PROGRESS REPORT
(January 1990 - June 1992)

Summary

Glu-tRNA^{Glu} is synthesized from glutamate and tRNA^{Glu} by glutamyl-tRNA synthetase (GluRS) (Lapointe and Söll, 1972). Recent work in *Synechocystis 6803* (Schneegurt and Beale, 1988; O'Neill and Söll, 1990a) and *Chlamydomonas reinhardtii* (Jahn *et al.*, 1992a) has demonstrated that Glu-tRNA^{Glu} has dual functions and is a precursor for protein and 5-aminolevulinate (ALA) synthesis. The currently published data do not provide compelling evidence for the notion that GluRS is regulated by chlorophyll precursors or in concert with the other enzymes of ALA synthesis (reviewed in Jahn *et al.*, 1992b). In light of this we have redefined the C5-pathway as a two-step route to ALA starting with Glu-tRNA^{Glu}. Only two enzymes, Glu-tRNA reductase (GluTR) and GSA-2,1-amino-mutase (GSA-AM), are specifically involved in ALA synthesis. We have purified these enzymatic activities from *Chlamydomonas* and demonstrated that the two purified proteins in the presence of their cofactors NADPH and pyridoxal phosphate are sufficient for the *in vitro* Glu-tRNA → ALA conversion (Chen *et al.*, 1990; Jahn *et al.*, 1991a). By complementation of an *Escherichia coli hemA* mutant we have cloned the genes encoding GluTR from *Synechocystis 6803* (Verkamp *et al.*, 1992) and *Arabidopsis thaliana* (M. Kumar, unpublished). The sequences of the GluTR proteins deduced from these genes share highly conserved regions with those of bacterial origin (Verkamp *et al.*, 1992). High-level overexpression of *hemA* genes has so far not yet been accomplished despite efforts to this effect (Verkamp *et al.*, 1992). We have also cloned and

analyzed the gene encoding GSA-AM from *Arabidopsis* (L. Ilag, unpublished). As in *Salmonella typhimurium*, there are indications which suggest the existence of an additional pathway for ALA formation in *E. coli*.

To shed light on the recognition of the single $tRNA^{Glu}$ by the chloroplast enzymes GluTR, GluRS and also by elongation factor EF-Tu, we characterized a chlorophyll-deficient mutant of *Euglena*. This strain contains $tRNA^{Glu}$ with a point mutation in the TΨC-loop. The altered tRNA is produced in lower amount and supports protein, but not ALA synthesis.

Complementation of an *E. coli hemA* mutation also led to the isolation of the *A. thaliana* alternative oxidase gene which endowed *E. coli* with the cyanide-resistant alternative respiration found in plants (Kumar and Söll, 1992). Not only is this an interesting case of heterologous pathway engineering, but the gene's active expression in *E. coli* will allow the unraveling of the many questions surrounding this heretofore rather inaccessible plant enzyme located in the mitochondrial membrane (Moore and Siedow, 1991).

As stated in our last grant application three years ago *"our final goal is the understanding of ALA formation in higher plants."* At that time most of our work was carried out in model systems (*E. coli*, cyanobacteria and *Chlamydomonas*). Since then we have made significant progress, as summarized below, towards our goal. With cloned and characterized *Arabidopsis* genes encoding the enzymes of ALA formation at hand, we are now in a position to study the regulation of this process in higher plants.

This work has led to 14 publications, and three additional papers are in press (see p. 7).

Glutamyl-tRNA reductase (GluTR)

The definitive characterization of the GluTR activity as well as the isolation of clones encoding this protein from *Synechocystis 6803* and *Arabidopsis* was critically dependent on our work in *E. coli*. Thus, a description of our *E. coli* results follows.

Escherichia coli: Purification of GluTR from *E. coli* K12 (Jahn *et al.*, 1991b) revealed the presence of two independent activities with relative molecular weights of approximately 45 (GluTR45) and 85 kDa. The reaction products formed by both enzymes from Glu-tRNA were found identical when analyzed by HPLC. Both enzymes were dependent in their activity on the presence of NADPH and showed clear substrate specificity for homologous Glu-tRNA when tested with heterologous Glu-tRNAs from other species like *C. reinhardtii*, *B. subtilis* and *Synechocystis*. Both enzymatic activities were found to be insensitive to heme. Using *in vivo* complementation of an *E. coli* mutant defective in heme biosynthesis the *E. coli hemA* gene was cloned (for review see O'Neill and Söll, 1990b). This gene encodes a protein of an $M_r \approx 45{,}000$. Although experiments suggested that the gene encodes GluTR activity (Avissar and Beale, 1990), direct proof was lacking. To identify the enzymatic basis of the HemA protein we overexpressed the protein in yeast, which cannot form ALA from $Glu\text{-}tRNA^{Glu}$. We were not able to overexpress the protein directly in *E. coli* using a variety of different systems probably due to its very stringent regulation. The functional analysis of the partially purified protein overexpressed in yeast revealed its GluTR activity. Subsequent gel filtration experiments identified the HemA protein as GluTR45. This is the first direct demonstration of GluTR activity for the HemA protein (Verkamp *et al.*, 1992).

Synechocystis 6803: By complementation of an *E. coli* strain with a disrupted *hemA* gene we cloned its functional analog from the *Synechocystis 6803*. The gene encoded a protein of 427 amino acids (molecular mass 47,525 Da) with significant homology to the known *hemA* genes from other prokaryotes (Verkamp *et al.*, 1992). Rieble and Beale (1991a) isolated from *Synechocystis 6803* the GluTR enzyme which possessed a homo-octameric structure (subunit $M_r \approx 39$ kDa). The amino-terminal sequence of the purified GluTR shows no homology to the known *hemA* sequences or to our *Synechocystis hemA* gene sequence (Verkamp *et al.*, 1992). This discrepancy may suggest the presence of two GluTR enzymes in cyanobacteria.

Arabidopsis thaliana: Over the past three years we spent a great deal of effort in unsuccessful attempts to clone the gene for a plant GluTR. The different approaches involved PCR (based on oligonucleotides corresponding to the conserved amino acid sequences), isolation of a plant enzyme (in order to prepare antibodies) and cross-hybridization with *hemA* genes from other organisms. However, the *in vivo* abundance of GluTR is so low that the final amounts of purified protein were insufficient for antibody preparation. Moreover, the nucleotide sequences of the *hemA* genes are sufficiently different so that hybridization-based techniques were not successful. In our desperation we turned to the method had allowed the isolation of the *Synechocystis* clone (Verkamp *et al.*, 1992), to functional complementation of an ALA-auxotrophic *E. coli hemA* strain with *Arabidopsis* cDNA. In addition to isolating a very interesting *Arabidopsis* gene (see below), Madan Kumar finally isolated an *hemA* homolog of *A. thaliana* recently. Although the characterization of the clone is not yet complete, the cDNA sequence-deduced amino acid sequence of the open reading frame (ORF) shows the characteristic conserved sequences of the known GluTR proteins (Verkamp *et al.*, 1992). Together with the fact that the clone complements *E. coli hemA* mutations these data establish that we have the correct gene.

GSA-2,1-aminomutase (GSA-AM)

Escherichia coli: The gene encoding the *E. coli* enzyme was cloned by complementation of an ALA-auxotrophic *E. coli hemL* strain. The gene was mapped to min 4 of the *E. coli* chromosome and found to be identical with the previously described *popC* locus. Overexpression of the gene gave an easily purified protein whose analysis revealed a dimeric structure of the enzyme. The activity was highly stimulated by the presence of PLP and PAP and inhibited by gabaculine and aminooxyacetic acid; thus the enzyme had characteristics of a classical aminotransferase (Ilag *et al.*, 1991). To learn more about the enzyme mechanism possible intermediates were tested with purified the enzyme and the reaction sequence was followed spectroscopically. 4,5-Dioxovaleric acid and 4,5-diaminovaleric acid were efficiently converted to ALA by either the PAP or PLP form of the enzyme, indicating their role as reaction intermediates. To understand the significance for catalysis of a lysine conserved in all GSA-aminomutase sequences known to date (Grimm *et al.*, 1991), K265 of the *E. coli* enzyme was changed to arginine by oligonucleotide-directed mutagenesis. The activity of mutant K265R GSA-AM enzyme was only 2% of that of the wild-type. The GSA-AM activity was also tested *in vivo* in a strain with a disrupted *hemL* gene. This *E. coli* strain has leaky ALA auxotrophy with glycerol as carbon source, indicating the presence of a compensatory pathway for ALA formation in *E. coli*. The same is observed in an *E. coli* strain with a *hemL* gene disruption (Ilag and Jahn, 1992).

Arabidopsis thaliana: The cDNA was cloned (Larry Ilag, unpublished) by functional complementation of the *E. coli hemL* mutant (Ilag *et al.*, 1991) for auxotrophic growth on glycerol with a plasmid-based *Arabidopsis* cDNA library derived from leaf mRNA. The enzyme shows high homology on the nucleotide and amino acid sequence level to other known GSA-amino-mutases. The cDNA sequence reveals an ORF of 474 amino acids with considerable homology (55-78% identity) to the sequences deduced for other known GSA-AM enzymes from barley (Grimm, 1990), *Synechococcus* and *E. coli* (Grimm *et al.*, 1991). Together with the fact that the clone complements *E. coli hemL* mutations these data establish that we have the correct gene (*GSA*). This is the first instance of this gene from a dicot. Like the other GSA-AM genes the *Arabidopsis* gene contains a transit peptide. The lysine residue which we have shown plays an important role in catalysis in the *E. coli* enzyme (Ilag and Jahn, 1992) is conserved also in the *Arabidopsis* sequence.

The genomic copy of the *Arabidopsis GSA* gene was isolated from a λ-based library. Several clones were found of which one was selected for sequencing. The gene has two short introns and appears to occur as a single copy in the haploid genome as revealed by Southern blot analysis. As there is no published genomic sequence of the GSA-AM from a eukaryote we

cannot say anything regarding the conservation of the position, size, and sequence of the introns for this gene. Mapping of the transcription start site is currently underway.

Analyses of initial Northern blots indicate that the *GSA* transcript is expressed in three week-old *Arabidopsis* plantlets grown in the light. As the gene encodes a chloroplast protein, it was no surprise to find that the gene is expressed in leaves. Interestingly, however, the gene seems to be expressed also in roots.

Currently Albert Crescenzo is engaged in overexpressing active *Arabidopsis* GSA-AM in *E. coli* so that we can prepare antibodies needed for *in vivo* expression studies by Western blots.

Glutamate tRNA ($tRNA^{Glu}$)

Given that tRNA is such a versatile molecule which interacts with many different proteins in a very specific fashion we wanted to shed light on the recognition of the single chloroplast $tRNA^{Glu}$ by the chloroplast proteins GluTR, GluRS and elongation factor EF-Tu. We surmised that it should be possible to isolate $tRNA^{Glu}$ mutants which will still be recognized by GluRS and EF-Tu and thus support protein synthesis, while they could be impaired in ALA synthesis because of loss of recognition by GluTR. Therefore we characterized a chlorophyll-deficient mutants of *Euglena* (in collaboration with Dr. H. Lyman, SUNY Stony Brook). One of the mutant strains contains a chloroplast $tRNA^{Glu}$ with a point mutation in the TΨC-loop. The altered tRNA is produced in near normal amounts and allows protein synthesis, but preliminary experiments show that ALA synthesis is impaired (Nicole Stange and Uli Thomann, unpublished).

Cloning and expression of *Arabidopsis* alternative oxidase

Hemes, important porphyrin derivatives, are essential components of redox enzymes, e.g., cytochromes. Thus, a *hemA E. coli* strain is deficient in cytochrome-mediated aerobic respiration. In our attempts to isolate the *Arabidopsis* GluTR gene by complementation of such a strain with an *A. thaliana* cDNA library we isolated a clone which permitted the *E. coli hemA* strain to grow aerobically. Analysis revealed that the clone encodes the gene for *Arabidopsis* alternative oxidase whose deduced amino acid sequence has 71% identity with that of the enzyme from the voodoo lily *Sauromatum guttatum* (Rhoads and McIntosh, 1991). This enzyme is missing in bacteria, and is much sought after by others (Moore and Siedow, 1991; Rhoads and McIntosh, 1991). The *Arabidopsis* enzyme is expressed as a 30 kDa protein in *E. coli*, and confers to this organism cyanide-resistant growth which in turn, is sensitive to salicyl hydroxamate. Based on these observations we proposed a model for the creation of a new respiratory pathway operative in the transformed *E. coli hemA* strain. The introduction of this pathway now opens the way to new genetic/molecular biological investigations of alternative oxidase and its cofactor. Upon completion of a manuscript on this work (Kumar and Söll, 1992) we have given the clone to Drs. J. Siedow, M. Hanson, R. Gennis and others, since we will not pursue this topic any further.

Publications (March 1990 - June 1992) supported fully or in part by this grant

Chen, M.W., Jahn, D., O'Neill, G. and Söll, D. (1990) Purification of the glutamyl-tRNA reductase from *Chlamydomonas reinhardtii* involved In δ-aminolevulinic acid formation during chlorophyll biosynthesis. *J. Biol. Chem.* 265, 4058-4063.

Chen, M.W., Jahn, D., Schön, A., O'Neill, G.P. and Söll, D. (1990) Purification and characterization of *Chlamydomonas reinhardtii* chloroplast glutamyl-tRNA synthetase, a natural misacylating enzyme. *J. Biol. Chem.* 265, 4054-4057.

Jahn, D., Kim, Y.C., Ishino, Y., Chen, M.W. and Söll, D. (1990) Purification and functional characterization of the Glu-$tRNA^{Gln}$ amidotransferase from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 265, 8059-8064.

O'Neill, G.P. and Söll, D. (1990) Transfer RNA and the formation of the heme and chlorophyll precursor, 5-aminolevulinic acid. *Biofactors* 2, 227-234.

O'Neill, G.P. and Söll, D. (1990) Expression of the *Synechocystis* 6803 $tRNA^{Glu}$ gene provides tRNA for both protein and chlorophyll biosynthesis *J. Bacteriol.* 172, 6363-6371.

O'Neill, G.P., Schön, A., Chow, H., Chen, M.W., Kim, Y.C. and Söll, D. (1990) Sequence of $tRNA^{Glu}$ and its genes from the chloroplast genome of *Chlamydomonas reinhardtii*. *Nucleic Acids Res.* **18**, 5893.

Jahn, D., Chen, M.W. and Söll, D. (1991) Purification and functional characterization of glutamate-1-semialdehyde aminotransferase from *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **266**, 161-167.

O'Neill, G.P., Thorbjarnardóttir, S., Michelsen, U., Pálsson, S., Söll, D. and Eggertsson, G. (1991) Δ-Aminolevulinic acid dehydratase deficiency can cause δ-aminolevulinate auxotrophy in *Escherichia coli*. *J. Bacteriol.* **173**, 94-100.

Jahn, D., Michelsen, U. and Söll, D. (1991) Two glutamyl-tRNA reductase activities in *Escherichia coli*. *J. Biol. Chem.* **266**, 2542-2548.

Ilag, L.L., Jahn, D., Eggertsson, G. and Söll, D. (1991) The *Escherichia coli hemL* gene encodes glutamate 1-semialdehyde aminotransferase. *J. Bacteriol.* **173**, 3408-3413.

O'Neill, G., Jahn, D. and Söll, D. (1991) Transfer RNA involvement in chlorophyll biosynthesis. *In* Subcellular Biochemistry, Vol. 17: Plant Genetic Engineering (Biswas, B.B. and Harris, J.R., eds). Plenum Press, New York. pp. 235-264.

Jahn, D., O'Neill, G.P., Verkamp E. and Söll, D. (1992) Glutamate tRNA: Involvement in protein synthesis and aminolevulinate formation in *C. reinhardtii*. *Plant Physiol. Biochem.* **30**, 245-253.

Verkamp, E., Jahn, M., Jahn, D., Kumar, A.M. and Söll, D. (1992) Glutamyl-tRNA reductase from *E. coli* and *Synechocystis* 6803: gene structure and expression. *J. Biol. Chem.* **267**, 8275-8280.

Jahn, D., Verkamp, E. and Söll, D. (1992) Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. *Trends Biochem. Sci.* **17**, 215-218.

Schön, A., Gough, S. and Söll, D. (1992) Nucleotide sequence and expression of two $tRNA^{Asp}$ species from barley chloroplasts. *Plant Mol. Biol.*, in press

Kumar, A.M. and Söll, D. (1992) *Arabidopsis* Alternative oxidase short-circuits *Escherichia coli* respiration. *Proc. Natl. Acad. Sci. USA*, in press

Ilag, L.L. and Jahn, D. (1992) Activity and spectroscopic properties of the *Escherichia coli* glutamate-1-semialdehyde aminotransferase and the putative active site mutant K265R. *Biochemistry*, in press.

END

DATE
FILMED
7/19/93