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IISc THESES ABSTRACTS

Thesis Abstract (Ph.D.)

Ribosomal RNA genes in rice by N. Hariharan.
Research supervisor: J. D. Padayatty.
Department: Biochemistry.

The hybridization of ^{32}P-labelled 25S, 17S and 5.8S rRNAs with the discrete *Eco*RI, *Bgl*II or *Bam*HI 8.8kb band from the rice variety IR20 DNA showed the presence of *Eco*RI, *Bgl*II or *Bam*HI families of tandemly repeated rDNA units. In the rice variety Mangetsumochi, the major rDNA unit is present in 7.8kb *Eco*RI DNA fragment. The *Bam*HI digestion of rice DNA also produced 3.8 and 5.0kb bands which are derived from the major 8.8kb *Bam*HI fragment. The 3.8kb DNA cloned in pBR322 at the *Bam*HI site, hybridized positively with 25S, 5.8S and 5S rRNAs separately, showing the presence of complementary sequences for these rRNAs.

A clone bearing the plasmid pBR322 carrying 0.3kb *Bam*HI rice DNA insert was selected by colony hybridization with ^{32}P-5S rRNA. The 0.3kb insert DNA may contain the 5S rRNA gene. The hybridization of the labelled 0.3kb DNA from the recombinant plasmid with 0.3kb genomic *Bam*HI DNA fragment and its multimers at 0.6 and 0.9kb shows that 5S rRNA genes are tandemly repeated. The minor bands of varying sizes suggest repeat length heterogeneity for 5S rRNA genes.

The 148-nucleotide partial sequence of 3.8kb DNA is identical to the 17S rRNA gene sequence of rice variety Mangetsumochi from 544 to 691 nucleotides except for the deletion of six nucleotides from 649 to 654 and an addition of two nucleotides at positions 662 and 663 in rice variety IR20. The partial 92- and 161-nucleotide sequence of 3.8kb DNA is identical to the known 25S rRNA gene sequence of rice variety Mangetsumochi from 1749 to 1909 and 1430 to 1521, respectively, except for the substitution of C for G at 1876, a six-nucleotide substitution from 1801 to 1806 and a two-nucleotide deletion at 1761 and 1762 in rice variety IR20. The 273-nucleotide sequence of 3.8kb DNA contains the 164 nucleotide of the 5.8S rRNA gene. However, the 5′-spacer shows substitution of 21 nucleotides from 47 to 67 in rice variety Mangetsumochi strain by a 12-nucleotide sequence in rice variety IR20. The nucleotide sequence analysis shows that the rRNA genes are organized in the usual order 17S-5.8S-25S.

The nucleotide sequence of 0.3kb *Bam*HI DNA shows that the DNA fragment is 303 bp long and carries 5S rRNA coding sequences from 31 to 119 and 1 to 30 nucleotides at the termini. There is a *Bam*HI site at 30/31 nucleotides in the 5S rRNA gene as predicted from the 5S rRNA sequence. The spacer DNA is 184 bp long and shows no homology except for the oligo (T) RNA-polymerase III transcription terminator at the 3′-end of the 5S rRNA gene. The coding sequences for 5S rRNA in the 0.303kb DNA are identical to the 5S rRNA species common to both ungerminated and germinated rice embryos.

The 5S rRNA is hybridized positively to the 3.8kb DNA containing 17S, 5.8S and 25S rRNA genes.

Computer analysis of the 5S and 5.8S rRNA gene sequences of rice variety IR20 and 17S and 25S rRNA gene sequences of rice variety Mangetsumochi reveals the presence of 39 hexa, four hepta and two octa nucleotide homologies between 5S and 17S rRNA genes and 47 hexa, 12 hecta, six octa and one deca nucleotide homologies between 5S and 25S rRNA genes. These homologous boxes are spread throughout the length of the genes. However, there is no hybridization between the 0.303kb *Bam*HI DNA carrying 5S rRNA genes and 5.8S rRNA, though hexa- and hepta-nucleotide sequence homologies are found between 5S and 5.8S rRNA genes. This suggests that more than one hepta-nucleotide sequence homology is required for RNA-DNA hybridization to occur. The computer analysis is supported by the positive hybridization between the restriction fragments of 3.8kb DNA with 5S rRNA. However, the 5S rRNA hybridized only with the 0.84kb fragment containing a ten-nucleotide sequence homology, suggesting that the minimum homology required for DNA-DNA hybridization is ten nucleotides.

The 119-nucleotide sequence obtained for 5S rRNAs purified from ungerminated and 48-h germinated embryos is identical to the 5S rRNA sequence of related members of the family Graminae namely, wheat, rye and maize except for two nucleotides at positions 107 and 117, where C and U residues are replaced by U and C, respectively, in rice. However, the presence of additional Gs on RNase T1 digestion of 5S rRNA from 48-h germinated embryos against C, A and U at positions 15, 19 and 21, respectively, suggested the presence of a second species of 5S rRNA. The 5S rRNA sequence contains a potential *Bam*HI site at position 30/31.

An insert DNA with a continuous 5S rRNA gene was constructed from the 0.303 bp DNA carrying discontinuous stretches of 5S rRNA genes and cloned in pBR322. The plasmid DNA carrying discontinuous and continuous 5S rRNA gene sequence was transcribed in an *in vitro* system derived from the nuclei of germinated rice embryos. Mature 5S rRNA like transcripts are produced from both the plasmid templates indicating the presence of an internal controlling region in rice 5S rRNA gene where the transcription factors and RNA polymerase III bind. The continuous 5S rRNA gene is about three fold more efficiently transcribed than the discontinuous gene where the 30 nucleotides at the 5'-end of the gene are substituted by pBR322 DNA sequence. This shows that the primary structure of the 5S rRNA gene and the 5'-flanking sequences are essential for efficient transcription.

Thesis Abstract (Ph.D.)

Biosynthesis of indole alkaloids in the higher plant—*Catharanthus roseus*: characterization of the microsomal *b*-type cytochrome system by N. Krishnamachary.
Research supervisor: K. M. Madyastha.
Department: Organic Chemistry.

1. Introduction

Indole alkaloids belong to a large family of structurally diverse compounds encompassing some of the most important plant medicinals discovered by man. The *in vivo* feeding experiments carried out with labelled precursors have resulted in the delineation of generally accepted pathway for their biosynthesis[1]. However, very little is known about the individual steps involved in the biosynthesis of these alkaloids at the cell-free level. Earlier studies have demonstrated the intermediacy of 8-hydroxy derivatives of geraniol and nerol in the biosynthesis of these natural compounds[2,3]. It has been shown that a cytochrome P-450 (cyt. P-450) system isolated from *C. roseus* converts geraniol and nerol to

their respective 8-hydroxylated derivatives in the presence of O_2 and NADPH[4]. The hydroxylase activity has been reconstituted using partially purified cyt. P-450, highly purified NADPH-cyt. P-450 reductase and lipid. However, the cyt. P-450 fraction used contained cytochrome b_{555} (cyt.b_{555}) and its reductase, NADH-cyt.b_{555} reductase[4].

In order to clarify the role of cyt.b_{555} system in NADPH-dependent cyt.P-450-mediated geraniol hydroxylation, an attempt was made to isolate and purify cyt.b_{555} and its reductase from the microsomal fraction of the higher plant, *C. roseus*. Using the purified proteins, antibodies were elicited in rabbit and their effects were tested on cyt.P-450-mediated geraniol hydroxylation reaction.

2. Methods

Cell-free extract was prepared from five-day-old *C. roseus* seedlings as reported earlier[5]. Microsomes were prepared and suspended in 0.1 M Tris-HCl (pH 7.8) containing 15% glycerol, 1 mM each of EDTA and DTT. After sonication, microsomes were solubilized in two steps, once using sodium cholate (protein:cholate as 1:1) and the second time using a mixture of deoxycholate and Triton X-100 (protein:deoxycholate:Triton X-10 as 1:0.5:1). Each time, the contents were centrifuged at 105,000 × g for 1h and the supernates were pooled. It was concentrated, dialysed against 0.1 M Tris-HCl (pH 7.8) containing 15% glycerol, 0.1 mM EDTA and 0.1 mM DTT (Buffer A) and subjected to DE-52 column chromatography. Cyt.b_{555} and NADH-ferricyanide reductase were eluted with Buffer A containing 0.3 M KCl. This preparation was subjected to DEAE-Sephadex A-50 column chromatography, and on differential elution, cyt.b_{555} and two forms of NADH-ferricyanide reductase were separated. The cyt.b_{555} was further purified to homogeneity by chromatography on Blue 2-Sepharose CL-6B, Sephadex G-75, DE-52 cellulose and $Ca_3(PO_4)_2$ gel treatment, to a specific content of 18.5 nmol per mg of protein. The two forms of ferricyanide reductase were partially purified further on Sephadex G-75 and Blue 2-Sepharose CL-6B column chromatography. Using the purified cyt.b_{555} and partially purified NADH-cyt.b_{555} reductase (F_{P_D}) (one of the ferricyanide reductases which catalysed the reduction of cyt.b_{555} could be designated as F_{P_D}) antibodies were raised in rabbit. Geraniol hydroxylation was carried out as reported earlier[4,5]. Heme content in the purified cyt.b_{555} was determined by a known method[6]. Estimations of cyt.b_{555} and NADH-ferricyanide reductase were carried out as reported[5,6]. Protein estimations were carried out by the method of Lowry *et al*[7].

3. Results

Purified cyt.b_{555} gave a single band when examined by SDS-PAGE and the molecular weight was estimated to be 16,500. The reduced form of the pigment has major peaks at 424, 525 and 555 nm. The α-band of the reduced form is asymmetric with a shoulder at 559 nm. The absorption spectrum of the pyridine ferrohemochrome has maxima at 557, 524 and 418 nm indicating that the prosthetic group of this pigment is protoheme. The cytochrome is autooxidizable and does not combine with CO, cyanide and azide. The cyt.b_{555} can be reduced (~75%) with NADH in the presence of one of the partially purified NADH-ferricyanide reductases. Hence, this form of the reductase could be designated as F_{P_D} of *C. roseus* microsomes. Under identical conditions, another form of the ferricyanide reductase failed to catalyse the reduction of cyt.b_{555}.

Anti-cyt.b_{555} serum significantly inhibited the microsomal NADH-cyt.c reductase activity and decreased the microsomal content of cyt.b_{555}, whereas anti-E_{P_D} antibodies and control serum did not affect the content of cyt.b_{555}. However, anti-F_{P_D} antibodies inhibited NADH-cyt.*c* and NADH-ferricyanide reductases to 70 and 60% respectively. But the geraniol hydroxylase activity in the microsomal fraction was not inhibited by both these antibodies.

4. Discussion

For the first time plant microsomal *b*-type cytochrome was purified to homogeneity. The molecular weight of cyt.b_{555} from *C. roseus* is close to detergent-solubilized rabbit liver microsomal cyt.b_5^s, but differs from cyt.b_{555} isolated from the soluble fraction of mung-bean seedlings[9]. The α-band of the reduced form of cyt.b_{555} is asymmetric with a shoulder at 559 nm. Similar observations have been made for cyt.b_{555} isolated from the soluble fraction of mung-bean seedlings[9]. One of the two forms of ferricyanide reductase could transfer electrons from NADH to the hemoprotein, cyt.b_{555}, indicating that it is a component of the microsomal cyt.b_{555} system. Since the anti-cyt.b_{555} and anti-F_{b_5} antibodies do not inhibit the microsomal geraniol hydroxylase, it appears that in *C. roseus*, cyt.b_{555} is not participating as one of the components of the cyt.P-450-mediated monoterpene 8-hydroxylase system.

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Thesis Abstract (Ph.D.)

Vanadate- and ubiquinone-dependent reduction of oxygen by microsomes and mitochondria
by Milind Shivajirao Patole.
Research supervisors: T. Ramasarma and C. K. Ramakrishna Kurup.
Department: Biochemistry.

1. Introduction

Vanadate, an essential micronutrient and a trace element has a number of physiological and metabolic effects[1]. However, the underlying basis for the essential nature of vanadium is not known. The demonstration that polyvanadate brings about SOD-sensitive NADH oxidation by plasma membranes and generates H_2O_2 is an important observation in this context[2,3]. Results of experiments

designed to elucidate the involvement of polyvanadate in transferring of reducing equivalents from NADH to oxygen in microsomes and of ubiquinone in H_2O_2 generation in mitochondria are presented in this thesis.

2. Materials and methods

Rat liver and brain microsomes were used as enzyme sources and were prepared by differential centrifugation. The changes in absorbance at 340 and 650 nm were followed to measure oxidation of NADH and reduction of vanadate, respectively. Oxygen uptake studies were carried out in oxygraph fitted with Clark oxygen electrode. Lipid peroxides were quantitated by TBA-reactive material.

3. Results and discussion

Orange-yellow colored polyvanadate and phosphate anions were required for the vanadate-stimulated NADH oxidation. High rates of the reaction were observed in the presence of small concentrations of polyvanadate whereas even with large concentrations of ortho- and meta-vanadate the rates were negligible. Metavanadate, in presence of a small amount of polyvanadate showed an additive rate that was higher than the sum of individual rates. SOD-sensitivity of this activation of metavanadate suggested that superoxide radicals were essential for the activation process.

In contrast to earlier observations with plasma membranes, it was observed that NADH oxidation was SOD-insensitive when microsomes were used as the enzyme source. Also the rate of NADH oxidation proceeded at the same rate even in the absence of oxygen. These results indicated that some component of reaction mixture acted as an electron sink. Vanadate being transition element can accept or donate electrons. A blue-color compound absorbing at 650 nm was detected in the assay mixture indicating the formation of a reduced vanadate species[4]. Involvement of cytochrome b_5 was also shown in this reduction of vanadate.

The disappearance of 650 nm-absorbing compound occurred at two different rates. The SOD-insensitive degradation of the blue compound indicates autooxidation of reduced vanadate. When concentration of NADH was in far excess than vanadate, higher rate of disappearance of blue-color compound was observed and this type of reductive change was accompanied by a high rate of oxygen uptake. Under this condition NADH disappearance, oxygen uptake and degradation of blue-color compound were found to be SOD-sensitive, indicating involvement of superoxide radicals.

Formation of complex between phosphate, reduced vanadate and oxygen such as peroxy-phospho-vanadate (P-V^{v}-$OO^{\cdot}$) type of radical species is indicated which is likely to be the primary oxidant of NADH, schematically shown in fig. 1. Such a complex polyvanadate was also found to induce NADH-dependent lipid peroxidation in microsomes. This was found to be the first demonstration that NADH, instead of NADPH, could serve as electron source for lipid peroxidation.

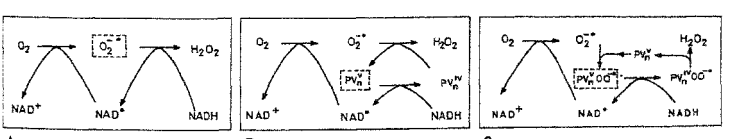

FIG. 1.

Brain mitochondria had been shown to possess the ubiquinone-dependent H_2O_2-generatir capacity. This resolved the ambiguity whether or not mitochondria from this tissue behave simil to other tissues in this respect.

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