nodules, suggested that the plant occupies a role in the synthesis of LB from ALA, be it as a source of enzyme activity, cofactor or as an acceptor for the haem produced. The primary structure of the globin moiety of leghaemoglobin has been shown to be determined by the plant genome (30), while bacteroids have been shown to be the site of haem synthesis (31). Bacteroids of soybean have lost much of their capacity to form porphobilinogen from ALA, when compared to the laboratory grown *R.* _japonicum_ (32), suggesting a indirect role of culture rhizobia in LB synthesis.
REFERENCES