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BARLEY GLUTAMYL tRNA^{Glu} REDUCTASE: MUTATIONS AFFECTING HAEM INHIBITION AND ENZYME ACTIVITY*

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Key Word Index—*Hordeum vulgare*; Gramineae; Poaceae; barley, 5-aminolevulinic acid; chlorophyll; haem; biosynthesis.

Abstract—Glutamyl tRNA^{Glu} reductase converts glutamate molecules that are ligated at their α-carboxyl groups to tRNA^{Glu} into glutamate 1-semialdehyde, an intermediate in the synthesis of 5-aminolevulinate, chlorophyll and haem. The mature plant enzymes contain a highly conserved extension of 31–34 amino acids at the N-terminus not present in bacterial enzymes. It is shown that barley glutamyl tRNA^{Glu} reductases with a deletion of the 30 N-terminal amino acids have the same high specific activity as the untruncated enzymes, but are highly resistant to feed-back inhibition by haem. This peptide domain thus interacts directly or indirectly with haem and the toxicity of the 30 amino acid peptide for *Escherichia coli* experienced in mutant rescue and overexpression experiments can be explained by extensive haem removal from the metabolic pools that cannot be tolerated by the cell. Induced missense mutations identify nine amino acids in the 451 residue long C-terminal part of the barley glutamyl tRNA^{Glu} reductase which upon substitution curtail drastically, but do not eliminate entirely the catalytic activity of the enzyme. These amino acids are thus important for the catalytic reaction or tRNA binding.

INTRODUCTION

Utilizing tRNA^{Glu} as a cofactor, higher plants, algae, cyanobacteria and several other bacteria synthesise 5-aminolevulinic acid from glutamic acid as an intermediate in the biosynthesis of chlorophyll and other natural tetrapyrroles [1, 2]. In these organisms, the enzyme glutamyl tRNA^{Glu} reductase, in the presence of NADPH, reduces glutamate molecules that are activated at their α-carboxyl groups by ligation to the 3′ OH of tRNA^{Glu}. Glutamate 1-semialdehyde is the product of this enzyme and it is subsequently converted into 5-aminolevulinate via 4,5-diaminovalerate by an aminotransferase.

Aminoacylated tRNAs are normally used for protein biosynthesis where they serve as substrates in the transfer of amino acids to develop the polypeptide chains following the codons of a given mRNA. The reaction catalysed by the glutamyl tRNA^{Glu} reductase is the only known example of a reduction of an aminoacylated tRNA to an α-aminoaldehyde. Control of chlorophyll and haem biosynthesis is thought to occur at the level of glutamyl-tRNA^{Glu} reductase by haem feedback inhibition [1]. Genes encoding glutamyl tRNA^{Glu} reductase have been obtained from several organisms and recombinant proteins prepared. By complementation of *hem A* mutants, the genes encoding glutamyl tRNA^{Glu} reductases have been isolated and sequenced from *Escherichia coli* [3, 4], *Bacillus subtilis* [5], *Synechocystis* PC6803 [6, 7], *Salmonella typhimurium* [8], *Chlorobium vibrioforme* [9, 10], *Pseudomonas aeruginosa* [11], *Xanthomonas campestris* pv phaseoli [12], *Coxiella burnetii* (GeneBank accession no. X78969), *Clostridium josui* [13], and *Arabidopsis thaliana* [14]. By screening cDNA libraries *Hem A* genes have been isolated and characterized from cucumber (GeneBank accession no. D67088) and barley [15]. Two *Hem A* genes, one expressed in leaves and the other in the root and flowers are found in *Arabidopsis* [16], cucumber [17] and barley [15]. These genes show a high sequence identity at the deduced amino acid level. The three mature plant enzymes are larger than the bacterial enzymes and on alignment show a highly conserved extension of 31–34 amino acids at the N-terminus (cf. Fig. 4). This

* This paper is dedicated to Professor Clarence (Bud) A. Ryan at the occasion of his 65th birthday in appreciation of his success in telling us how plants sense their enemies.

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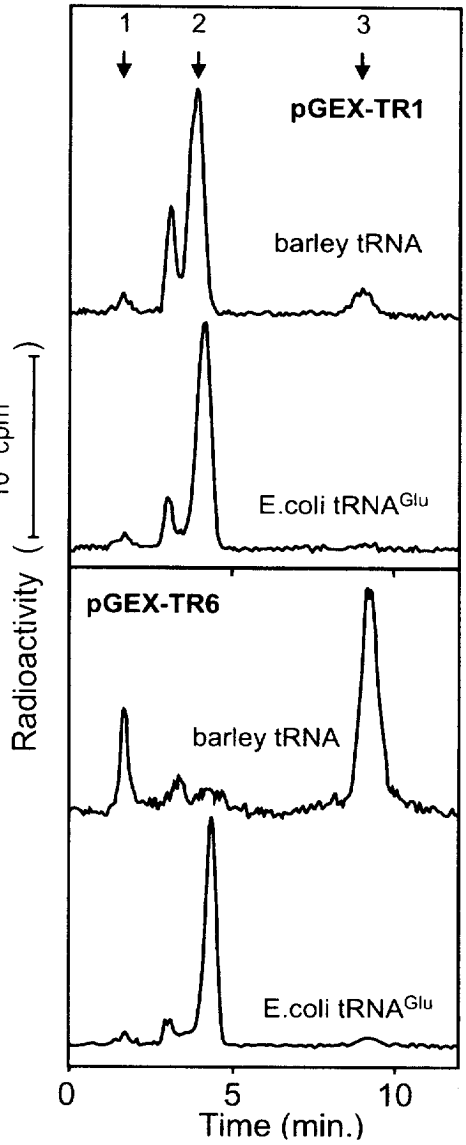

Fig. 1. Enzyme assays with purified GST fusion proteins harbouring truncated barley glutamyl tRNA^{Glu} reductase. (Top) Assays performed with the fusion protein expressed from plasmid pGEX-TR1 using barley chloroplast tRNA and *E. coli* tRNA^{Glu}. (Bottom). Corresponding assays with the fusion protein expressed from pGEX-TR6. Arrows point to the R_fs of authentic glutamyl tRNA^{Glu} (1), glutamic acid (2) and glutamate 1-semialdehyde (3).

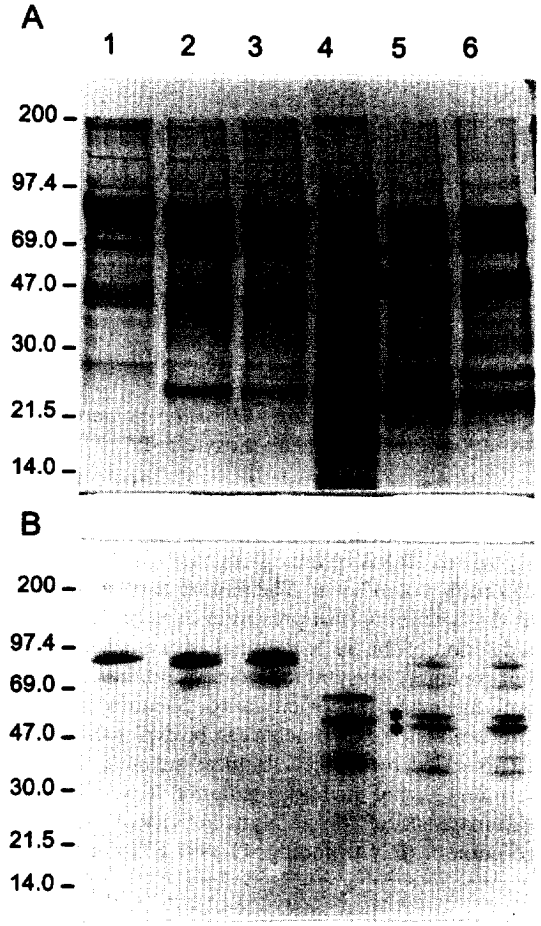

Fig. 2. Peptide mapping of thrombin digested purified GST fusion proteins carrying the TR 6 and TR9 truncated and UV4 non-truncated glutamyl tRNA^{Glu} reductases. The peptides are monitored by SDS-PAGE (A) and Western blot analysis using an antibody against the reductase (B). The undigested fusion proteins contain a full-length reductase (lanes 1) or truncated versions with low (TR9, lanes 2), respectively, high enzymatic activity (TR6, lanes 3). Thrombin digestion cleaves in an internal site of the barley glutamyl tRNA^{Glu} reductase and accordingly yields, from all 3 fusion proteins a 52 kDa peptide from the C-terminal part of the protein (lower asterisk, lanes 4, 5, 6). The full-length reductase is seen as a 60 kDa band (UV4, lane 4), whereas both truncated reductases migrate with a molecular mass of 55 kDa (upper asterisk, lane 5 = TR9 and lane 6 = TR6). The intense band at 70 kDa in lanes 1–3 of Fig. 2(A) is the *E. coli* heat shock protein dnaK which co-purifies with the recombinant protein during glutathione-sepharose affinity chromatography.

extension is not a part of the transit peptide for transport into chloroplasts, which has been identified as a further extension of 43 amino acid residues by cDNA sequencing [15] and N-terminal sequencing of the mature enzyme purified from barley [18]. The catalytically active barley glutamyl tRNA^{Glu} reductase, whether isolated from chloroplasts or produced as a fusion protein with *Schistosoma japonicum* glutathione S-transferase (GST) is inhibited by haem and utilises glutamate activated by barley chloroplast tRNA^{Glu} preferentially to that activated by *E. coli* tRNA^{Glu} [18, 19]. The *E. coli hem A* mutant remains auxotrophic for 5-aminolevulinate, when the full length mature enzyme protein is expressed in its cells, but it is rescued by complementation with a truncated form of the barley glutamyl tRNA^{Glu} reductase lacking 19 amino terminal amino acids [15]. In order to elucidate the function of the amino terminal extension of the higher plant enzymes and the apparent toxicity of this peptide for *E. coli*, we have expressed 7 truncated barley glutamyl tRNA^{Glu} reductases with a

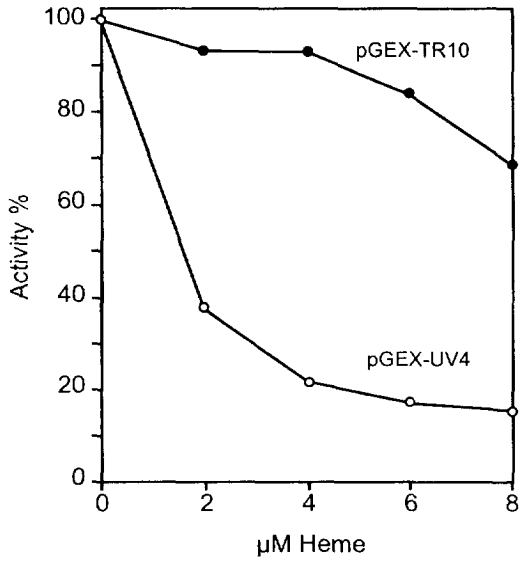

Fig. 3. Differential effects of haem on the GST fusion proteins harbouring truncated and non-truncated barley glutamyl tRNA^{Glu} reductases. Assays contain 3.5 μg of either the truncated fusion protein TR10 (.) or the fusion protein with non-truncated reductase UV4 (o).

deletion of 30 N-terminal amino acids as glutathion S-transferase fusion proteins. The truncated form turned out to be resistant to haem inhibition. Additionally the effect on specific enzyme activity was analysed for several amino acid substitutions in the part of the molecule with shared conserved domains among the higher plant and bacterial enzymes.

RESULTS

Isolation and expression of truncated barley glutamyl tRNA^{Glu} reductase cDNAs

A DNA fragment of expected size was obtained, when PCR was performed on a barley leaf cDNA library using synthetic primers based on the N-terminal amino acid sequence of the mature protein [19] and a sequence internal in the *hem A* gene, indicating that the cDNA for a full-length barley glutamyl tRNA^{Glu} reductase is present in the library. As detailed under 'Experimental', complementation of the *E. coli hem A* mutant AN344 with plasmids of the library yielded 18 clones containing the same insert with identity to the barley *Hem A* gene sequence [15] except for deletion of 30 codons for the N-terminal domain of the mature protein. Since the truncated *Hem A* cDNA was able to convert the 5-aminolevulinate auxotrophy of the *E. coli hem A* mutant to prototrophy, the protein produced by it must be catalytically active utilising glutamate ligated to *E. coli* tRNA^{Glu}.

Accordingly the truncated barley *Hem A* gene was amplified and inserted into the vector pGEX-2T yielding the expression plasmid pGEX-TR, in which the truncated *Hem A* gene was joined at its 5'end to the GST gene with the linker containing a thrombin cleavage site [19]. *E. coli* cells were transformed with the plasmid. Seven transformants contained the plasmid with an insert and produced high amounts of protein that immunoreacted with the glutamyl tRNA^{Glu} reductase specific antibody, when grown and induced with isopropyl β-D-thiogalactoside (IPTG). Truncated proteins were prepared from them in large scale and purified. The expression plasmids in the seven transformants are designated pGEX-TRI, pGEX-TR5, pGEX-TR6, pGEX-TR7, pGEX-TR9, pGEX-TR10, and pGEX-TR-11. All plasmids were isolated and their inserts sequenced. The gene inserts were with 1362 bp of the same size and started with the 5' GAA AAG AGT AGC ATC sequence, i.e. with codon 31 of the open reading frame for the mature enzyme. Four of the inserts had base changes expected to translate into amino acid substitutions.

Properties of the truncated GST-glutamyl tRNA^{Glu} reductase fusion proteins

The truncated glutamyl tRNA^{Glu} reductase fusion proteins isolated from the seven transformants showed differences in enzymatic activity when tested with both barley chloroplast tRNA and *E. coli* tRNA^{Glu} in the reconstitution assay (Fig. 1). The specific activities of three of the truncated fusion proteins produced by plasmids pGex-TR6, pGEX-TR7 and pGEX-TR10 varied from 1.6 to 2.4 nkat mg^{-1} and are considered not significantly different from the specific activity of the fusion protein with the non-truncated glutamyl tRNA^{Glu} reductase (2 nkat mg^{-1}). Like the non-truncated enzyme they also had very low but measurable activities with *E. coli* tRNA^{Glu} in the assays (Table 1). The fusion proteins expressed by pGEX-TR1, pGEX-TR11, pGEX-TR9 and pGEX-TR-5 produced glutamate 1-semialdehyde at greatly reduced rates (0.02 to 0.19 nkat mg^{-1}) with barley chloroplast tRNA and showed no detectable activity with *E. coli* tRNA^{Glu}.

In order to determine if the highly active and weakly active truncated fusion proteins were of the same size, they were peptide mapped by thrombin cleavage. As is exemplified for the feeble TR9 and the active TR6 fusion proteins in Fig. 2, they had a slightly smaller molecular mass than the full-length glutamyl tRNA^{Glu}-GST fusion protein (Fig. 2 lanes 2, 3 *vs* 1). Thrombin cleavage of both fusion proteins with the truncated reductase component yielded a 52 kDa peptide with the determined N-terminal amino acid sequence A_{64}-I-S-E-L-T-S-L_{71}. It results from the internal thrombin cleavage site in the barley glutamyl tRNA^{Glu} reductase and is also obtained by cleavage of the full-length reductase-GST fusion protein (Fig. 2 lanes 4, 5, 6). The polypeptides with an apparent molecular mass of 55 kDa are the truncated reductases (Fig. 2 lanes 5, 6), while the full-length reductase has the expected 60 kDa molecular mass (Fig. 2 lane 4). It is concluded that the enzymatically highly and weakly active trunc-

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                                                ↓
Hvu  .DAGGDAQAA SKAASITALE QFKISA.DRY MKEKSSIAVI GLSVHTAPVE MREKLAVAEE LWPRAISELT
Csa  QVDEIDPPKS SN...LSALE QLKTSAVDRY TKERSSIVVI GLSIHTTPVE MREKLAIPEA EWPRAIGELC
Ath  CVLSASSDSA SNAASISALE QLKNSAADRY TKERSSIVVI GLSIHTAPVE MREKLAIPEA EWPRAIAELC
Syn  .......... .......... .......... ....MNIAVV GLSHKTAPVE IREKLSIQEA KLEEALTHLR
Eco  .......... .......... .......... ....MTLLAL GINHKTAPVS LRERVSFSPD KLDQALDSLL
Bsu  .......... .......... .......... ....MHILVV GVDYKSAPIE IREKVSFQPN ELAEAMVQLK
Cvi  .......... .......... .......... ....MNIISV GVNHKTAPIE IRERIALSEV QNKEFVTDLV

                                                                    K122
                                                                     ↑
Hvu  SLNHIEEAAV LSTCNRMEIY VVALSWNRGI REVVDWMSKK SGIP.ASELR EHLFMLRDSD ATRHLFEVSA
Csa  GLNHIEEAAV LSTCNRMEIY VVALSQHRGV KEVTEWMSKT SGIP.VSEIC QHRFLLYNND ATQHIFEVSA
Ath  GLNHIEEAAV LSTCNRMEIY VLALSQHRGV KEVTEWMSNT SGIP.VSEIC QHRFLLYNKD ATQHIFEVSA
Syn  SYPHIEEVTV ISTCNRLEIY AVVTDTEKGV VEITQFLSET GNIP.LATLR RYLFTLLHED AVRHLMRVAA
Eco  AQPMVQGGVV LSTCNRTELY LSVEEQDNLQ EALIRWLCDY HNLN.EEDLR KSLYWHQDND AVSHLMRVAS
Bsu  EEKSILENII VSTCNRTEIY AVVDQLHTGR YYIKKFLADW FQLS.KEELS PFLTFYESDA AVEHLFRVAC
Cvi  SSGLASEAMV VSTCNRTELY VVPGMPEVNC DYLKDYIISY KDAR.NAVRP EHFFNRFYCG TARHLFEVSS

                    N154
                     ↑
Hvu  GLDSLVLGEG QILAQVKQVV RNGQNSGGLG KNIDRMFKDA ITAGKRVRCE TNISAGAVSV SSAAVELAMM
Csa  GLDSLVLGEG QILAQVKQVV KVGQGVAGFG RNISGLFKHA ITVGKRVRTE TNIAAGAVSV SSAAVELALM
Ath  LGDSLVLGEG QILAQVKQVV KVGQGVNGFG RNISGLFKHA ITVGKRVRTE TNIASGAVSV SSAAVELALM
Syn  GLESLVLGEG QILAQVRTTH KLGQKYKGVG RLLDRLFKQA ITAGRRVRTE TDIGTGAVSI SSAAVELVHR
Eco  LGDSLVLGEP QILGQVKKAF ADSQKGHMKA SELERMFQKS FSVAKRVRTE TDIGASAVSV AFAACTLARQ
Bsu  GLDSMVIGET QILGQVRDSF KTAQQEKTIG TIFNELFKQA VTVGKRTHAE TDIGSNAVSV SYAAVELAKK
Cvi  AIDSLVLGEG QILGQVKDAY RIAAEVGTAG ILLTRLCHSP FSVAKKVKTR TKLMEGAVSV SYAAVELAQK

Hvu  KLPKSECLSA RMLLIGAGKM GKLVVKHLIA KGCKKVVVVN RSVERVDAIR EEMKDIEIV. .YRPLTEMYE
Csa  KLPEPSHATA RMLVIGAGKM GKLVIKHLVA KGCTKMVVVN RSEERVTAIR EEMKGVEII. .YKPLTEMLS
Ath  KLPQSSNVSA RMCVIGAGKM GLLVIKHLMA KGCTKVVVVN RSEERVSAIR EEMPGIEII. .YRPLDEMLA
Syn  QVDLS...SQ KTVIIGAGKM ACLLVKHLLA KGATDITIVN RSQRRSQDLA NQFPQAQLT. .LCPLTDMFT
Eco  IFESLS..TV TVLLVGAGET IELVARHLRE HKVQKMIIAN RTRERAQILA DEVG.....A EVIALSDIDE
Bsu  IFGNLS..SK HILILGAGKM GELAAENLHG QGIGKVTVIN RTYLKAKELA DRFS.....G EARSLNQLES
Cvi  IFSNLSM..K KVLLIGAVKQ S......WQQ STCTPRTPGT SSSPTGRNPR PRACEELGTN RVLPYESYKE

                                  S302                  L318 G322
                                   ↑                     ↑   ↑
Hvu  AAADADVVFT STASESLLFT KEHAEALPPI SLAMGGVR.. LFVDISVPRN VGACLSEVEH ARVYNVDDLK
Csa  CTAEADVIFT STASESLLFT KEQVKDLPPV GHDVGGLR.. LFIDISVPRN VGACINNLED VRVYNVDDLK
Ath  CASEADVVFT STASETPLFL KEHVENLPQA SPEVGGLR.. HFVDISVPRN VGSCVGEVET ARVYNVDDLK
Syn  AIAAGDIVFT STGATEPIL. ..NCENLTGC VINRKSL... MLVDISVPRN VAADVHAMEQ VRAFNVDDLK
Eco  RLREADIIIS STASPLPIIG KGMVERALKS RRN....QPM LLVDIAVPRD VEPEVGKLAN AYLYSVDDLQ
Bsu  ALAEADILIS STGASEFVVS KEMMENANKL RKG....RPL FMVDIAVPRD LDPALNDLEG VFLYDIDDLE
Cvi  HLHEFDIIIT AVSTKEYILN AAEMQQSMAK RR....LKPV IILDLGLPRN VDPEVGALQN MFLKDIDALK

                                  L371                  H387          K400
                                   ↑                     ↑             ↑
Hvu  EVVEANKEDR VRKAMEAQTI ITQELKRFEA .......WRD SLETVPTIKK LRSYAD.... RIRASELEKC
Csa  EVVAANKEDR LRKAMEAQSI ITEESKQFEA .......WRD SLETVPTIKK LRAYAE.... RIRTAELEKC
Ath  EVVAANKEDR MRKAMEAQTI ITEESTQFEA .......WRD SLETVPTIKK LRAYAE.... RIRVAELEKC
Syn  EVVAQNQASR RQMARQAEAL LEEEIAAFDL .......WWR SLETVPTISS LRSKVE.... DIREQELEKA
Eco  SIISHNLAQR KAAAVEAETI VAQETSEFMA .......WLR AQSASETIRE YRSQAE.... QVRDELTAKA
Bsu  GIVEANMKER RETAEKVELL IEETIVEFKQ .......WMN TLGVVPVISA LREKAL.... AIQSETMDSI
Cvi  HIIDKNLERR RAELPKVKSI IDEELIASAS GSTPSRYVRP SLTCNPSSSK SRRKNS.... SVPPQGERRG

                                                                D454    P464
                                                                 ↑       ↑
Hvu  LQKIGEDNLN KKMRRSIEEL STGIVNKLLH GPLQHLRCDG SDSRTLDETL ENMHALVRIF SLDTEKAVLE
Csa  LSKMGDD.IP KKTRRAVDDL SRGIVNKLLH GPMQHLRCDG SDSRTLSETL ENMHALNRMF SLETEIAVE.
Ath  MSKMGDD.IN KKTTRAVDDL SRGIVNRFLH GPMQHLRCDG SDSRTLSETL ENMHALNRMY GLEKDILEEK
Syn  LSRLGSE.FA KHQEVIEAL. TRGIVNKILH EPMVQLRAQ. ...QDIEARK QCLRSLKMLF DLEVEEQFGE
Eco  LAAL..EQ.G GDAQAIMQDL AWKLTNRLIH APTKSLQQAA RDGDNERLN. ...IL...RD SLGLE.....
Bsu  ERKL..PHLS TREKKLLNKH TKSIINQMLR DPILKVKELA ADADSEEKLA LFMQIFDIEE AAGRQMMKTV
Cvi  VEAHGTPDRQ DPEKNPASSY QDAQGS.... GRYRRQHPQQ SQPRQEHLRS .......... ..........

Hvu  QKIKAKVEKT QS
Csa  QKIRAKVEQN QK
Ath  LKAMAEQQHK ..
Syn  .......... ..
Eco  .......... ..
Bsu  ESSQKVHSFK KA
Cvi  .......... ..
```

Fig. 4. Location of the amino acid substitutions in the mutant truncated glutamyl $tRNA^{Glu}$ reductases. The primary structure of the enzymes deduced from the *hem A* gene sequences of barley (Hvu), cucumber (Csa), *Arabidopsis* (Ath), *Synechocystis* (Syn), *E. coli* (Eco), *B. subtilis* (Bsu), and *Chlorobium vibrioforme* (Cvi) were aligned. The arrow points to the N-terminus of the truncated reductases and the amino acid substitutions are given in bold above the barley sequence.

Table 1. Deduced amino acid substitutions and glutamyl tRNA^{Glu} reductase activities of truncated GST-glutamyl tRNA^{Glu} reductase fusion proteins

Expression plasmid	Amino acid substitution*	Activity with barley chloroplast tRNA†	Activity with *E. coli* tRNA^{Glu}†
pGEX-UV4	none	2.0	0.02
pGEX-TR6	none	1.98	0.01
pGEX-TR7	none	1.63	0.01
pGEX-TR10	none	2.43	0.02
pGEX-TR1	$I_{464} \rightarrow P$	0.02	nd
pGEX-TR11	$L_{387} \rightarrow H$, $L_{302} \rightarrow S$	0.19	nd
pGEX-TR9	$I_{318} \rightarrow L$, $R_{322} \rightarrow G$, $N_{454} \rightarrow D$	0.1	nd
pGEX-TR5	$M_{122} \rightarrow K$, $K_{154} \rightarrow N$, $F_{371} \rightarrow L$, $E_{400} \rightarrow K$	0.08	nd

The non-truncated barley glutamyl tRNA^{Glu} reductase was expressed from pGEX-UV4. The truncated enzymes with 30 N-terminal amino acids deleted were expressed from the other pGEX-TR plasmids.

* Amino acid substitutions are deduced from nucleotide sequencing and numbered from the N-terminus of the non-truncated mature reductase of barley.

† The enzyme activities are in nkat mg^{-1}; nd = not detected.

ated barley tRNA^{Glu} reductases have the same size and peptide structure.

Haem at 2 μM causes a 63% inhibition of the activity of non-truncated barley glutamyl tRNA^{Glu} reductase fusion protein. At this concentration haem caused only 7 to 18% inhibition of activity in the truncated reductase fusion proteins TR6, TR7, and TR10. Figure 3 illustrates the effect of increasing amounts of haem on glutamate 1-semialdehyde synthesis by the truncated fusion protein TR 10 and the full length protein UV4. The concentration of haem (1.5 μM) which causes a 50% inhibition of the non-truncated reductase fusion protein barely affects the activity of the truncated glutamyl tRNA^{Glu} reductase with a deletion of 30 N-terminal amino acid residues.

The *Hem A* cDNA inserts in the four plasmids pGEX-TR1, pGEX-TR11, pGEX-TR9 and pGEX-TR-5 contain nucleotide substitutions which cause 1 to 4 amino acid substitutions in the truncated proteins as listed in Table 1. The amino acid substitutions in these four proteins with greatly reduced specific activity are expected to reveal regions in the primary structure involved in glutamyl-tRNA^{Glu} substrate recognition and/or participating in catalysis. The mutations in the proteins were analysed as to their location in the alignment of the amino acid sequences of the glutamyl tRNA^{Glu} reductases from seven different organisms (Fig. 4). Identical amino acids are highlighted and the amino acid substitutions are indicated above the barley sequence. The single amino acid change of $L_{464} \rightarrow P$ in the TR1 protein concerns a leucine conserved in all glutamyl tRNA^{Glu} reductases analysed except in *B. subtilis*, where the residue is an alanine. The substitution with a proline is likely to cause an abnormal bending of the protein back bone and thereby an altered fold. The two amino acid substitutions, $L_{302} \rightarrow S$ and $L_{387} \rightarrow H$ in the TR11 protein are located in positions conserved in the higher plant and cyanobacterial enzymes. Of the three amino acids changed in the TR 9 protein, the residue R_{322} which is mutated to G is conserved in all sequences analysed. The mutation $I_{318} \rightarrow L$ is in a position conserved in six of the seven analysed sequences; but in the *Chlorobium* protein there is a leucine aligning at this position. The third change from N_{454} to D has taken place at a residue conserved in the three plant but not in the microbial sequences. In the gene encoding the TR5 protein five nucleotide substitutions were found, four of which led to amino acid changes: $M122 \rightarrow K$, $K_{154} \rightarrow N$, $F_{371} \rightarrow L$ and $E_{400} \rightarrow K$. It cannot be decided whether all four mutations or only those placed in the conserved positions, K_{154}, F_{371}, and E_{400} contribute to the reduction in specific activity of the truncated reductase fusion protein.

From these observations it is concluded that several amino acid substitutions affect the catalytic activity of the barley glutamyl tRNA^{Glu} reductase, but that a 30 amino acid N-terminal deletion has no detrimental effect on the catalytic activity of the enzyme.

DISCUSSION

Haem is considered as a feed-back inhibitor regulating chlorophyll and other tetrapyrrole biosynthesis at the level of glutamyl tRNA^{Glu} reductase [1, 2]. A truncated barley glutamyl tRNA^{Glu} reductase lacking the 19 N-terminal amino acids was recently found to functionally complement the *E. coli hem A* mutant AN344 [15]. In the present study a truncated *Hem A* barley cDNA with a 30 codon deletion at the 5'end of the reading frame for the mature enzyme complemented the same mutant. The results in this paper demonstrate that 30 N-terminal amino acids can be deleted without affecting the specific activity of the enzyme expressed as a fusion protein. The remaining 454 residue C-terminal part of the barley glutamyl tRNA^{Glu} reductase which is homologous to the bacterial enzymes is therefore involved in catalysis and glutamyl tRNA^{Glu} binding. Four independently induced mutants with one to four amino acid sub-

stitutions in this part of the polypeptide chain curtail drastically but do not eliminate entirely the catalytic activity of the reductase. This may imply that nine of the ten mutated amino acid residues are important for tRNA binding rather than being decisive for the catalytic reaction.

A resistance to haem inhibition was conferred on the barley enzyme when the 30 N-terminal amino acids were deleted. The N-terminal region of the plant protein therefore probably binds haem and/or mediates in the inhibitory process. Interestingly, the glutamyl $tRNA^{Glu}$ reductase enzymes purified from *E. coli* are insensitive to haem inhibition [20] even though their activity in crude cell extracts is strongly inhibited by haem [21] suggesting that a component external to the reductase can introduce haem sensitivity to the *E. coli* enzyme. Whether or not the N-terminal region of the barley enzyme mediates in the inhibitory process has to be checked, preferably by synthesising the N-terminal region separately and restoring with it the haem sensitivity in the truncated barley glutamyl $tRNA^{Glu}$ reductase fusion protein.

Sensitivity to haem inhibition caused by the N-terminal 19 to 30 amino acids of the barley glutamyl-$tRNA^{Glu}$ reductase may be the reason why enzymatically active full length protein could not be overproduced in *E. coli*. It could be a consequence of extensive haem removal from the metabolic pools, which cannot be tolerated by the cell. Only transformants with either the deleted N-terminal domain or a strongly reduced activity of the enzyme can survive and are selected in the complementation assays. The fact that the GST fusion protein containing the full-length glutamyl $tRNA^{Glu}$ reductase can be overexpressed in highly active form would then imply that the fusion protein protects against excessive haem withdrawal.

EXPERIMENTAL

Isolation of a truncated barley glutamyl $tRNA^{Glu}$ reductase gene. Plasmids were prepd from a barley leaf cNDA library and used to complement the *E. coli hem A* mutant AN344. Eighteen colonies were obtained that complemented the *E. coli hem A* mutant. The plasmids of these colonies were isolated and the inserts sequenced. All plasmids contained inserts with a truncated *hem A* gene starting with GAA AAG AGT AGC ATC GCT.

Construction of the expression plasmid pGEX-TR. PCR was performed using two primers 5′ CGC GGA TCC GAA AAG AGT AGC ATC GCT GTA AT and 3′ CCG GAA TTC GCT TTG GGT CTT CTC TAC that contained restriction sites to allow the directional cloning of the PCR product into pGEX-2T (Pharmacia Biotech. Sweden) in frame with the gene for GST, to give plasmid pGEX-TR. Cells of *E. coli* JM109 were transformed with this plasmid and selected on Luria-Bertani plates containing 100 $\mu g\ ml^{-1}$ ampicillin [22]. Fourteen ampicillin resistant colonies were isolated and their plasmids analysed. Cells containing plasmids with an insert of the correct size were checked for the expression of the fusion protein by SDS-PAGE and Western blot analysis using an antibody raised against the barley glutamyl $tRNA^{Glu}$ reductase.

Large scale isolation of the truncated fusion proteins. 2 × YT [22] medium (1.5 l) containing 0.2% glucose and 100 $\mu g\ ml^{-1}$ ampicillin were inoculated with 250 μl glycerol stock of *E. coli* cells harbouring a pGEX-TR plasmid. Cells were allowed to grow overnight at 37° and expression of the fusion protein was induced by adding 1 mM IPTG. After 2 hr induction period the cells were harvested, disrupted and centrifuged as described earlier [19]. The fusion proteins were purified by affinity of GST to glutathione. The soluble protein extract containing the fusion protein was applied onto a Sepharose G-50 column equilibrated with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 1 mM dithiothreitol, pH 7.4). The eluate of this column was applied directly to a glutathione Sepharose 4B column equilibrated with the same buffer. Unbound protein was washed off with PBS and the column was further washed with 0.1 M Tricine–NaOH, pH 9.0, 0.3 M glycerol, 25 mM $MgCl_2$ and 1 mM dithiothreitol. Bound protein was eluted with 10 mM glutathione in the Tricine-buffer. All steps were performed at 4°.

Assay for glutamyl $tRNA^{Glu}$ reductase activity. Glutamyl $tRNA^{Glu}$ reductase activity of the fusion proteins were measured in a reconstitution assay with glutamyl-$tRNA^{Glu}$ synthetase and glutamate 1-semialdehyde aminotransferase. In a total vol. of 50 μl, the assays contained 10 μg purified glutamyl $tRNA^{Glu}$ synthetase, 30 μg glutamate 1-semialdehyde aminotransferase, 20 μM [0.5 μCi][U-^{14}C] glutamate, 40 μg total chloroplast tRNA [20], 0.1 M Tricine–NaOH, pH 7.9, 0.3 M glycerol, 25 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 1 mM NADPH and 3.5 μg fusion protein. After a 20 min incubation at 30° the assays were stopped by adding 7 μl of 7% perchloric acid. The ppts formed were removed by centrifugation for 5 min at 21 000 *g* and 25 μl of the supernatants were analysed by HPLC as described previously [18]. For the assays performed with *E. coli* $tRNA^{Glu}$ 20 μg tRNA (Sigma) and 50 μg fusion protein were used. Unlabelled glutamate (40 μM) was added to avoid glutamate limiting conditions in some assays.

Other methods. Thrombin cleavage of the fusion proteins was performed as previously described [19]. Protein was estimated using the protein assay kit from Biorad USA. SDS-PAGE was performed as described in [23] using a Tris–Tricine running buffer system [24]. Gels were stained with colloidal Coomassie brilliant blue [25]. Western blot analysis was carried out using the Western Exposure™ Chemiluminescent Detection System (Clontech, U.S.A.) following the manufacturer's instructions. Sequencing was performed using the dye terminator method on an Applied Biosystem 373A sequencing machine. PCR, cloning, plas-

mid prepn and other methods were done following procedures described in [22].

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