Ciba Foundation Symposium 180

THE BIOSYNTHESIS OF THE TETRAPYRROLE PIGMENTS

1994

JOHN WILEY & SONS

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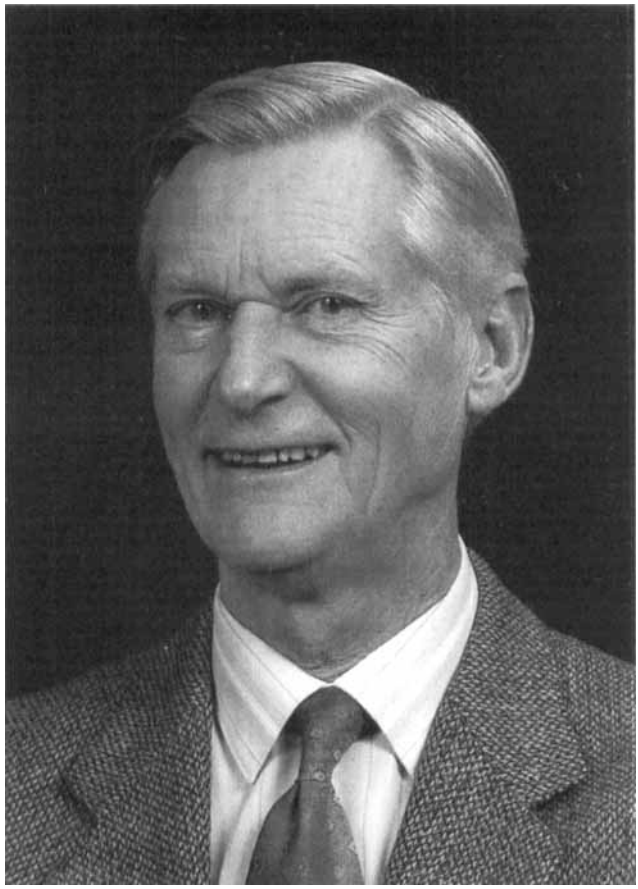

Sir Alan Battersby FRS

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Preface

The Ciba Foundation's 311th symposium (number 180 in the New Series of publications), on the biosynthesis of the tetrapyrrole pigments, was organized in honour of Professor Sir Alan Battersby FRS. Professor Battersby joined the Executive Council of the Ciba Foundation in 1977 but his association with the Foundation predates this by some two years as a member of the Scientific Advisory Panel. He was Chairman of the Council from 1983 to 1990 and became a Trustee in 1993. His seminal contributions to bio-organic chemistry and, in particular, to the elucidation of the biosynthesis of alkaloids and, more recently, vitamin B_{12}, are well known, and his personal distinction has been marked worldwide by a multitude of awards, honorary degrees and other accolades. The Foundation is deeply indebted to him for the support and help he has unstintingly given us during almost 20 years and we offer this book as a public expression of our appreciation and thanks. It is also a testament to an exquisitely successful collaboration between chemists and biologists which is the kind of cooperation that the Ciba Foundation is committed to fostering.

Derek J. Chadwick
Director, The Ciba Foundation

Introduction

Duilio Arigoni

Laboratorium für Organische Chemie, ETH-Zentrum, Universitätstrasse 16, CH-8092 Zürich, Switzerland

I welcome you to this meeting on The Biosynthesis of the Tetrapyrrole Pigments. Albert Eschenmoser and I happen to be two old-timers of the Ciba Foundation, having participated in the symposium on Biosynthesis of Terpenes and Sterols held here in 1958 (Ciba Foundation 1959); as I was reminiscing about the old days, I remembered that during this meeting Konrad Bloch had drawn my attention to the common origin of studies on the biosynthesis of sterols and terpenes and on the biosynthesis of the tetrapyrrole pigments. Everything started in Rittenberg's laboratories when it was found that feeding deuterated acetic acid to rats resulted in time-dependent incorporation of deuterium into fatty acids. It was soon realised that the labelled precursor was a magnificent new tool for biosynthetic investigations and a decision was taken to focus attention on cholesterol and haem. As junior members of Rittenberg's group, David Shemin and Konrad Bloch drew straws to establish who would tackle which area. *Bloch got the cholesterol and Shemin got the haem.*

One may wonder, in retrospect, why Shemin's contribution seemed for quite a while to have less impact on the chemical community than Bloch's work on steroid biosynthesis. Steroids were more fashionable than the tetrapyrrole pigments in the 1950s and this is probably why the discovery of mevalonic acid in Folkers' group and Woodward's new mechanistic interpretation of the old Robinson postulate linking the structures of squalene and cholesterol appealed to the organic chemists more than Shemin's discovery that 5-aminolaevulinic acid and porphobilinogen were intermediates on the pathway to tetrapyrroles. In my opinion, Shemin's work stands out as equally revolutionary and seminal and we would not have been able to gather together here today had he not made his pioneering contributions.

Although Shemin's main interest was in the biosynthesis of haem, his results were later extrapolated to the biosynthesis of chlorophyll, but it was not until the late 1970s that organic chemists started to be attracted to the area. It is fair to say that the structure of vitamin B_{12} was largely responsible for this change in mood. The B_{12} coenzyme had already taught organic chemists several unexpected lessons when it was discovered that it can act as a reservoir of biological radicals playing an important role in hitherto unprecedented biological

reactions. Vitamin B_{12} has been referred to as the Everest of organic synthesis and was soon to play a similar role in biosynthetic investigations. Tackling the problem posed by the biosynthesis of this complex molecule was not an easy task in the early 1970s. The soluble enzyme cocktail responsible for the generation of the compound produced the material in disappointingly low yields and the problem of the degradation of the biosynthetic material was not helped by the scanty knowledge of the compound's chemistry. The development of ^{13}C NMR spectroscopy provided the necessary breakthrough and paved the way for studies by different groups. By the end of the 1970s, these studies had led to the identification of three different intermediates beyond uroporphyrinogen III. In the words of one of the protagonists of such studies, development in this area has been 'agonizingly slow' ever since.

In the mean time, the pathway to uroporphyrinogen III has been refined in remarkable detail and it is now possible to discuss at the molecular level the mode of action of selected enzymes.

A revitalization of the biosynthetic studies has come about in more recent years as a result of the efforts of geneticists and microbiologists who have succeeded in isolating the entire set of genes encoding all the proteins involved in B_{12} biosynthesis. This caused a somewhat paradoxical situation in which there was access to the enzymes but no specific knowledge of the reactions they were catalysing!

This situation is now changing rapidly and there has been spectacular recent progress in identifying long-sought new intermediates. All of this makes it appropriate to refresh the subject last discussed here at the Ciba Foundation in 1955 at the meeting on Porphyrin Biosynthesis and Metabolism (Ciba Foundation 1955), to review the new results, to discuss them critically and, if possible, to outline directions for future research.

It is a pleasure for me to be here as your chairman, and it adds to my pleasure that this meeting is also intended to be a tribute to the unfailing leadership of one of the scientists in this group, my good friend Alan Battersby. I am looking forward to three exciting and rewarding days.

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Enzymic and mechanistic studies on the conversion of glutamate to 5-aminolaevulinate

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Abstract. Higher plants, algae, cyanobacteria and several other photosynthetic and non-photosynthetic bacteria synthesize 5-aminolaevulinate by a $tRNA^{Glu}$-mediated pathway. Glutamate is activated at the α-caboxyl by ligation to $tRNA^{Glu}$ with an aminoacyl-tRNA synthetase. An NADPH-dependent reductase converts glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde, which is finally converted to 5-aminolaevulinate by an aminotransferase. These components are soluble and in plants and algae are located in the chloroplast stroma. In plants and algae the $tRNA^{Glu}$ is encoded in chloroplast DNA whereas the enzymes are encoded in nuclear DNA. The $tRNA^{Glu}$ has a hypermodified 5-methylaminomethyl-2-thiouridine-pseudouridine-C anticodon and probably plays a role in the light–dark regulation of 5-aminolaevulinate synthesis. Ligation of glutamate to $tRNA^{Glu}$ requires ATP and Mg^{2+} and proceeds via a ternary intermediate. Glutamyl-$tRNA^{Glu}$ reduction appears to involve formation of a complex. Glutamate 1-semialdehyde non-enzymically synthesized by reductive ozonolysis from γ-vinyl GABA is used as substrate by the last enzyme. Glutamate-1-semialdehyde aminotransferase contains pyridoxal phosphate as a prosthetic group. The enzyme is converted to spectrally different forms by treatment with 4,5-diaminovalerate or 4,5-dioxovalerate. The pyridoxamine 5′-phosphate form of the enzyme converts (*S*)-glutamate 1-semialdehyde to 5-aminolaevulinate via 4,5-diaminovalerate through a bi-bi ping-pong mechanism.

1994 The biosynthesis of the tetrapyrrole pigments. Wiley, Chichester (Ciba Foundation Symposium 180) p 3–25

Natural tetrapyrrole pigments are chlorophylls, haems, phycobilins (including the phytochrome chromophore) and corrinoids. The pyrrole rings in these pigments are synthesized from 5-aminolaevulinate, a non-protein amino acid produced primarily for this purpose. After studying tetrapyrrole pigments for nearly 20 years, Sir Alan Battersby rated chlorophyll as the fundamental pigment of life and placed haem, the red pigment of blood, in second place (Battersby 1988). Interestingly, 5-aminolaevulinate is biosynthesized in two completely

different ways for these two major tetrapyrrole pigments. Succinyl-CoA and glycine are condensed in a single step catalysed by the enzyme-5-aminolaevulinate synthase (EC 2.3.1.37) to produce 5-aminolaevulinate for biosynthesis of mammalian haem. For the synthesis of chlorophyll in plants, glutamate is converted to 5-aminolaevulinate by a multi-enzyme pathway.

In higher plants, 5-aminolaevulinate synthesis is stimulated by light. Only small amounts of 5-aminolaevulinate are made in darkness to provide for the synthesis of haem and trace amounts of protochlorophyllide, a chlorophyll precursor. Rapid synthesis of chlorophyll occurs when etiolated seedlings are placed in light. Treatment of such greening leaves with laevulinate inhibits chlorophyll synthesis, leading to accumulation of 5-aminolaevulinate. Laevulinate is a competitive inhibitor of 5-aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) and prevents further metabolism of 5-aminolaevulinate. Beale and Castelfranco treated greening etiolated plant tissues with laevulinate and ^{14}C-labelled glutamate, glutamine, α-ketoglutarate, succinate or glycine, and found that the accumulated 5-aminolaevulinate was poorly labelled from glycine or succinate in comparison with ^{14}C incorporation from the five-carbon compounds (Beale & Castelfranco 1974). Using specifically labelled [^{14}C]glutamate together with laevulinate, by analysing the distribution of ^{14}C in the 5-aminolaevulinate, Beale et al (1975) and Meller et al (1975) demonstrated that the entire carbon skeleton of the five-carbon precursor is incorporated intact into 5-aminolaevulinate. The enzymes, cofactors and intermediates involved in the conversion of glutamate to 5-aminolaevulinate have been isolated and studied at the Carlsberg Laboratory (von Wettstein 1991).

Conversion of glutamate to 5-aminolaevulinate involves several interesting reactions (Fig. 1). Glutamate is first activated at the α-carboxyl by ligation to $tRNA^{Glu}$. An aminoacyl-tRNA synthetase catalyses the ligation in the presence of ATP and Mg^{2+}. Activated glutamate is then reduced to glutamate 1-semialdehyde in an NADPH-dependent reaction. Finally, glutamate 1-semialdehyde is converted to 5-aminolaevulinate in a reaction catalysed by the enzyme glutamate-1-semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase, EC 5.4.3.8). All higher plants, algae and cyanobacteria that have been analysed synthesize 5-aminolaevulinate for their tetrapyrrole pigments by the $tRNA^{Glu}$-mediated pathway from glutamate, as do several photosynthetic and non-photosynthetic bacteria.

Preparation of $tRNA^{Glu}$ and the enzymes involved in 5-aminolaevulinate biosynthesis

All the components involved in 5-aminolaevulinate synthesis in greening barley are soluble and localized in the plastid stroma. The 5-aminolaevulinate-synthesizing activity of crude preparations is extremely labile, the enzyme involved in the conversion of glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde

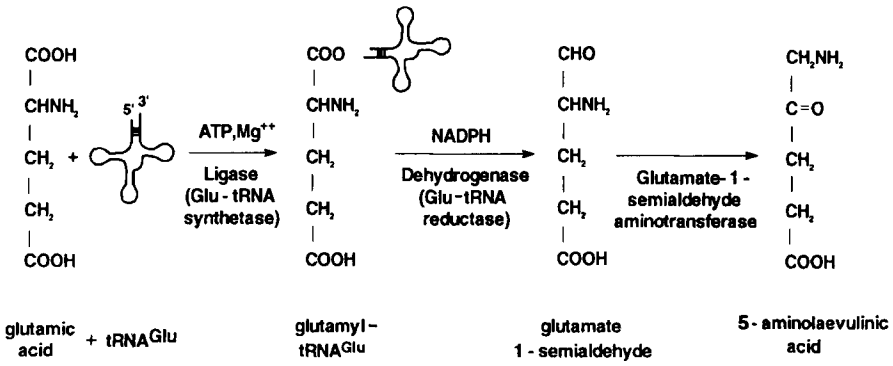

FIG. 1. The pathway of biosynthesis of 5-aminolaevulinic acid from glutamic acid. Glutamic acid combines with $tRNA^{Glu}$ in a ligation reaction catalysed by glutamyl-tRNA synthetase (glutamyl-tRNA ligase, EC 6.1.1.17). The activated glutamate is then reduced to glutamate 1-semialdehyde, which is then converted to 5-aminolaevulinic acid in a reaction catalysed by glutamate-1-semialdehyde aminotransferase (glutamate-1-semi aldehyde 2,1-aminomutase, EC 5.4.3.8).

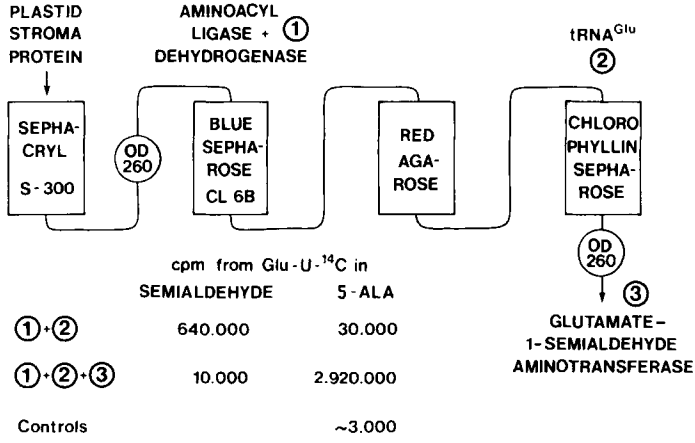

FIG. 2. The enzymes and $tRNA^{Glu}$ required for converting glutamate to 5-aminolaevulinate (5-ALA) in the chloroplast stroma can be partially separated and purified by gel filtration and serial affinity chromatography. (1) Cibacron Blue–Sepharose binds glutamate–$tRNA^{Glu}$ ligase and glutamyl-$tRNA^{Glu}$ reductase (dehydrogenase). (2) Chlorophyllin–Sepharose binds the tRNA complement of the chloroplast. Glutamate-1-semialdehyde aminotransferase is collected in the run-off fraction (3).

being the most unstable component. In greening barley the components are more stable when kept at pH 9–10, rather than at pH 8 where the enzymes show their optimal activities. Rapid partial purification of active preparations by gel filtration and affinity chromatography in glycerol-containing Tricine buffers at pH 9 is a convenient way to obtain the $tRNA^{Glu}$, and the enzymes retain high activities (Fig. 2).

The components required for conversion of glutamate to 5-aminolaevulinate, and subsequently to uroporphyrinogen, are routinely partially purified from the stroma of greening barley plastids by Sephacryl S-300 gel filtration and affinity chromatography with, sequentially, Cibacron Blue–Sepharose, Procion Red–agarose and chlorophyllin–Sepharose or haem–Sepharose (Wang et al 1981). This procedure separates the components into three fractions which must be combined for the conversion of glutamate to 5-aminolaevulinate. Blue Sepharose binds the ligase and the reductase, and Procion Red binds several unwanted proteins. Chlorophyllin or haem Sepharose binds the tRNA complement of the chloroplasts (Kannangara et al 1984). Glutamate-1-semialdehyde aminotransferase is not retained by the affinity columns and is collected in the run-off fraction. When the aminotransferase fraction is omitted from the reconstituted mixture, the product formed is glutamate 1-semialdehyde and not 5-aminolaevulinate.

The role of $tRNA^{Glu}$

The intriguing feature of the conversion of glutamate to 5-aminolaevulinate is the involvement of $tRNA^{Glu}$. Our experiments, and those of others, indicate that all organisms investigated which use glutamate to synthesize 5-aminolaevulinate do so via the $tRNA^{Glu}$-mediated pathway (Avissar et al 1989, Beale & Weinstein 1990, Huang & Wang 1986, Jahn et al 1992, Jordan 1990, Kannangara 1991, Kannangara et al 1988, O'Neill et al 1991). The involvement of $tRNA^{Glu}$ in the biosynthesis of 5-aminolaevulinate has been demonstrated in several ways: (1) ribonuclease A or snake venom phosphodiesterase sensitivity of 5-aminolaevulinate synthesis (see below); (2) reconstitution of 5-aminolaevulinate synthesis by the addition of $tRNA^{Glu}$; (3) conversion of glutamyl-$tRNA^{Glu}$ to 5-aminolaevulinate with purified reductase.

The nucleotide sequences of the tRNAs involved in 5-aminolaevulinate synthesis can be fitted into the clover leaf structure. At their 3′ ends they have the C-C-A sequence characteristic of all tRNAs. The anticodon sequence U-U-C identifies them as glutamate-specific tRNAs. The 3′ C-C-A, as well as the anticodon sequence, is required for the ligation. Removal of the C-C-A by digestion with snake venom phosphodiesterase leads to complete loss of the tRNA's ability to ligate glutamate. Replacing the C-C-A sequence with nucleotidyl transferase (polyribonucleotide nucleotidyltransferase, EC 2.7.7.8) restores this ability (Schön et al 1986).

The first position of the anticodon of barley chloroplast $tRNA^{Glu}$ is occupied by 5-methylaminomethyl-2-thiouridine (Fig. 3). This hypermodified nucleotide can be oxidized under mild conditions with iodine. The oxidized $tRNA^{Glu}$ is unable to function in the ligase reaction but its activity can be restored by reduction with thiosulphate. This reversible oxidative inactivation of $tRNA^{Glu}$ suggests a mechanism for the photostimulation of 5-aminolaevulinate and

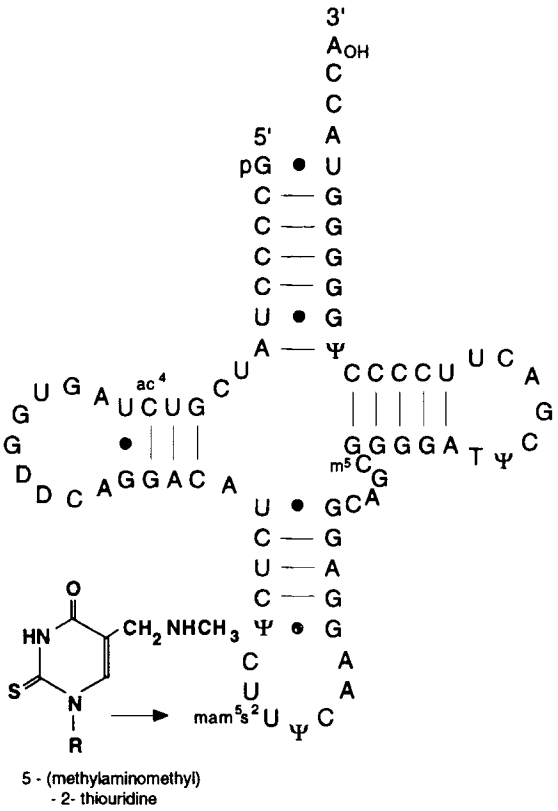

FIG. 3. Nucleotide structure of barley $tRNA^{Glu}$, which participates in 5-aminolaevulinate biosynthesis, and the structure of the hypermodified nucleotide, 5-methylaminomethyl-2-thiouridine, in the first position of its anticodon. ψ, pseudouridine.

protein synthesis observed in higher plants and algae. Oxidation of $tRNA^{Glu}$ markedly curtails 5-aminolaevulinate and protein synthesis in plastids in darkness, but in light the inactive $tRNA^{Glu}$ is rapidly reduced to its active form. The oxidation of $tRNA^{Glu}$ probably involves a conversion of the mercapto group of the 5-methylaminomethyl-2-thiouridine at the anticodon to a disulphide form. The loss of the ability to regulate 5-aminolaevulinate synthesis by light could be lethal to the plant, as it is in some *tigrina* mutants of barley. These mutants overproduce 5-aminolaevulinate in the dark, leading to accumulation of excessive amounts of protochlorophyllide. When these mutants are grown in day and night conditions they develop a primary leaf with alternating bleached/necrotic and green bands, the *tigrina* phenotype, because the regions of leaf that grow and differentiate at night are bleached by light because of the excess of protochlorophyllide whereas those which

grow and differentiate in the day accumulate chlorophyll instead of protochlorophyllide.

In higher plants, the $tRNA^{Glu}$ involved in 5-aminolaevulinate synthesis is encoded by the chloroplast DNA. The barley gene is closely linked to the genes encoding $tRNA^{Tyr}$ and $tRNA^{Asp}$ on a 1.6 kb HindIII-EcoRI restriction fragment. The three genes are transcribed as a single precursor RNA which is processed and modified to give the functional tRNA molecules. The $tRNA^{Glu}$ gene is transcribed both in light and in darkness, but, in cucumber, the levels of expression of this gene and other plastid tRNA genes are higher in greening cotyledons than in etiolated cotyledons (Masuda et al 1992). Application of the hormone benzyladenine stimulates the synthesis of 5-aminolaevulinate in etiolated cucumber cytoledons *in vivo*, apparently through a general increase in production of plastid RNA.

Glutamate–$tRNA^{Glu}$ ligase (glutamyl-tRNA synthetase)

There are two glutamate–tRNA ligases in higher plants and algae, one located in the cytoplasm and the other inside the chloroplast. The latter enzyme is responsible for activating glutamate for 5-aminolaevulinate and plastid protein synthesis. The chloroplast enzyme also catalyses the ligation of glutamate to $tRNA^{Gln}$ for its subsequent conversion to glutamine by amidophosphoribosyltransferase (Schön et al 1988). There must be a mechanism for balancing the needs of protein and chlorophyll synthesis for glutamyl-$tRNA^{Glu}$ to ensure the orderly development of the chloroplast. Glutamate–$tRNA^{Glu}$ ligase probably participates in this regulatory process by forming a multi-enzyme complex which directs the path of glutamyl-$tRNA^{Glu}$.

The barley chloroplast $tRNA^{Glu}$ ligase is encoded in the nuclear DNA. The protein comprises two identical subunits, both synthesized in the cytoplasm, each with a 34 amino acid pre-sequence and molecular mass of 58 kDa. The subunits are then transported into the chloroplast, processed to give the mature subunits of 526 amino acids (molecular mass 54 kDa) and assembled to give the functional enzyme. The barley chloroplast enzyme shows 40% amino acid sequence identity to other known glutamate–$tRNA^{Glu}$ ligases (Andersen 1992).

Aminoacyl-tRNA synthetases ligate their amino acids to their cognate tRNAs in two steps. In the first step, the enzyme catalyses a reaction between the amino acid and Mg^{2+}–ATP to give an aminoacyl-adenylate and pyrophosphate. The aminoacyl moiety is then transferred from aminoacyl-adenylate to either the 2′ or the 3′ OH of the tRNA, and AMP is released. The release of pyrophosphate is not observed with glutamate–$tRNA^{Glu}$ ligase in the absence of $tRNA^{Glu}$, in contrast to most other aminoacyl-tRNA synthetases; the synthetase's mechanism of catalysis is therefore likely to involve the formation of a ternary intermediate, as illustrated in Fig. 4.

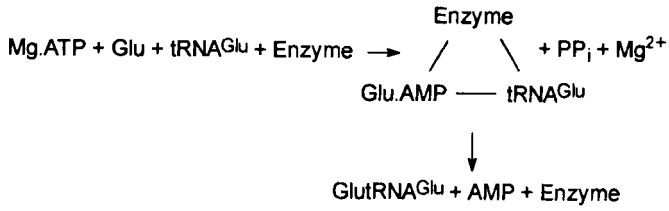

FIG. 4. The mechanism of glutamate–tRNA^{Glu} ligase (EC 6.1.1.17, glutamyl-tRNA synthetase) involves the formation of a ternary complex as an intermediate.

The properties of glutamyl-tRNA^{Glu} reductase

The enzyme responsible for the conversion of glutamyl-tRNA^{Glu} to glutamate 1-semialdehyde appears to differ in subunit composition and molecular mass between different organisms (Jahn et al 1992). A monomeric protein of 130 kDa purified from *Chlamydomonas reinhardtii* forms a stable complex with glutamyl-tRNA^{Glu} and reduces the bound glutamate 1-semialdehyde in the presence of NADPH (Jahn 1992). Two monomeric glutamyl-tRNA^{Glu} reductases have been found in *Escherichia coli*, with molecular masses of 45 kDa and 85 kDa. The glutamyl-tRNA^{Glu} reductase purified from *Synechocystis* 6803 is a multimeric protein of 350 kDa composed of 39 kDa subunits. An oligomeric glutamyl-tRNA^{Glu} reductase made up of 50 kDa subunits has also been indicated in *Bacillus subtilis*. The enzyme in greening barley appears to be a dimer of two identical 60 kDa subunits and carries a 450 nm light-absorbing chromophore. Glutamyl-tRNA^{Glu} is unstable at neutral pH, which makes some of its kinetic parameters difficult to measure. Haem, at 3 μM and above, inhibits the activity of glutamyl-tRNA^{Glu} reductase purified to homogeneity from *Synechocystis*. In *E. coli* cell extracts glutamyl-tRNA^{Glu} reductase activity is inhibited by haem, but both *E. coli* enzymes are insensitive to haem when purified (cf. Jahn et al 1992). Other factors are probably required for haem to inhibit the *E. coli* reductases. Partially purified enzyme preparations from greening barley convert over 90% of the added glutamate into glutamate 1-semialdehyde in coupled assays with the ligase, tRNA^{Glu}, NADPH, ATP, GTP and Mg^{2+} (Kannangara et al 1988). Increasing concentrations of haem, up to 50 μM, progressively inhibit glutamate 1-semialdehyde formation in the coupled assay, and glutamyl-tRNA^{Glu} accumulates in the reaction mixture. Haem is therefore thought to be a feedback regulator of chlorophyll biosynthesis.

The *hemA* gene product—glutamyl-tRNA^{Glu} reductase?

The *E. coli* K12 strain, which has mutations in the *hemA* gene, requires 5-aminolaevulinate for growth. *hemA* genes have been cloned and sequenced from several bacteria and higher plants and these genes complement the *E. coli hemA*

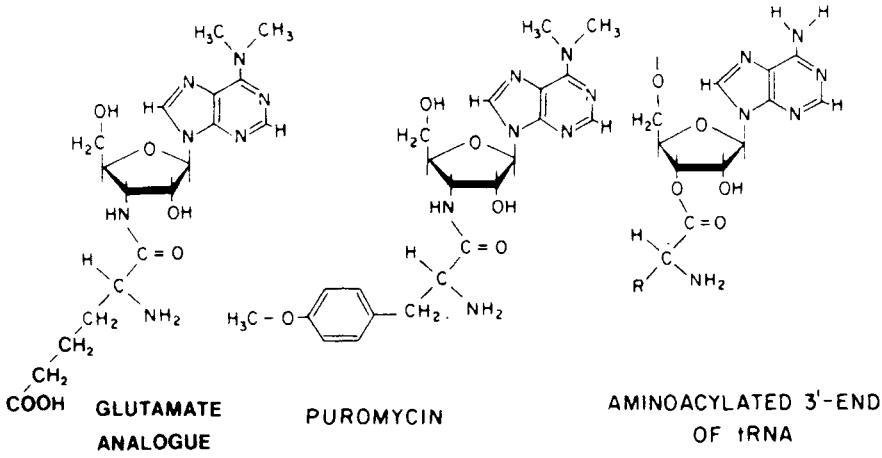

FIG. 5. Puromycin and its glutamate analogue resemble the 3′ end of an aminoacylated tRNA.

mutation. Furthermore, *E. coli* cells overproduce uroporphyrinogen when they are engineered to overexpress the *hemA* gene. Cell-free extracts from such transformed *E. coli* cells have a significantly higher capacity to convert glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde than those from untransformed cells. However, it has not been possible to purify the proteins to demonstrate that reductase activity is associated with the overexpressed *hemA* protein. The *hemA* gene and the recently identified *hemM* gene (Murakami et al 1993) probably encode the proteins required for glutamyl-$tRNA^{Glu}$ reduction.

Mechanism of conversion of glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde

The conversion of glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde probably occurs on a protein complex (Jahn 1992, Kannangara et al 1988). Glutamyl-$tRNA^{Glu}$ forms stable complexes in the presence of GTP, glutamate–$tRNA^{Glu}$ ligase and the proteins involved in glutamyl-$tRNA^{Glu}$ reduction. A somewhat similar complex is formed during the initial step of polypeptide chain elongation, where GTP, the elongation factor EF-Tu and aminoacylated tRNA combine prior to participation of the ribosome. Puromycin, which inhibits protein synthesis and causes premature termination of polypeptide chain synthesis, and the glutamate analogue of puromycin resemble the 3′ end of an aminoacylated tRNA (Fig. 5). The glutamate analogue inhibits the conversion of glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde, whereas puromycin itself has no effect. On the basis of this and other observations, a likely mechanism is nucleophilic attack by the α-amino group of glutamate on the esterified carboxyl carbon of glutamyl-$tRNA^{Glu}$ to give azeridinone propionic acid which is then reduced and hydrolysed to glutamate 1-semialdehyde (Fig. 6).

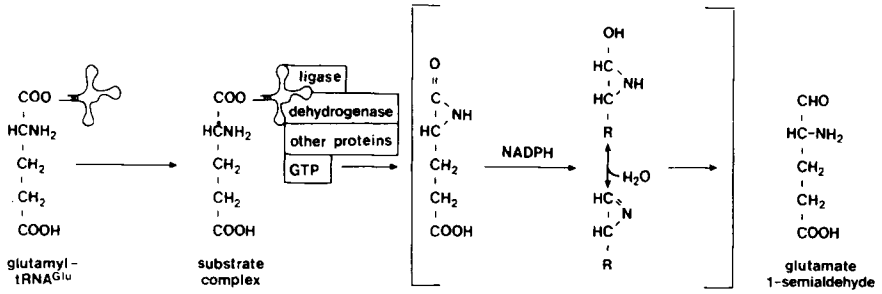

FIG. 6. The conversion of glutamyl-tRNA^{Glu} to glutamate 1-semialdehyde probably involves the formation of a substrate–enzyme complex and proceeds in several steps.

Glutamate-1-semialdehyde aminotransferase

Glutamate-1-semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase, EC 5.4.3.8) is an abundant protein in the stroma of greening barley chloroplasts. It is encoded by nuclear DNA and has a molecular mass of 92 kDa. It is made up of two identical subunits, each of which carries a pyridoxamine 5′-phosphate cofactor. Glutamate-1-semialdehyde aminotransferase catalyses the last step of the C_5 pathway of 5-aminolaevulinate biosynthesis. Studies of this enzyme have been facilitated by the chemical synthesis of glutamate 1-semialdehyde.

Chemical synthesis of glutamate 1-semialdehyde

Three methods are available for synthesis of glutamate 1-semialdehyde. Two of these methods use *N*-carbobenzoxy-L-glutamate γ-benzyl ester as starting material; these two methods are quite cumbersome and give poor yields (5–10%) of (*S*)-glutamate 1-semialdehyde. The most convenient method starts from 4-amino-5-hexenoic acid (Gough et al 1989), also called γ-vinyl GABA, which is available as a mixture of *R* and *S* enantiomers from Merrell Dow (Cincinnati, USA). An aqueous solution containing equimolar amounts of 4-amino-5-hexenoic acid and HCl is treated with ozone to give the ozonide (Fig. 7), which is then reduced by passage through a Dowex 50X8 column in its H^+ form. This method gives pure (*RS*)-glutamate 1-semialdehyde in yields of up to 90%.

Glutamate 1-semialdehyde hydrochloride is stable, as a solid. We at the Carlsberg Laboratory, and Peter Jordan and co-workers at the University of London, have analysed the structure of glutamate 1-semialdehyde by NMR and infrared spectroscopy (Gough et al 1989, Hoober et al 1988, Jordan 1990). The two groups obtained identical spectra but interpreted them differently. Our interpretation is that glutamate 1-semialdehyde is a linear molecule existing as

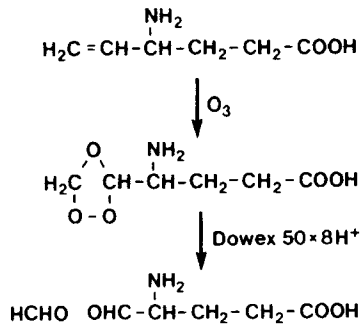

FIG. 7. Chemical synthesis of glutamate 1-semialdehyde by reductive ozonolysis of γ-vinyl-γ-aminobutyric acid (γ-vinyl GABA, 4-amino-5-hexenoic acid).

a hydrate in solution. Peter Jordan's group, however, think that the spectra represent a cyclic molecule, hydroxyaminotetrahydropyranone. Stock solutions (50 to 100 mM) of glutamate 1-semialdehyde when neutralized turn yellow/brown owing to the formation of the dihydropyrazine and pyrazine (Fig. 8). A measurable amount of glutamate 1-semialdehyde is converted spontaneously to 5-aminolaevulinate at pH 8.0. Titration of (*RS*)-glutamate 1-semialdehyde hydrochloride with NaOH indicates the presence of a free carboxyl group (Fig. 9). Glutamate 1-semialdehyde condenses with ethyl acetoacetate or acetylacetone to give a pyrrole (Fig. 10). A vitamin B_6 enzyme converts glutamate 1-semialdehyde to 5-aminolaevulinate. These properties lead us to conclude that glutamate 1-semialdehyde has a linear structure and to discount the cyclic hydroxyaminotetrahydropyranone as a substrate for glutamate 1-semialdehyde aminotransferase.

Properties of glutamate-1-semialdehyde aminotransferase

Glutamate-1-semialdehyde aminotransferase has an exceptionally high affinity for (*S*)-glutamate 1-semialdehyde ($K_m = 12\,\mu M$). The enzyme is irreversibly inhibited by gabaculine (3-amino-2,3-dihydrobenzoic acid), methylgabaculine, 2-hydroxy-3-amino-3,5-cyclohexadiene-1-carboxylic acid, 4-amino-5-hexynoic acid (acetylenic GABA), 4-amino-5-fluoropentanoic acid and glutamic acid 5-monohydroxamate (Gough et al 1992). These compounds inhibit chlorophyll synthesis in greening barley leaves causing accumulation of glutamate 1-semialdehyde. A significant reduction in the enzyme activity and a parallel reduction in chlorophyll accumulation are observed in transgenic tobacco plants carrying a DNA sequence antisense to the glutamate-1-semialdehyde aminotransferase gene.

The structural gene for glutamate-1-semialdehyde aminotransferase has been isolated and characterized from barley (Grimm 1990), *E. coli*, *B. subtilis*,