Glutamyl-tRNA reductase activity in *Bacillus subtilis* is dependent on the *hemA* gene product

Ingrid SCHRÖDER,* Lars HEDERSTEDT,* C. Gamini KANNANGARA† and Simon P. GOUGH†

*Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund, Sweden,

and †Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

The *Bacillus subtilis* *hemAXCDBL* operon encodes enzymes for the synthesis of 5-aminolaevulinic acid via the C₅ pathway (*hemA* and *hemL*) and uroporphyrinogen III (*hemB*, *hemC* and *hemD*). *B. subtilis* HemA protein (molecular mass 50 kDa) was overexpressed in *hemA* mutants of both *Escherichia coli* and *B. subtilis*. A mutant *B. subtilis* HemA protein with a Cys to Tyr change at position 105 was also overexpressed. Both wild-type and mutant HemA proteins migrated as oligomers (molecular mass ≥ 230 kDa) on gel-filtration columns. All column fractions containing wild-type HemA protein had glutamyl-tRNA reductase activity. No glutamyl-tRNA reductase activity was found with the mutant HemA protein. It is concluded that the *B. subtilis* *hemA* gene product is identical to, or part of, the glutamyl-tRNA reductase of the C₅ pathway.

INTRODUCTION

5-Aminolaevulinic acid (ALA) is an important early precursor in tetrapyrrole and haem biosynthesis. In higher plants, algae and many bacteria, including *Bacillus subtilis* [1] and *Escherichia coli* [1,2], ALA is synthesized from glutamate via the C₅ pathway (for review see [3,4]). This synthetic route radically differs from that operating in animals, yeast and some bacteria, e.g. *Rhodobacter* [5] and *Rhizobium* [6] species, where ALA formation from glycine and succinyl-CoA is catalysed by one enzyme, ALA synthase (EC 2.3.1.37) [4].

The C₅ pathway involves at least three enzymic steps (Fig. 1). First, tRNA^{Glu} is charged with glutamate by glutamyl-tRNA synthase (EC 6.1.1.17). This step is probably identical with that used in protein synthesis and requires ATP and Mg²⁺. Next, the glutamyl-tRNA is reduced to yield glutamate 1-semialdehyde (GSA). This step is catalysed by glutamyl-tRNA reductase, with NADPH as reductant. Finally, ALA is formed from GSA by the action of GSA 2,1-aminomutase (EC 5.4.3.8). It is not known how the C₅ pathway is regulated in bacteria, but modulation of the reductase activity seems likely. The aspect of regulation and also the unusual substrate, glutamate activated by tRNA, makes glutamyl-tRNA reductase an enzyme of particular interest.

Evidence for two glutamyl-tRNA reductases has been presented in *E. coli* [7]. Both are described as monomeric proteins, one of 45 kDa and the other, more abundant, enzyme of 85 kDa. In *Chlamydomonas reinhardtii* the reductase was shown to be a

monomeric protein of 130 kDa [8]. The reductase from *Synechocystis* was recently purified and found to be a homo-oligomer of 350 kDa, with subunits of 39 kDa [9]. *B. subtilis* [10], *E. coli* [11] and *S. typhimurium* [12] cells mutated in the *hemA* gene are ALA auxotrophs. The *hemA* gene product in these bacteria (as derived from the DNA sequence) is a 46–51 kDa polypeptide, and alignment of the deduced amino acid sequences of the three HemA proteins shows 34% identical amino acid residues with two gaps [10]. Avissar & Beale [13] have shown that extracts from an *E. coli* *hemA* mutant cannot catalyse GSA or ALA synthesis from glutamyl-tRNA unless the extract is supplemented with a preparation from *Chlorella vulgaris* cells containing glutamyl-tRNA reductase activity. These results and the presence of a putative dinucleotide [NAD(P)]-binding site in the *hemA* gene product [10] suggest that HemA is the reductase of the C₅ pathway. However, it can not be unambiguously concluded from the above results that the HemA protein itself has glutamyl-tRNA reductase activity, e.g. the *hemA* gene product could be required for the expression or assembly of a functional reductase or be a subunit of the reductase.

In *B. subtilis* the *hemA* and *hemL* (encoding the GSA 2,1-aminomutase) genes are parts of the *hemAXCDBL* operon which is proposed to encode the enzymes for synthesis of uroporphyrinogen III from glutamyl-tRNA ([14] Fig. 2). The *hemC*, *-D* and *-B* genes are essential for uroporphyrinogen III synthesis from ALA. Deletion of *hemX* (unknown gene product) does not affect ALA or haem synthesis (I. Schröder, unpublished work).

Fig. 1. The C₅ pathway for ALA synthesis

Abbreviations used: ALA, 5-aminolaevulinic acid; GSA, glutamate 1-semialdehyde; DTT, dithiothreitol.

† To whom correspondence should be addressed.

Fig. 2. Genetic map of the *hemAXCDBL* operon in the *B. subtilis* chromosome [14] (a), DNA from the *hemA* region in different plasmids (b) and deletions of *hemA* in the chromosomes of two different *B. subtilis* mutants (c)

P indicates the location of one identified promoter, * indicates the location of the *hemA20* mutation; *cat* is a chloramphenicol resistance gene that replaces the deleted region. Restriction sites: B, *Bam*HI; Bc, *Bcl*I; H, *Hind*III; M, *Mlu*I; Ps, *Pst*I; S, *Sal*I. The orientation of the *cat* gene in *B. subtilis* Δ401R and Δ404R was not correctly indicated in previous publications [10,14].

The *hemA20* mutation of one *B. subtilis* ALA-requiring mutant has been identified as a single base pair transition in the *hemA* gene, resulting in a Cys to Tyr substitution at position 105 in the HemA protein [10]. The aim of the present study was to determine if the HemA protein has glutamyl-tRNA reductase activity, and also to analyse some structural properties of this protein. The experimental approach was to purify the wild-type HemA and the mutant HemA20 proteins and use them in a coupled assay for reductase with glutamyl-tRNA synthase and tRNA isolated from a *B. subtilis* *hemA* deletion mutant. The results show that glutamyl-tRNA reductase activity is associated with the wild-type HemA protein. Furthermore, our data suggest that *B. subtilis* HemA produced in *E. coli* or *B. subtilis* is in the form of homo-oligomers, predominantly of four or five 50 kDa polypeptides.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains used were JM83 [*ara rpsL* Δ(*lac-proAB*) Φ80 *lacZ*ΔM15] [15], MM294 (*pro thi endA hsr_k⁻ hsr_m⁺*) [16] and AN344 (*pro leu hemA*) [17]. *B. subtilis* strains used were 3G18 (*ade met trpC2*) (obtained from G. Venema, University of Gröningen), KA11 (*met trpC2 hemA20*) [10,18], Δ401R [*ade met trpC2* Δ(*hemA'*) *cat*] [10], Δ404R [*ade met trpC2* Δ(*hemAX*) *cat*] [10]. Plasmids used were pHV32 [19], pUC18 and pUC19 [15], pHP13 [20], pLUP512 [10], pLUP512A20 (this work, see Fig. 2), pLUP510 [10], pLUP507 [10], pLUP508 [10] and pHEMA1 (this work, see Fig. 2).

Plasmid pLUP512A20 was constructed by replacing the 890 bp *Nsi*I-*Eco*RV fragment in pLUP512 with the corresponding fragment from the chromosome of KA11 containing the *hemA20* mutation [10]. pHEMA1 was constructed by inserting the 1.5 kbp *Eco*RI fragment from pLUP512 containing the *hemA* gene into pHP13.

Growth media

E. coli strains were grown on LA plates or in LB medium [21].

B. subtilis strains were grown on tryptose blood agar base (TBAB; Difco), in Spizizen's minimal medium [22], in LB or in nutrient sporulation medium (NSMP) [23]. The following antibiotics and concentrations were used: ampicillin (50 mg/l), chloramphenicol (*B. subtilis*, 5 mg/l; *E. coli*, 12.5 mg/l) and erythromycin (5 mg/l). ALA was used at 1.5 mg/l or 5 mg/l for *B. subtilis* and 20 mg/l for *E. coli*. Required amino acids were used at 10 mg/l.

DNA techniques

Competent *E. coli* cells were prepared as described by Mandel & Higa [24]. *B. subtilis* was grown to competence as described by Arwert & Venema [25]. Plasmid DNA from *E. coli* and *B. subtilis* was prepared by the method of Ish-Horowitz & Burke [26]; *B. subtilis* was treated with lysozyme (5 mg/l at 37 °C for 30 min) prior to the alkaline lysis.

General DNA techniques were as described in Sambrook *et al.* [21]. Restriction endonucleases and T4 DNA ligase were from Boehringer.

Isolation of soluble fractions from *B. subtilis* Δ401R

B. subtilis Δ401R was grown in portions of 1 litre of NSMP containing 1.5 mg of ALA/l in 5 litre indented flasks in a rotary (200 rev./min) incubator at 37 °C. When the culture reached stationary phase, the cells were harvested by centrifugation at 6000 *g* for 15 min at 4 °C. The pellets were washed once in 50 mM-sodium phosphate buffer, pH 7.9, and once with 0.1 M-Tricine/NaOH, pH 9.0, and then stored at -20 °C until used. The cells (20 g wet wt.) were resuspended in 50 ml of 0.1 M-Tricine/NaOH, pH 9.0, 0.3 M-glycerol, 25 mM-MgCl₂ and 1 mM dithiothreitol (DTT), after which 0.2 g of lysozyme was added and the suspension was incubated on ice for 30 min. The suspension was then passed once through a French pressure cell and a high-speed supernatant was obtained by centrifugation at 300 000 *g* for 20 min at 2 °C.

The high-speed supernatant was fractionated by chromatography at 4 °C using four different affinity columns in sequence as described by Wang *et al.* [27] and Kannangara *et al.* [28]. Briefly, the soluble cell extract was first passed through a Sephacryl S-300 column (5 cm × 49 cm) to remove very high- and low-molecular-mass components. A Cibacron Blue Sepharose column (2.5 cm × 12 cm) was then used to adsorb the glutamyl-tRNA synthase. The material that did not bind to this column was passed through a Procion Red-agarose column (2.5 cm × 8 cm). The flow-through from this column was finally applied to a chlorophyllin-Sepharose column (2.5 cm × 12 cm). Two fractions were used: (1) 1M_b, the material bound to the Blue Sepharose column and eluted with 1 M-NaCl which contains glutamyl-tRNA synthase; and (2) 1M_c, the material eluted with 1 M-NaCl from the chlorophyllin-Sepharose column and from which tRNA was isolated by phenol extraction followed by ethanol precipitation.

Preparation of wild-type and mutant HemA proteins

E. coli AN344/pLUP512 and AN344/pLUP512A20 were grown overnight with shaking (200 rev./min) at 37 °C in 150 ml of LB containing ampicillin. For AN344/pLUP512A20 the medium was supplemented with ALA. The cells were harvested by centrifugation at 7000 *g* for 15 min at 4 °C and washed once with 30 mM-Tris/HCl (pH 8.0)/50 mM-NaCl. The pellet was resuspended in 1 ml of the same buffer and sonicated while chilled on ice. High-speed supernatants were obtained by centrifugation at 100 000 *g* for 60 min at 4 °C.

An overnight culture of *B. subtilis* Δ404R/pHEMA1 grown in LB containing erythromycin was used to inoculate 1 litre of fresh medium in 5 litre indented flasks to an *A*₆₀₀ of 0.1. The

Fig. 3. Analysis of wild-type and mutant *B. subtilis* HemA protein in different strains by Coomassie Brilliant Blue R250 staining and immunoblotting

Soluble proteins from bacterial lysates were fractionated on SDS/PAGE (12% gel) followed by Coomassie Blue staining (a), or were transferred to nitrocellulose membranes and probed with antiserum raised against denatured HemA protein (b and c). A 8 μ g sample of total protein was applied in each slot in (a), 1 μ g of protein was applied per slot in (b), and 5 μ g was applied per slot in (c). Lanes: 1, *E. coli* AN344/pLUP512; 2, *E. coli* AN344/pLUP512A20; 3, *E. coli* JM83/pLUP507; 4, *E. coli* JM83/pLUP508; 5, *E. coli* JM83/pLUP510; 6, *E. coli* JM83/pUC19; 7, *B. subtilis* Δ 404R/pHP13; 8, *B. subtilis*/pHEMA1. The DNA fragments cloned in different plasmids are described in Fig. 2.

cultures were grown at 37 °C with shaking (200 rev./min) and harvested when entering stationary phase. The cells were centrifuged at 6000 *g* for 10 min at 4 °C, washed once in 30 mM-Tris/HCl (pH 8.0)/50 mM-NaCl, and resuspended in 4 ml of the same buffer containing 0.1 mg of lysozyme. The cells were incubated for 30 min at 37 °C and then sonicated while chilled on ice. High-speed supernatants were obtained after centrifugation at 100 000 *g* for 60 min at 4 °C.

Samples of 2 ml of the respective supernatants (approx. 40 mg of protein) were loaded on a Sephacryl S-400 HR column (1.6 cm $\times$ 90 cm) equilibrated with 30 mM-Tris/HCl (pH 8.0)/50 mM-NaCl (flow rate 10 ml/h). Fractions of 2.5 ml were collected and analysed for protein and HemA antigen by SDS/PAGE and immunoblotting.

Glutamyl-tRNA reductase activity assay

Glutamyl-tRNA reductase activity was measured as described in [29]. The assay mixture contained, if not stated otherwise, 75 μ g of partially purified (approx. 50% pure; see Fig. 4b) wild-type HemA or mutant HemA20 protein fraction, 0.88 mg of protein of fraction 1M₀ (glutamyl-tRNA synthase), 7 A₂₆₀ units of total *B. subtilis* tRNA, 25 μ Ci of L-[3,4-³H]glutamic acid (0.5 nmol, NEN), 1 mM-NADPH, 1 mM-ATP and 10 mM-laevulinic acid in 1 ml of 0.1 M-Tricine/NaOH, pH 7.9, 0.3 M-glycerol, 25 mM-MgCl₂ and 1 mM-DTT. The mixture was incubated at 37 °C for 40 min, if not stated otherwise, and the reaction was terminated by transferring the tubes to ice. An excess (100 μ g) of ALA was then added as internal standard followed by 20 μ l of 70% HClO₄. The product, radioactive GSA or ALA, was quantified by scintillation counting after purification involving ion-exchange chromatography, reaction with ethylacetoacetate to obtain ALA- and GSA-pyrroles, and subsequent t.l.c. [29]. The recovery of the added unlabelled ALA was 45–65%, as determined using the Ehrlich reagent [30]. The glutamyl-tRNA reductase activities found in the different sets of experiments show substantial variation, and the reason for this is unclear (it could be due to freezing and thawing of the different components used, or the use of different batches of [³H]glutamate). However, qualitative results were fully reproducible.

Antisera against HemA

An AN344/pLUP512 high-speed supernatant containing large amounts of HemA protein (see Fig. 3a) was further fractionated by gel filtration on an Ultragel AcA-34 (IBF, Villeneuve-la-Garenne, France) (1.6 cm $\times$ 90 cm) column (flow rate 7.5 ml/h). Fractions of 2.5 ml were collected and analysed for HemA protein by SDS/PAGE and Coomassie Brilliant Blue R250 staining. Peak fractions were dialysed against water, and after freeze-drying were dissolved in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate, pH 7.2) and electrophoresed on a 10% gel by SDS/PAGE. The HemA protein band (50 kDa) was cut out from the gel and electroeluted in 0.4 M-Tris/HCl (pH 9.18)/0.1% SDS, using a Biotrap (Schleicher & Schuell). Rabbits were injected subcutaneously with 0.3 mg of the freeze-dried material or 0.1 mg of the electroeluted protein. The antigen was mixed with an equal volume of Freund's complete adjuvant at the first injection. Injections were repeated at intervals with the same amount of antigen in Freund's incomplete adjuvant. The rabbits were bled and immunoglobins were purified and stored as described by Harboe & Ingild [31], except that the DEAE-Sephadex step was omitted.

Other methods

SDS/PAGE was done as described by Neville [32], and proteins were stained with Coomassie Brilliant Blue R250. The following proteins were used as markers: β -galactosidase (130 kDa), BSA (68 kDa), catalase (58 kDa), fumarase (48 kDa), carbonic anhydrase (29 kDa) and myoglobin (17 kDa). For identification of the HemA protein on immunoblots, the proteins were electroblotted as described by Towbin *et al.* [33] on to nitrocellulose membranes (BA 85; Schleicher & Schuell). The filter was blocked, probed with primary antibody and developed according to Blake *et al.* [34]. Goat anti-(rabbit IgG) conjugated to alkaline phosphatase (BioZac AB) was used as secondary antibody. Rocket immunoelectrophoresis for quantification of antigen was done in ME agarose (SeaKem) as described in detail elsewhere [35]. Protein was determined as described by Lowry *et al.* [36] using BSA as standard. Fluorescence was measured at room temperature using a Shimadzu UV-3000 spectrophotometer with a fluorometer attachment device (excitation wavelength 400 nm, with a 500 nm emission cut-off filter). Tritium was quantified by scintillation counting in Ready Safe (Beckman). For estimation of the molecular mass of *B. subtilis* wild-type HemA protein expressed in *E. coli* AN344/pLUP512, an analytical gel-filtration column (Sephacryl S-300, 80 cm $\times$ 2.6 cm, flow rate 0.5 ml/min) was used; 40 mg of protein was applied. For calibration of the column the following proteins were applied separately: thyroglobin (670 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa).

RESULTS AND DISCUSSION

Overexpression of *B. subtilis* wild-type and mutant HemA proteins in *E. coli* AN344

HemA is expressed in large amounts in the *E. coli* ALA auxotrophic strain AN344 (*hemA*⁻) containing the cloned *B. subtilis hemA* gene on the multicopy plasmid pLUP512 (Fig. 2). The transformed cells no longer required ALA for growth, and the HemA protein was found predominantly in the soluble cell fraction ([10]; Fig. 3a). To express the mutant HemA20 protein, plasmid pLUP512A20 was constructed. This plasmid was identical to pLUP512 except that it contained the *hemA20* mutation. *B. subtilis* KA11 (*hemA20*) and *E. coli* AN344 respectively were transformed with pLUP512A20 and, as a positive control, pLUP512. Transformants prototrophic for ALA were for each

Fig. 4. Gel filtration of an *E. coli* AN344/pLUP512 cytoplasmic cell fraction

A high-speed supernatant of cell lysate (40 mg of protein) was applied to the Sephacryl S-400 HR column and 2.5 ml fractions were collected. (a) Fractions were analysed for protein, HemaA antigen and fluorescence (excitation at 400 nm); the relative amount of HemaA antigen was determined by rocket immunoelectrophoresis using antiserum directed against native HemaA protein. Fractions 31, 41, 47, 55 and 61 were analysed on SDS/PAGE (12% gel), followed by Coomassie Brilliant Blue R250 staining (b) or by immunoblotting using antiserum directed towards denatured HemaA protein (c). Samples of 5 μ l of each fraction were applied on the Coomassie-stained gel, whereas 5 μ l of fractions 31 and 61, and only 0.2 μ l of fractions 41, 47 and 55 was analysed in the immunoblot.

strain obtained only with pLUP512, confirming that pLUP512A20 contains the *hemaA20* mutation.

In order to detect and quantify wild-type and mutant HemaA proteins, antisera were used. The specificity of the antisera was analysed in immunoblots using soluble cell extracts from *E. coli* strains carrying different plasmids (Fig. 3b). HemaA antigen was detected only in strains containing plasmids with an intact *hemaA* gene; i.e. an in-frame *SalI* deletion in *hemaA* corresponding to amino acid residues 60–277 (pLUP510; see Fig. 2) resulted in no detectable HemaA antigen in the extract (Fig. 3b, lane 5). Extracts of wild-type *E. coli* carrying pUC19 did not contain any antigen in the 50 kDa region which reacted with antisera against HemaA (Fig. 3b, lane 6). Furthermore, the expression of HemaA was independent of the orientation in the plasmid of the DNA fragment containing the *hemaA* gene (pLUP507 and pLUP508). These results demonstrate that the antisera are specific for the *B. subtilis* HemaA protein and either that *E. coli* HemaA is not recognized by the antibodies directed against *B. subtilis* HemaA or that the *E. coli* HemaA protein is present in very low amounts in

the extract. Compared with the antisera raised against SDS-denatured HemaA, the serum raised against native HemaA was less specific but gave more distinct immunoprecipitates in agarose immunoelectrophoresis.

As can be seen in Fig. 3, the *B. subtilis* HemaA20 protein is expressed in *E. coli* AN344/pLUP512A20 to about the same extent and has the same size as the wild-type HemaA protein expressed in AN344/pLUP512. Since *E. coli* AN344/pLUP512A20, but not AN344/pLUP512, requires ALA for growth, it is concluded that wild-type *B. subtilis* HemaA protein can cause the formation of ALA in *E. coli* AN344 (the mutant HemaA20 protein is functionally inactive).

Overexpression of wild-type HemaA protein in *B. subtilis*

In cell extracts of wild-type *B. subtilis* 3G18 no HemaA antigen was detected in immunoblots or by rocket immunoelectrophoresis. This suggests that the HemaA protein is present in very low amounts in wild-type *B. subtilis* cells. To be able to analyse HemaA synthesized in *B. subtilis*, it was necessary to overexpress the protein. A derivative of the low-copy-number *B. subtilis* plasmid pHP13 containing the same *hemaA* fragment as in pLUP512 was constructed and named pHEMA1 (Fig. 2). This plasmid was used to transform the ALA-requiring *B. subtilis* Δ 404R, which has the *hemaA* promoter region, *hemaA* and *hemaX* deleted from the chromosome (Fig. 2; [10]). *B. subtilis* Δ 404R/pHEMA1 grows without supplementation with ALA and contains HemaA antigen of the same size as that of *E. coli* AN344/pLUP512 (Fig. 3c, lane 8). *B. subtilis* Δ 404R carrying the vector pHP13 requires ALA for growth and does not contain detectable amounts of HemaA antigen (Fig. 3c, lane 7). The *B. subtilis* *hemaA* gene product is therefore also required for ALA formation in *B. subtilis*.

B. subtilis glutamyl-tRNA reductase activity measurements

Glutamyl-tRNA reductase activity was measured in a coupled assay consisting of a [3 H]glutamyl-tRNA-generating system to which was added partially purified HemaA protein. The partially purified HemaA preparation was obtained by gel filtration of a high-speed supernatant from *E. coli* AN344/pLUP512 (Fig. 4). Each complete assay contained the 1M_b fraction from *B. subtilis* Δ 401R (as a source of glutamyl-tRNA synthase), unfractionated *B. subtilis* tRNA, wild-type HemaA or mutant HemaA20 protein fraction, cofactors and substrate. In *B. subtilis* Δ 401R the *hemaA* gene is deleted, and this strain was used to assure that the 1M_b fraction lacked HemaA protein. An outline of how the different components were prepared is presented in Fig. 5.

We found that it was necessary to measure the GSA/ALA formed by a procedure (with internal unlabelled ALA standard) which first separates glutamate from GSA/ALA by Dowex cation chromatography, with subsequent derivatization of the GSA/ALA to the pyrrole, extraction with ether and t.l.c. [29]. Separation of glutamate from GSA by an h.p.l.c. method identical to that originally described in [27], but using a higher salt concentration (100 mM-NaH₂PO₄) in the running buffer, did not separate GSA from unidentified products formed during the assays.

Glutamyl-tRNA reductase activities of cloned *B. subtilis* wild-type HemaA and mutant HemaA20 proteins in *E. coli* AN344

Glutamyl-tRNA reductase was first analysed in one sample (fraction no. 55; Fig. 4) enriched for wild-type *B. subtilis* HemaA protein expressed in *E. coli* AN344/pLUP512. An analogous sample containing the HemaA20 protein served as a negative control, i.e. the two types of sample were identical except that they contained a functional and a defective HemaA protein respectively (Fig. 5). In a time course experiment, glutamyl-

Fig. 5. Outline for preparation of the different components used in the coupled glutamyl-tRNA reductase activity assay

See the Materials and methods section for experimental details. the superscripts *B.s.* and *E.c.* indicate *B. subtilis* and *E. coli* respectively.

tRNA reductase activity was only found with the wild-type HemA protein (Fig. 6). After 40 min incubation, about 1% of the added radioactively labelled glutamate was recovered as GSA/ALA. The activity was correlated with the amount of wild-type HemA protein added; with five times less fraction 55 the amount of reaction product decreased to one-third (40 min incubation; results not shown). Furthermore, with half the amount of wild-type HemA together with the same amount of HemA20 protein fraction the amount of reaction product decreased to about half (results not shown). The activity was negligible at 4 °C (Fig. 6). Adding twice the amount of glutamyl-tRNA synthase (1M_b from *B. subtilis* Δ401R) to the assay did not affect the activity obtained, showing that the glutamyl-tRNA synthase (or other proteins contained in this fraction) was not

Fig. 6. Time course of glutamyl-tRNA reductase activity at 37 °C with *B. subtilis* wild-type HemA (●) and mutant HemA20 (○) protein expressed in an *E. coli* hemA⁻ mutant

The assay mixture used was the same as described in Table 1. The same amounts of wild-type and mutant HemA (fraction 55, Fig. 4) antigen were used in the assay. ▼, Result of an assay run with wild-type HemA incubated on ice for 40 min.

limiting for the conversion of glutamate to GSA/ALA (results not shown).

GSA as the product of the assay

From the assay used, we could not distinguish whether the product was GSA or ALA. We therefore compared the relative activities obtained in the absence and the presence of gabaculin (3-amino-2,3-dihydroxybenzoic acid), an inhibitor of *B. subtilis* GSA 2,1-aminomutase [1,14]. The activity was not sensitive to this inhibitor at a concentration of 10 μM, indicating that the product is predominantly GSA (Table 1). In agreement with this, we could not detect any GSA 2,1-aminomutase activity in the 1M_b fraction used as glutamyl-tRNA synthase (results not shown).

Table 1. Glutamyl-tRNA reductase activity

[³H]Glu-tRNA was measured as HClO₄-precipitated radioactivity, to indicate Glu-tRNA charging. The specific activity of glutamyl-tRNA reductase was calculated from the c.p.m. in GSA-pyrrole divided by the relative amount of HemA antigen as determined by rocket immunoelectrophoresis. The assay mixture contained 75 μg of HemA^{E.c.} protein fraction (no. 55, Fig. 4), 1 mM-NADPH, 1 mM-ATP, 25 μCi of L-[3,4-³H]glutamic acid (0.5 nmol), 10 mM-laevulinic acid, 7 A₂₆₀ units of *B. subtilis* tRNA and 0.88 mg of 1M_b protein (Glu-tRNA-synthase-containing fraction) from *B. subtilis* Δ401R in 1 ml of 0.1 M-Tricine/NaOH, pH 7.9, 0.3 M-glycerol, 25 mM-MgCl₂ and 1 mM-DTT. The superscripts *E.c.* and *B.s.* indicate protein from *E. coli* and *B. subtilis* respectively.

	10 ⁻³ × [³ H]Glu-tRNA (c.p.m.)	Glutamyl-tRNA reductase activity	
		10 ⁻³ × [³ H]GSA-pyrrole formation (c.p.m.)	Specific activity (pmol of GSA/h per HemA)
Mix	211	661	19
Mix + 10 μM-gabaculin	265	640	18
Mix - ATP	6	40	1
Mix - Mg ²⁺ *	17	43	1
Mix - 1M _b	15	54	2
Mix - NADPH	230	36	1
Mix + NADH†	202	114	3
Mix + HemA ^{B.s.} ‡	134	1620	843

* The assay contained 1.25 mM-MgCl₂.

† 1 mM-NADH instead of NADPH.

‡ 75 μg of HemA^{B.s.} protein fraction no. 55 from *B. subtilis* Δ404R/pHEMA1 instead of HemA^{E.c.} protein fraction no. 55 from *E. coli* AN344/pLUP512 (see Fig. 5)

Table 2. tRNA specificity of *B. subtilis* glutamyl-tRNA reductase

[³H]Glu-tRNA was measured as HClO₄-precipitated radioactivity, to indicate Glu-tRNA charging. [³H]GSA-pyrrole formation represents Glu-tRNA reductase activity. The assay mixture contained cofactors and 110 µg of protein of Hema^{E.c.} fraction 55 (Fig. 4). 1M_b^{B.s.} indicates that an affinity fraction from *B. subtilis* Δ401R was used as the source of Glu-tRNA synthase; 0.88 mg of protein was added to the mixture. 1M_b^{E.c.} is total aminoacyl-tRNA synthase from *E. coli* (Sigma); 0.16 mg of protein was added to the assay mixture.

Additions	10 ⁻³ × [³ H]Glu-tRNA (c.p.m.)	10 ⁻³ × [³ H]GSA pyrrole formation (c.p.m.)
1M _b ^{B.s.} (no tRNA)	9	14
1M _b ^{B.s.} + 10 A ₂₆₀ units of tRNA ^{B.s.}	98	223
1M _b ^{B.s.} + 10 A ₂₆₀ units of tRNA ^{E.c.}	35	49
1Mb ^{E.c.} (no tRNA)	5	8
1Mb ^{E.c.} + 10 A ₂₆₀ units of tRNA ^{B.s.}	4	6
1Mb ^{E.c.} + 10 A ₂₆₀ units of tRNA ^{E.c.}	28	120

Cofactor requirements for the formation of GSA by the *B. subtilis* Hema protein

The cofactor requirements for activity were as expected for the C₅ pathway (Fig. 1). As seen in Table 1, charging of the tRNA with glutamate requires the 1M_b fraction containing glutamyl-tRNA synthase, ATP and Mg²⁺. As anticipated, these factors were also required for glutamyl-tRNA reductase activity in the coupled assay. In assays conducted without NADPH the charging of tRNA is not affected, but no GSA is formed. Substitution of NADPH for NADH gave a decreased activity.

The cofactor requirements for activity (Tables 1 and 2) and the procedure used to isolate the reaction product(s) make us confident that our assay has measured the activity of the glutamyl-tRNA reductase of the C₅ pathway of *B. subtilis*.

tRNA specificity of the *B. subtilis* Hema protein

B. subtilis glutamyl-tRNA synthase can charge both *B. subtilis* and *E. coli* tRNA, whereas the *E. coli* synthase is inactive with *B. subtilis* tRNA (Table 2; [7]). The tRNA specificity of the *B. subtilis* Hema protein was analysed by comparing the activities obtained using *E. coli* and *B. subtilis* total tRNAs. Significant activities were obtained with *E. coli* tRNA charged with the homologous synthase as well as with the *B. subtilis* synthase. These results show that the *B. subtilis* Hema protein (glutamyl-tRNA reductase) also can use loaded *E. coli* glutamyl-tRNA, albeit less efficiently. This result agrees with the finding that *B. subtilis* Hema *in vivo* complements the defect of *E. coli* AN344 (*hema*⁻).

Molecular mass of the Hema protein determined by gel filtration: quaternary structure

Hema peak fraction no. 55 and catalase (232 kDa) comigrated on the Sephacryl S-400 HR column (Fig. 4a). The mutant Hema20 protein expressed in *E. coli* AN344/pLUP512A20 behaved as wild-type Hema protein, i.e. it eluted with a peak at the same position and comigrated with a fluorescent chromophore. To further analyse the molecular mass of Hema, a diluted high-speed supernatant from *E. coli* AN344/pLUP512 was applied to a Sephacryl S-300 gel-filtration column equilibrated with 0.1% Thesit (Boehringer)/20 mM-Mops, pH 7.4. On this

Table 3. Glutamyl-tRNA reductase activity, using the same volume of each fraction, obtained by gel filtration of an *E. coli* AN344/pLUP512 soluble extract

Activity was calculated as the c.p.m. in [³H]GSA (corrected for background) per ml, i.e. the same volume of each fraction was used. The specific activity was calculated as the c.p.m. in [³H]GSA (corrected for background) relative amount of HemaA antigen. The total assay mixture was the same as in Table 1, except that 10 A₂₆₀ units of *B. subtilis* tRNA was used. The numbers indicate the HemaA-containing fractions from Sephacryl S-400 HR gel filtration of a high-speed supernatant from *E. coli* AN344/pLUP512 (see Fig. 4); 0.1 ml of each fraction was used in the assay.

Glutamyl-tRNA reductase activity			
Hema from fraction no.	10 ⁻³ × [³ H]GSA- pyrrole (c.p.m.)	Activity (pmol of GSA/h per ml)	Specific activity (pmol of GSA/h per HemaA)
None	3	—	—
31	17	7	29
41	70	31	15
47	25	11	3
55	39	17	2
61	7	3	6

analytical column HemaA again appeared in all high-molecular-mass fractions, but peaked in a fraction corresponding to a molecular mass of 230 kDa.

Glutamyl-tRNA reductase activity of Hema protein fractionated by gel filtration

Although HemaA protein expressed in both *E. coli* and *B. subtilis* tends to migrate as a protein of about 230 kDa during gel filtration, it also forms homo- or hetero-oligomers of larger molecular mass (Fig. 4). When soluble extracts of *E. coli* AN344/pLUP512 were subjected to preparative isoelectric focusing, HemaA protein was also found in all fractions from pH 3.0 to 9.0 (results not shown). This again suggests that oligomeric forms of different molecular mass can exist. It was therefore of interest to analyse the glutamyl-tRNA reductase activity in different fractions. The results from such analysis of fractions 31, 41, 47, 55 and 61 (Fig. 4) are presented in Table 3. It is considered significant that activity was found in all fractions assayed and that they all contained HemaA protein (Fig. 4). HemaA was the only Coomassie-stained protein common to all of the assayed fractions (Fig. 4b). However, the glutamyl-tRNA reductase activity was not proportional to the amount of HemaA protein, i.e. the specific activity varied. Therefore an assay was run with different HemaA-containing fractions (fractions 31, 41, 47, 55 and 61) which had been diluted to give the same amount of HemaA antigen (Table 4). In this experiment the specific activity increased, but there was still a variation in specific activity between the different fractions used. We conclude that the HemaA protein forms inactive aggregates at high concentrations and this is the reason for the inability to correlate glutamyl-tRNA reductase activity and amount of HemaA protein. In addition, it is possible that the red fluorescent chromophore which comigrates with the HemaA peak fraction no. 55 (Fig. 4a) has inhibitory effect. The fluorescent chromophore was extractable into ethyl acetate at pH 4.0 and could be re-extracted from the organic phase by 2% (0.54 M) HCl. Absorption maxima were found at 405.5, 552 and 593 nm (uroporphyrin 405.5, 552 and 594 nm; [37]). The solubility properties and spectral data suggest that the chromophore is uroporphyrin. This is a small

Table 4. Glutamyl-tRNA reductase activity, using the same amount of HemA antigen, in different fractions obtained by gel filtration of an *E. coli* AN344/pLUP512 soluble extract

Specific activity was calculated as described in Table 3, and the total assay mixture was the same as described in Table 3. The numbers indicate the HemA-containing fractions from Sephacryl-S-400 HR gel filtration of a high-speed supernatant from *E. coli* AN344/pLUP512 (see Fig. 4). The same amount of HemA antigen was used in the assay; i.e. fraction nos. 41, 47, 55 and 61 were diluted 8, 14, 40 and 2 times respectively.

HemA from fraction no.	Glutamyl-tRNA reductase activity	
	$10^{-3} \times [^3\text{H}]\text{GSA pyrrole}$ (c.p.m.)	Specific activity (pmol of GSA/ h per HemA)
None	3	—
31	17	29
41	33	57
47	5	8
55	7	13
61	12	20

water-soluble molecule, and it most probably must be bound to protein in order to migrate in the high-molecular-mass range.

The distribution of the uroporphyrin in the column fractions does not correspond to that of the HemA protein, suggesting that it is not specifically bound to this protein. This conclusion is supported by the following. Soluble extracts of *B. subtilis* $\Delta 404\text{R}/\text{pHEMA1}$ also contained uroporphyrin. However, upon gel filtration, uroporphyrin (peaking at fraction 61) did not comigrate with the HemA protein (peaking at fraction 55).

Uroporphyrin may inhibit the enzymic activity or inactivate the HemA protein by means such as photo-oxidation. Accordingly, protein fractions enriched in HemA that did not contain uroporphyrin [fractions 31, 41 and 61 from *E. coli* AN344/pLUP512 (Table 3) and fraction 55 from *B. subtilis* $\Delta 404\text{R}/\text{pHEMA1}$ (Table 1)] showed the highest specific glutamyl-tRNA reductase activity. The specific activity of HemA in this fraction 55 preparation from *B. subtilis* $\Delta 404\text{R}/\text{pHEMA1}$ was found to be about 40 times higher than that prepared from *E. coli* AN344/pLUP512 (Table 1).

Conclusion

From the following findings we conclude that the *B. subtilis* HemA protein constitutes glutamyl-tRNA reductase, or is at least part of the reductase. First, *E. coli* and *B. subtilis* strains mutated in *hemA* require ALA as a growth factor in order to synthesize haem [10,11,18]. Furthermore, the *hemA* gene in *B. subtilis* is localized in the same operon as *hemL*, which encodes the GSA 2,1-aminomutase of the C_5 pathway for ALA synthesis [14]. Thus the structural genes (*hemA* and *hemL*) for the two unique enzymes of the C_5 pathway are closely linked, and are probably cotranscribed. It has also been shown that *E. coli* containing the *E. coli hemA* gene or the *B. subtilis hemA* gene on multicopy plasmids excretes ALA [2] or overproduces tetrapyrroles (the present work). Finally, glutamyl-tRNA reductase activity is correlated with *B. subtilis* HemA protein (the present work). Such activity is not found with the *B. subtilis* mutant HemA20 protein containing a point mutation (Cys to Tyr) in a conserved sequence of the polypeptide.

The 45 kDa glutamyl-tRNA reductase recently found in *E. coli* [7] could be identical with HemA, which has a predicted mass of 46 kDa (cf. [38]). This purified enzyme was found to be monomeric, as determined by gel filtration and density gradient

centrifugation. In contrast, the *B. subtilis* HemA protein is predominantly present as oligomers, as determined by gel filtration under various conditions.

The identification of HemA as glutamyl-tRNA reductase and the available expression systems for the production of *B. subtilis* HemA will allow purification of this interesting but low-abundance enzyme for more detailed studies on regulation, functions and structure.

This work was supported by grants from the Swedish Medical Research Council, the Swedish Natural Science Research Council and Wera och Emil Cornells Stiftelse, and by a grant by the Danish Biotechnology Programme 1991–1995 to Diter von Wettstein. We are grateful to K. Tsiobanelis for technical assistance.

REFERENCES

- O'Neill, G. P., Chen, M.-W. & Söll, D. (1989) FEBS Microbiol. Lett. **60**, 255–260
- Li, J.-M., Brathwaite, O., Cosloy, S. D. & Russel, C. S. (1989) J. Bacteriol. **171**, 2547–2552
- Kannangara, C. G., Gough, S. P., Bruyant, P., Hooper, J. K., Kahn, A. & von Wettstein, D. (1988) Trends Biochem. Sci. **13**, 139–143
- Jordan, P. M. (1990) in Biosynthesis of Heme and Chlorophylls (Daily, H. A. ed.) pp. 55–121, McGraw-Hill, New York
- Hornberger, U., Liebetanz, R., Tichy, H.-V. & Drews, G. (1990) Mol. Gen. Genet. **221**, 371–378
- Stanley, J., Dowling, D. N. & Broughton, W. J. (1988) Mol. Gen. Genet. **215**, 32–37
- Jahn, D., Michelsen, U. & Söll, D. (1991) J. Biol. Chem. **266**, 2542–2548
- Chen, M.-W., Jahn, D., O'Neill, G. P. & Söll, D. (1990) J. Biol. Chem. **265**, 4058–4063
- Rible, S. & Beale, S. I. (1991) J. Biol. Chem. **266**, 9740–9745
- Petricek, M., Rutberg, L., Schröder, I. & Hederstedt, L. (1990) J. Bacteriol. **172**, 2250–2258
- Sasarman, A., Surdeanu, M. & Horodniceanu, T. (1968) J. Bacteriol. **96**, 1882–1884
- Elliott, T. (1989) J. Bacteriol. **171**, 3948–3960
- Avissar, Y. J. & Beale, S. I. (1989) J. Bacteriol. **171**, 2919–2924
- Hansson, M., Rutberg, L., Schröder, I. & Hederstedt, L. (1991) J. Bacteriol. **173**, 2590–2599
- Yanish-Perron, C., Viera, J. & Messing, J. (1985) Gene **33**, 103–119
- Amann, E., Brosius, J. & Ptashne, M. (1983) Gene **25**, 167–178
- MacGregor, C. H. (1976) J. Bacteriol. **126**, 122–131
- Holmgren, E., Hederstedt, L. & Rutberg, L. (1979) J. Bacteriol. **138**, 377–382
- Niaudet, B., Goze, A. & Ehrlich, S. D. (1982) Gene. **19**, 277–284
- Haima, P., Bron, S. & Venema, G. (1987) Mol. Gen. Genet. **209**, 335–342
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
- Spizizen, J. (1958) Proc. Natl. Acad. Sci. U.S.A. **44**, 1072–1078
- Fortnagel, P. & Freese, E. (1968) J. Bacteriol. **95**, 1431–1438
- Mandel, M. & Higa, A. (1979) J. Mol. Biol. **53**, 159–162
- Arwert, F. & Venema, G. (1973) Mol. Gen. Genet. **123**, 185–198
- Ish-Horowitz, D. & Burke, J. F. (1981) Nucleic Acids Res. **9**, 2989–2998
- Wang, W.-Y., Gough, S. P. & Kannangara, C. G. (1981) Carlsberg Res. Commun. **46**, 243–257
- Kannangara, C. G., Gough, S. P., Oliver, R. P. & Rasmussen, K. (1984) Carlsberg Res. Commun. **49**, 417–437
- Friedmann, H. C., Thauer, R. K., Gough, S. P. & Kannangara, C. G. (1987) Carlsberg Res. Commun. **52**, 363–371
- Mauzerall, D. & Granick, S. (1956) J. Biol. Chem. **219**, 435–446
- Harboe, N. & Ingild, A. (1973) in A Manual of Quantitative Immunoelectrophoresis (Axel, N. H. & Weeke, B., eds.), pp. 161–164, Universitetsforlaget, Oslo
- Neville, D. M., Jr. (1971) J. Biol. Chem. **246**, 6328–6334
- Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
- Blake, M. S., Johnston, K. H., Russel-Jones, G. J. & Gotschlich, E. C. (1984) Anal. Biochem. **136**, 175–179

35. Rutberg, B., Hederstedt, L., Holmgren, E. & Rutberg, L. (1978) *J. Bacteriol.* **136**, 304–311
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
37. Fuhrhop, J.-H. & Smith, K. M. (1975) *Laboratory Methods in Porphyrin and Metalloporphyrin Research*, Elsevier Scientific Publishing Company, Amsterdam
38. Verkamp, J. G. & Chelm, B. K. (1989) *J. Bacteriol.* **171**, 4728–4735

Received 2 August 1991/20 September 1991; accepted 25 September 1991