Regulation of a glutamyl-tRNA synthetase by the heme status

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Glutamyl-tRNA (Glu-tRNA), formed by Glu-tRNA synthetase (GluRS), is a substrate for protein biosynthesis and tetrapyrrole formation by the C₅ pathway. In this route Glu-tRNA is transformed to δ -aminolevulinic acid, the universal precursor of tetrapyrroles (e.g., heme and chlorophyll) by the action of Glu-tRNA reductase (GluTR) and glutamate semialdehyde aminotransferase. GluTR is a target of feedback regulation by heme. In *Acidithiobacillus ferrooxidans*, an acidophilic bacterium that expresses two GluRSs (GluRS1 and GluRS2) with different tRNA specificity, the intracellular heme level varies depending on growth conditions. Under high heme requirement for respiration increased levels of GluRS and GluTR are observed. Strikingly, when intracellular heme is in excess, the cells respond by a dramatic decrease of GluRS activity and the level of GluTR. The recombinant GluRS1 enzyme is inhibited *in vitro* by hemin, but NADPH restores its activity. These results suggest that GluRS plays a major role in regulating the cellular level of heme.

gene expression | protein biosynthesis | tetrapyrrole biosynthesis

In addition to their crucial role in aminoacyl-tRNA formation during protein synthesis (1) aminoacyl-tRNA synthetases have acquired additional functions including regulation of gene expression, RNA splicing, or cytokine activity (2, 3).

Glutamyl-tRNA (Glu-tRNA) synthetase (GluRS), which forms Glu-tRNA by the esterification of glutamate to the 3' end of tRNA, is found in different organisms in two forms: a discriminating enzyme (D-GluRS) that recognizes exclusively tRNA^{Glu} and a relaxed specificity nondiscriminating enzyme that recognizes both tRNA^{Gln} and tRNA^{Glu} (ND-GluRS). This ND-GluRS produces Glu-tRNA^{Gln} in the many organisms (most bacteria and all archaea and eukaryotic organelles) that lack GluRS; the mischarged Glu-tRNA^{Gln} is then transformed by a tRNA-dependent amidotransferase to Gln-tRNA^{Gln} (4). Genomewide analysis revealed that many bacteria contain duplicated GluRSs (GluRS1, GluRS2) (5). In addition, some organisms contain a truncated version of GluRS (GluQRS) that is involved in the modification of tRNA^{Asp} (6, 7). Thus, the GluRS enzymes form a family of diverse and versatile proteins that synthesize multiple Glu-tRNAs with different functions.

Apart from its role in protein biosynthesis, Glu-tRNA^{Glu} is also the substrate for the biosynthesis of tetrapyrroles (heme, chlorophyll, and other derivatives) by the C₅ pathway found in plants, archaea, and most bacteria (8–10). Thus, the glutamate moiety of Glu-tRNA is reduced by the Glu-tRNA reductase (GluTR) to glutamate semialdehyde (GSA) (11). GSA is then converted to δ amino levulinic acid (ALA) by the transamination of the ammonium group from position 1 to position 2 by GSA aminomutase (12, 13). ALA is transformed to tetrapyrroles by a universally conserved pathway (14).

The biosynthesis of tetrapyrroles is a finely tuned process because of the many functions and the potential toxicity of its products. Heme plays a central role in the regulation of tetrapyrrole biosynthesis as a regulator of ALA formation. Several lines of evidence indicate that either the intracellular concentration and/or the activity of GluTR are regulated by heme.

Conditional proteolysis occurs in a direct heme-dependent process in *Salmonella* (15) or through the interaction with FLU/FLP proteins in plants (16, 17). Increased transcription of *hemA* (the gene encoding GluTR) also controls the level of GluTR in *Pseudomonas* (18). Alternatively, feedback regulation of GluTR activity by heme (19) is also a mechanism that regulates the formation of ALA. Because of these regulatory features and the fact that in model organisms only a small fraction of Glu-tRNA is transformed to tetrapyrroles, GluTR is regarded as the first enzyme committed to tetrapyrrole biosynthesis.

Heme is the cofactor of cytochromes in respiratory chains (20). This porphyrin molecule also plays roles in iron homeostasis (21), protection against reactive oxygen species (14), and as cofactor of PAS (common domain in period circadian protein, aryl hydrocarbon receptor nuclear translocator, single-mind protein) and globin-like proteins for bacterial signal transduction (22). A fine balance between the content of protoheme and iron to form active heme is critical for cell viability because an excess of any of the two components might be toxic to cells because of oxidative damage of biomolecules (23).

Acidithiobacillus ferrooxidans, an acidophilic γ -proteobacterium that uses ferrous ion (Fe²⁺), sulfur (S⁰), or reduced sulfur compounds as electron donors (24), is a suitable model to study the molecular mechanisms involved in the regulation of heme content. In fact, *A. ferrooxidans* growing in different substrates is able to modulate the content of cytochromes, reaching up to 10% of the total proteins in cells grown in Fe²⁺ medium (24, 25). Our working hypothesis is that some of the components of the complex Glu-tRNA formation system from *A. ferrooxidans* might have a regulatory role in the biosynthesis of heme. This system is formed by several tRNAs and two paralog GluRS enzymes; GluRS1 is specific for tRNAs with augmented D stem (tRNA^{Glu} and tRNA^{Gln}), and GluRS2 preferentially aminoacylates a tRNA^{Gln}, which has only 3 bp at the D stem (5, 26). The fact that *A. ferrooxidans* Glu-tRNA^{Glu}₃ is not substrate for GluTR suggests that some components of the system are committed to specific roles in either protein or heme biosynthesis (27). The data presented here reveal that the levels of both GluRS1 and GluTR are regulated by the heme status. Strikingly, the activity of GluRS1 was also modified under heme excess. These data led us to propose that GluRS1 and GluTR in *A. ferrooxidans* both are committed to the regulation of heme biosynthesis.

Results

Aminoacylation of tRNA^{Glu} Correlates with the Demand of Heme. Cytochromes represent up to 10% of total protein from *A. ferrooxidans* grown in Fe²⁺, most of them being higher than in

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Abbreviations: Glu-tRNA, glutamyl-tRNA; GluRS, Glu-tRNA synthetase; GluTR, Glu-tRNA reductase; ALA, amino levulinic acid.

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Fig. 3. Immunodetection of GluTR and GluRSs. Whole exponentially growing cells cultured in Fe^{2+} or S^0 (A) or Fe^{2+} supplemented with various concentrations of ALA (B) were electrophoresed under denaturing conditions, transferred to a solid matrix, and probed with antibodies against *A. ferrooxidans* GluRS1 (Top), GluRS2 (Middle), or GluTR (Bottom). Each lane was loaded with 1 μg of protein except for GluRS2 detection (from a separate experiment) where 20 μg was used.

decrease) when the cells were cultured in the presence of the precursor of heme biosynthesis.

The steady-state level of both GluRS1 and GluRS2, measured by Western blot analysis, was slightly higher in cells grown in Fe^{2+} compared with S^0 grown cells (Fig. 3A Top and Middle, respectively). The level of the GluRS1 and GluRS2 mRNAs was tested by RT-PCR. Whereas the mRNA encoding GluRS1 showed a slight increase in Fe^{2+} compared with S^0 , no variation in the mRNA of GluRS2 was observed (Fig. 4). These data suggest that a transcriptional mechanism might control the level of GluRS1 in cells grown in Fe^{2+} and/or S^0 . Similar results were obtained when the expression of GluTR at the level of protein or mRNA was measured (Figs. 3A Bottom and 4). Conversely, no significant variation of both GluRS1 and GluRS2 was observed when the cells were cultured in Fe^{2+} supplemented with increasing concentrations of ALA (Fig. 3B Top and Middle, respectively). Under these culture conditions, we observed a marked variability in the level of the mRNA encoding GluRS1 (from no mRNA to the same level as in cells cultured in Fe^{2+}) measured by RT-PCR (data not shown). We believe that the presence of ALA in the culture medium induces an unknown event in the cells that led to instability of the mRNA during the isolation procedure. We concluded that the activity of GluRS1, rather than the steady-state level of the enzyme, is affected by the presence of ALA in the culture medium. Semiquantitative analysis by Western blot revealed a relatively low amount of GluRS2 (5–10% depending on the cell growth condition) compared with GluRS1. Thus, together these data indicate that GluRS1 is the major GluRS in *A. ferrooxidans* and suggest that it is mostly dedicated to heme biosynthesis because a reduction of the activity of GluRS1 had no effect on cell growth.

Hemin Inhibits the Activity of GluRS1. Because the intracellular heme content correlates with the activity of the endogenous

GluRS1, it is possible that heme or an intermediate of its synthesis might affect the enzymatic activity. To test this hypothesis, the effect of hemin, an analog of heme containing Fe^{3+} , was determined on the activity of each recombinant GluRS1 fused to GST (GST-GluRS1), recombinant GluRS1 released from GST (rGluRS1), or the endogenous GluRS1 (in extracts from cells grown in Fe^{2+}). As shown in Fig. 5A, a dose-dependent inhibition by hemin of all tested GluRSs was observed. A

Fig. 5. Inhibition of GluRS1 by hemin. (A) Relative activity of GST-GluRS1 (▲), rGluRS1 (●), and endogenous GluRS1 (■) treated with variable concentrations of hemin is shown. Initial velocities without added hemin measured as Glu-tRNA formed in 15 min were GST-GluRS1 (61.8 pmol/50 nM), rGluRS1 (46.8 pmol/50 nM), and endogenous GluRS1 (458 pmol/ μg of total protein). (B) rGluRS1 was preincubated with hemin then incubated with 2 mM NADPH or NADP $^+$, and the aminoacylation activity was measured after 15 min of incubation. Lines on the top of the bars indicate values range.

Fig. 4. Level of mRNAs encoding GluRSs and GluTR. Total mRNA was extracted from exponentially growing cells cultured in Fe^{2+} , Fe^{2+} /ALA, or S^0 and subjected to RT-PCR using specific primers for GluRS1, GluRS2, or GluTR. CyD is the representative amplification of a loading control.

recruitment of heme by GST that is known to bind heme (28, 29) might explain the ≈ 10 -fold higher sensitivity of GST-GluRS1 to the inhibitor. GluRS2 was also inhibited by heme to a similar extent as GluRS1 (data not shown). Binding of heme to GST-GluRS1, rGluRS1, and GST-GluRS2 at concentrations as low as 10 μ M was observed (data not shown). These findings strongly suggest that, upon culture of *A. ferrooxidans* in the presence of ALA, the reduced activity of GluRS1 is the result of an interaction of the enzyme with heme or any other intermediate tetrapyrrole (ALA added to the reaction mixture had no effect in the activity of GluRSs).

Heme causes oxidative damage to cellular macromolecules (23). Some damage can be reversed by general antioxidant systems that depend on NADPH or GSH (29, 30). We tested whether NADPH counteracts the inhibition of GluRS1 by heme. GluRS1 was preincubated with heme, then incubated with either NADPH or NADP⁺, and subsequently, the charging activity was measured. As shown in Fig. 5B, incubation of the enzyme in the presence of NADPH almost completely abolished the ability of heme to inhibit GluRS1. This effect was not observed when NADP⁺ was added, suggesting that a role of NADPH as an antioxidant (30) is involved in the protection against the inhibition by heme. Interestingly, the presence of GSH in the Fe²⁺/ALA culture medium of *A. ferrooxidans* led to a slight decrease in the inhibition of GluRS1 (data not shown). These data are consistent with the notion that a redox reaction is involved in the inhibition of GluRS1 by heme. It is not clear, however, if the effect is a result of the tetrapyrrole or the Fe³⁺ present in heme.

Heme Regulates the Level of GluTR. Regulation of GluTR at the protein level or the enzymatic activity is the major mechanism to control the heme status in cells that use the C₅ pathway (15–17). To test whether there is also such a mechanism in *A. ferrooxidans*, we inspected the cellular levels of the enzyme by Western blot analysis. The relative amount of GluTR was significantly higher in the cells grown in Fe²⁺ compared with S⁰ grown cells (Fig. 3A Lower). The *hemA* mRNA (encoding GluTR) was higher in Fe²⁺ compared with S⁰ (Fig. 4), suggesting a transcriptional control of the expression under these culture conditions as was observed for GluRS1. However, when the culture medium contained Fe²⁺/ALA, the concentration of GluTR decreased to almost undetectable levels (Fig. 3B Lower). The steady state of *hemA* mRNA showed the same level as in cells grown in Fe²⁺ (Fig. 4), suggesting that with an excess of heme (Fe²⁺/ALA) the amount of GluTR is controlled at the posttranscriptional level by a proteolytic mechanism. As in other organisms (19), heme binds and inhibits the activity of recombinant GluTR at concentrations as low as 5 μ M (data not shown). Hence, these data suggest that heme may be an intracellular effector that controls the function of GluTR at the transcriptional level or by specific degradation depending on the heme status, as observed in other organisms (15, 18, 19). Taken together, these results reveal that in *A. ferrooxidans* the function of both GluRS1 and GluTR is regulated by mechanisms that involve the steady-state level of the proteins or the activity of the enzymes in response to the cellular heme status.

Discussion

Regulation of Heme Biosynthesis. Insertion of iron into protoporphyrin IX is the final step in the biosynthesis of heme. Accumulation of either iron (21) or protoporphyrin is toxic to cells and leads to oxidative stress (31). These effects imply that a fine balance between these two components is required to avoid cell damage (32). Upon induction of cytochrome *c* synthesis in *A. ferrooxidans* grown in Fe²⁺, an increased heme demand must be fulfilled. Cells respond to heme requirements in part by modulating the level of GluTR, the first enzyme committed to the

production of ALA. Because the steady state of *hemA* mRNA is higher in these cells, it is likely that a transcriptional mechanism accounts for the different level of GluTR. Conversely, in cells grown in Fe²⁺/ALA, a higher amount of heme is produced that parallels a dramatic decrease in the steady state of GluTR. Probably the excessive heme produced triggers the proteolytic degradation of GluTR, as described in *Salmonella* (15). Other alternatives, including the control of translation of the mRNA by antisense RNA, cannot be ruled out.

A dual response in both the activity and the steady-state level of GluRS1 that correlates with the heme status is described here. With elevated heme requirements (cells grown in Fe²⁺), the level of GluRS1 increases probably through an increase in transcription. Conversely, with excess heme synthesis (cells grown in Fe²⁺/ALA), a dramatic reduction in the intracellular specific activity of GluRS1 occurs without any change in the protein level of this enzyme. Thus, upon increase of heme triggered by growth conditions (S < Fe²⁺ < Fe²⁺/ALA), the activity of GluRS1 correspondingly decreases. Whether this is the result of a more general cellular response, such as oxidative stress caused by elevated heme, needs to be investigated. Other synthetases (except TyrRS) did not respond in the same way. Because heme binds and inhibits GluRS, we believe that heme or any other precursor tetrapyrrole is the intracellular effector that triggers this regulatory mechanism.

Protection against the inhibition of GluRS1 by NADPH led us to consider that the effect of heme on the activity of this enzyme might be the result of a redox process. Interestingly, *E. coli* GluRS is substrate of DsbA, a protein involved in the restoration of the reduced state of cysteines in proteins upon oxidation (33). Several cysteines, equivalent to the SWIM domain (34) of *E. coli* GluRS (35) involved in zinc binding, are found in the catalytic domain of *A. ferrooxidans* GluRS1. Whether these cysteines are sensitive to cellular oxidative stress caused by elevated heme content is not known.

Regulation of the GluRS activity by the interaction with other proteins has been described. *Bacillus subtilis* GluRS copurifies with the adenosuccinate lyase, and the complex formed increased the thermostability of GluRS and decreased the *K_M* for glutamate and ATP (36). Also, the formation of a ternary complex between GluRS, MetRS, and the Arc1p protein has been described in *Saccharomyces cerevisiae*. The interaction of Arc1p with GluRS increased the binding affinity of the enzyme for tRNA by 100-fold (37). An interaction between GluRS and GluTR might occur in *A. ferrooxidans* like has been observed in *Chlamydomonas reinhardtii* (38). We hypothesize that GluRS1 and GluTR interact in the cell and that the decrease in the level of GluTR upon growing the cells in Fe²⁺/ALA might expose the GluRS to heme or any other effector that reduces the activity of the enzyme.

Is GluRS1 Dispensable for Protein Biosynthesis? Because of the high demand of heme in cells grown in ferrous ions, a deviation to heme biosynthesis could result in shortening of Glu-tRNA for protein biosynthesis. In the case of *A. ferrooxidans*, the existence of three tRNA^{Glu} species and two GluRS enzymes can be interpreted as an evolutionary adaptation, rather than redundancy, to the requirements of Glu-tRNA to produce high levels of heme. Under growth conditions in which cells do not require Glu-tRNA, as precursor for heme biosynthesis, up to 85% of GluRS1 is dispensable, but no major detrimental effect in the cell growth was observed. Thus, GluRS2 and the remaining 15% of the activity of GluRS1 are sufficient to provide the Glu-tRNA substrates for protein synthesis. In addition, the formation by GluRS1 of Glu-tRNA^{Glu₃}, which is not a substrate of GluTR (26), might ensure the provision of Glu-tRNA for protein biosynthesis. Thus, these data suggest that most of the activity of

GluRS1 is committed to heme biosynthesis, and protein biosynthesis might be a secondary role of this enzyme.

Duplication of GluRS has been proposed as an evolutionary event to ensure the specific decoding of glutamine codons during translation of the genetic information (5, 39). The data presented here suggest that a physiological role other than protein synthesis might explain the duplication of GluRS. In the same vein, an interesting hypothesis to explain the conservation of a reduced plastid genome in nonphotosynthetic plants and algae has been proposed. These organisms also have two GluRSs (one cytoplasmic and one organellar) and the plastid-encoded tRNA^{Glu} might be essential for heme biosynthesis (40). Thus, complex Glu-tRNA formation systems might have evolved to ensure the provision of heme.

Materials and Methods

Strains and Cell Growth Conditions. *A. ferrooxidans* ATCC 23270 was cultured at 30°C in 9K medium (41) supplemented with 33.3 g/liter Fe₂SO₄ at pH 1.6 or 5 g/liter S⁰ at pH 3.5. When necessary ferrous medium was supplemented with 50–200 µg/ml of ALA. *E. coli* DH5, used for plasmid propagation, was grown at 37°C in LB medium. *E. coli* BL21 (DE3) was used for overexpression and purification of proteins. When necessary, the LB medium was supplemented with 100 µg/ml ampicillin.

DNA Manipulations. General DNA manipulations were carried out according to standard procedures (42). Plasmid DNAs were obtained by using the Qiagen (Valencia, CA) extraction kit. Cloned DNA was sequenced at the Centro de Biotecnología, Facultad de Ciencias, Universidad de Chile. The preliminary sequence of *A. ferrooxidans* ATCC23270 genome was obtained from The Institute for Genomic Research (www.tigr.org).

Expression and Purification of Recombinant Proteins. Overproduction and purification of the recombinant proteins GST-GluRS1 and His(6)-GluTR was carried out as described (27). When required GST-GluRS1 purified from *E. coli* was digested with thrombin (Sigma, St. Louis, MO) according to the manufacturer's instructions to remove the GST. GST was overexpressed from pGEX2T vector (Amersham, Piscataway, NJ) and purified according to the manufacturer's instructions. His(6)-fGluRS2 was obtained by cloning of the nucleotides encoding the 190 carboxyl-terminal residues of GluRS2 into pET15b. Overexpression and purification from inclusion bodies was performed as described (27).

Production of Specific Antisera. His(6)-GluTR, GST-GluRS1, and His(6)-fGluRS2 were electrophoresed in SDS/PAGE, detected by staining with Coomassie blue, and excised from the gel. Gel strips were crushed and used to immunize rabbits (BiosChile, Santiago, Chile). Polyclonal antibodies obtained against GluTR were further purified by affinity chromatography on immobilized GluTR-agarose as described (43).

Western Blot Analysis. Samples were heated to 100°C for 10 min before gel loading. Protein samples were separated on 12% SDS/PAGE Tris-glycine gels and transferred to nitrocellulose membranes. Membranes were blocked with TBS (Tris-borate-NaCl) containing 5% nonfat dry milk and subjected to Western blot analysis by standard procedure. Polyclonal antibodies against GluRS1, fGluRS2, or GluTR were used at 1:10,000 dilutions. HRP-conjugated secondary antibodies (Pierce, Rockford, IL) were used at 1:30,000 dilution. Reacting protein were visualized by using the SuperSignal West Pico Chemiluminescent detection kit (Pierce).

Analysis of mRNA Expression. Total RNA from *A. ferrooxidans* was prepared according to Hangen and Young (44) and reverse-transcribed with the primers pGI-INR (5'-aagtttctgatcgccatgg-3'), pGII (5'-gaaccgcagggttaggaataac-3'), or pGluTRAf6 (5'-tgccgaacaccttcacac-3') for mRNAs encoding *gluX1* (GluRS1), *gluX2* (GluRS2), or *hemA* (GluTR), respectively and reverse-transcribed with RevertAid M-MuLV (Fermentas, Vilnius, Lithuania). PCR amplifications of *gluX1*, *gluX2*, and *hemA* cDNAs were carried out with primers pGI-INR and pGI-INF (5'-attgctattgcagtcgggaag-3'), pGII-INR and pGII-INF (5'-gaaccgcagggttaggaataac-3'), or pGluTRAf6 and pGluTRAF8 (5'-aacatcaggcagcatagcg-3'), respectively. PCRs were carried out by using conventional procedures. The primers *cydA1* (5'-aagctggcgccatggaagc-3') and *cydA4* (5'-actgcatggccaggaaaccg-3') were used to amplify the mRNA encoding *cydA*, a constitutively transcribed cytochrome, as loading control.

Preparation of tRNA. *In vivo*-expressed tRNAs [*A. ferrooxidans*'s tRNA^{Gln3} (UUG) and *E. coli* total tRNA] were obtained as described (5). tRNA^{Glu2} (CUC) transcript was synthesized by *in vitro* transcription (27).

Detection of *in Vivo* Aminoacylated/Deacylated tRNA. To determine the level of aminoacylated/deacylated tRNAs *in vivo* total tRNA was extracted from *A. ferrooxidans* as described (5). Briefly, periodate oxidation followed by β-elimination, electrophoresis on denaturing gels, and subsequent Northern blot were carried out as described (6). Northern blot probes were designed complementary to nucleotides 15–39 for tRNA^{Glu1}, nucleotides 14–37 for tRNA^{Gln2}, and nucleotides 13–37 for tRNA^{Gln3}.

Preparation of Cellular Extracts from *A. ferrooxidans*. Cells were harvested, washed with 10 mM H₂SO₄ for Fe³⁺ removal, and subsequently washed with 30 mM Hepes, pH 8.0. The bacterial pellet was resuspended in lysis buffer (30 mM Hepes, pH 8.0/150 mM NaCl/1 mM DTT), and cells were disrupted by cycles of freezing at –78°C and thawing by sonication. Extracts were centrifuged at 20,000 × *g* for 30 min. The supernatant was ultracentrifuged at 150,000 × *g* for 90 min. When necessary the cellular extract was dialyzed against lysis buffer containing 50% glycerol. Protein concentration was measured with Bradford reagent, and aliquots were stored at –80°C.

Determination of GluRS Activity. GluRS activity was determined according to Salazar *et al.* (5). Enzymatic activity was measured in 100 µl of reaction mixture, and the results are expressed as pmol of Glu-tRNA formed in 15-min incubation (initial velocity) relative to protein amount. Inhibition of activity by hemin was carried out by 15-min preincubation at 37°C. For the reversion assay, inhibited GluRS1 was further incubated with the corresponding reagent for 30 min at 37°C. The reaction was started by the addition of 0.2 µg/µl transcript tRNA^{Glu2} or 1 µg/µl total *E. coli* tRNA. Transcript tRNA^{Glu2} was previously denatured at 90°C for 3 min and cooled down slowly to facilitate RNA folding. The determination of the activity of GluRS1 from cellular extracts was carried out as described (5) with 0.1 µg of the cellular extract.

Heme Quantification. Cellular heme was quantified as described (45) with minor modifications. Harvested *A. ferrooxidans* (washed with 10 mM H₂SO₄) and *E. coli* cells were washed with 30 mM Hepes, pH 8.0. Thirty milligrams of the bacterial pellet was resuspended in 90 µl of a solution containing 0.5 M NaOH and 2.5% Triton X-100. Disrupted cells were sonicated to reduce the viscosity of the medium and centrifuged at 20,000 × *g* for 30 min. Formed haemetine was measured spectrophotometrically by the absorbance at 575 nm. Bovine hemin (Sigma) was used as standard for heme measurements (39).

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1. Ibba M, Söll D (2004) *Genes Dev* 8:731–738.
2. Park SG, Ewalt KL, Kim S (2005) *Trends Biochem Sci* 30:569–574.
3. Ryckelynck M, Giege R, Frugier M (2005) *Biochimie* 87:835–845.
4. Feng L, Tumbula-Hansen D, Min B, Namgoong S, Salazar J, Orellana O, Söll D (2005) in *Aminoacyl-tRNA Synthetases*, eds Ibba M, Cusack S (Landes, Georgetown, TX), pp 314–319.
5. Salazar JC, Ahel I, Orellana O, Tumbula-Hansen D, Krieger R, Daniels L, Söll D (2003) *Proc Natl Acad Sci USA* 100:13863–13868.
6. Salazar JC, Ambrogelly A, Crain PF, McCloskey JA, Söll D (2004) *Proc Natl Acad Sci USA* 101:7536–7541.
7. Blaise M, Becker HD, Lapointe J, Cambillau C, Giege R, Kern D (2005) *Biochimie* 87:847–861.
8. Beale SI, Gough SP, Granick S (1975) *Proc Natl Acad Sci USA* 72:2719–2723.
9. Schön A, Krupp G, Gough S, Berry-Lowe S, Kannangara CG, Söll D (1986) *Nature* 322:281–284.
10. Jahn D, Verkamp E, Söll D (1992) *Trends Biochem Sci* 17:215–218.
11. Schauer S, Chaturvedi S, Randau L, Moser J, Kitabatake M, Lorenz S, Verkamp E, Schubert WD, Nakayashiki T, Murai M, et al. (2002) *J Biol Chem* 277:48657–48663.
12. Smith MA, Kannangara CG, Grimm B (1992) *Biochemistry* 31:11249–11254.
13. Ilag LL, Jahn D (1992) *Biochemistry* 31:7143–7151.
14. Beale S (1996) in *Escherichia coli and Salmonella typhimurium*, ed Neilhardt FC (Am Soc Microbiol, Washington, DC), pp 731–748.
15. Wang L, Elliott M, Elliott T (1999) *J Bacteriol* 181:1211–1219.
16. Goslings D, Meskauskienė R, Kim C, Lee KP, Nater M, Apel K (2004) *Plant J* 40:957–967.
17. Falcatore A, Merendino L, Barneche F, Ceol M, Meskauskienė R, Apel K, Rochaix JD (2005) *Genes Dev* 19:176–187.
18. Krieger R, Rompf A, Schobert M, Jahn D (2002) *Mol Genet Genomics* 267:409–417.
19. Vothknecht U, Kannangara CG, von Wettstein D (1998) *Phytochemistry* 47:513–519.
20. Thöny-Meyer L (1997) *Microbiol Mol Biol Rev* 61:337–376.
21. Andrews SC, Robinson AK, Rodríguez-Quinones F (2003) *FEMS Microbiol Rev* 27:215–237.
22. Galperin M (2004) *Environ Microbiol* 6:552–567.
23. Graca-Souza AV, Maya-Monteiro C, Paiva-Silva GO, Braz GR, Paes MC, Sorgine MH, Oliveira MF, Oliveira PL (2006) *Insect Biochem Mol Biol* 36:322–335.
24. Ingledew WJ (1982) *Biochim Biophys Acta* 683:89–117.
25. Yarzabal A, Brasseur G, Bonnefoy V (2002) *FEMS Microbiol Lett* 209:189–195.
26. Núñez H, Lefmíl C, Min B, Söll D, Orellana O (2004) *FEBS Lett* 557:133–135.
27. Levicán G, Katz A, Valezuela P, Orellana O (2005) *FEBS Lett* 579:6383–6387.
28. Arias IM (1979) *Med Biol* 57:328–334.
29. Vincent SH, Grady RW, Shaklai N, Zinder JM, Muller-Eberhard U (1988) *Arch Biochem Biophys* 265:539–550.
30. Kirsch M, De Groot H (2001) *FASEB J* 15:1569–1574.
31. Winyard PG, Moody CJ, Jacob C (2005) *Trends Biochem Sci* 30:453–461.
32. Stojilkovic I, Perkins-Balding D (2002) *DNA Cell Biol* 21:281–295.
33. Leichert LI, Jacob U (2004) *PLoS Biol* 2:e333.
34. Makarova KS, Aravind L, Koonin EV (2002) *Trends Biochem Sci* 27:384–386.
35. Banerjee R, Dubois DY, Gauthier J, Lin SX, Roy S, Lapointe J (2004) *Eur J Biochem* 271:724–733.
36. Gendron N, Breton R, Champagne N, Lapointe J (1992) *Proc Natl Acad Sci USA* 89:5389–5392.
37. Graindorge JS, Senger B, Tritch D, Simos G, Fasiolo F (2005) *Biochemistry* 44:1344–1352.
38. Jahn D (1992) *FEBS Lett* 314:77–80.
39. Skouloubris S, Ribas de Pouplana L, De Reuse H, Hendrickson TL (2003) *Proc Natl Acad Sci USA* 100:11297–11302.
40. Barbrook AC, Howe CJ, Purton S (2006) *Trends Plant Sci* 11:101–108.
41. Silverman MP, Lundgren DG (1959) *J Bacteriol* 77:642–647.
42. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), 2nd Ed.
43. Salazar JC, Zúñiga R, Lefmíl C, Söll D, Orellana O (2001) *FEBS Lett* 491:257–260.
44. Hangen GS, Young E (1978) *J Virol* 26:793–804.
45. Zander R, Lang W, Wolf U (1984) *Clin Chem Acta* 136:83–93.