Expression of Functional Plasmodium falciparum Enzymes Using a Wheat Germ Cell-Free System

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One decade after the sequencing of the Plasmodium falciparum genome, 95% of malaria proteins in the genome cannot be expressed in traditional cell-based expression systems, and the targets of the best new leads for antimalarial drug discovery are either not known or not available in functional form. For a disease that kills up to 1 million people per year, routine expression of recombinant malaria proteins in functional form is needed both for the discovery of new therapeutics and for identification of targets of new drugs. We tested the general utility of cell-free systems for expressing malaria enzymes. Thirteen test enzyme sequences were reverse amplified from total RNA, cloned into a plant-like expression vector, and subjected to cell-free expression in a wheat germ system. Protein electrophoresis and autoradiography confirmed the synthesis of products of expected molecular masses. In rare problematic cases, truncated products were avoided by using synthetic genes carrying wheat codons. Scaled-up production generated 39 to 354 μg of soluble protein per 10 mg of translation lysate. Compared to rare proteins where cell-based systems do produce functional proteins, the cell-free yields are comparable or better. All 13 test products were enzymatically active, without failure. This general path to produce functional malaria proteins should now allow the community to access new tools, such as biologically active protein arrays, and lead to the discovery of new chemical functions, structures, and inhibitors of previously inaccessible malaria gene products.

The contribution of enzymology to a major global health problem is severely limited by an unusual general problem with protein expression. Malaria is a major life-threatening disease (1, 2). It is estimated to cause up to 1 million deaths annually, mostly in African children under 5 years of age, out of approximately 247 million total clinical cases. In spite of intense efforts to control malaria, the emergence of resistant parasites poses a major challenge. Therefore, it is necessary to discover novel targets and novel knowledge-based strategies for controlling malaria.

Despite the availability of complete coding sequences of Plasmodium falciparum (3), after a decade, only a small fraction of malaria parasite gene products have been functionally annotated and even fewer have potent, target-selective inhibitors. The leading global antimalarial drug discovery enterprise, Medicines for Malaria Venture (MMV [http://www.mmv.org/research-development]), has about five advanced projects with defined targets, but only two of these projects have access to recombinant, functionally active, pure protein targets for lead improvement.

The lack of defined biochemical systems for malaria drug discovery is directly related to the lack of a reliable heterologous expression system for malaria proteins. Previous attempts to express recombinant malaria proteins showed that overexpression of malaria proteins is a general, challenging task. Conventional Escherichia coli cell-based protein expression systems successfully express recombinant proteins from many genomes (4, 5). Yet when similar systems were applied to the expression of P. falciparum proteins on a single gene or on a genome scale (6, 7), the success rates, even loosely defined merely as the ability to make visible new protein, were less than 5%. In contrast, corresponding genes from human counterparts (8–10) or even from other parasitic sources are often readily overexpressed (11).

Various ideas have been offered to explain the challenges of expressing malaria proteins in cell-based systems. These explanations include the high AT content of the malaria coding regions, internal start and stop sites, autologous feedback loops, and non-specific nucleic acid binding (6, 12, 13). Efforts to overcome these potential issues, using tRNA augmentation, optimized codons, and alteration of host expression, have led to occasional successes (7, 12–18) but no universal malaria protein expression strategy.

One potentially useful approach with predictable success is to conduct expression in an auxotrophic host cell, where survival of the host is dependent on successful expression of the desired malaria gene (6, 12, 19). Due to limited availability of appropriate knockout host cells and to the absence of information about the functions of new genes, this is also not a practical solution for future genome-wide protein expression. In addition, many different protein production protocols have to be tested for optimum expression of each desired protein, again making it difficult to select one general system for new malaria proteins.

In contrast to many of the challenges of cell-based systems, cell-free systems are both open and flexible (Table 1). Among the available options, the wheat germ system is robust, it quickly produces high yields, and it is scalable and cost-effective. Previously, we demonstrated the successful cell-free expression of very challenging parasite enzymes in fully functional form: P. falciparum dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) and Cryptosporidium parvum thymidine kinase (20, 21). The cell-free-produced DHFR-TS had all three known functions of native protein: DHFR catalytic activity, TS catalytic activity, and autologous RNA binding (20).

Since then, cell-free systems derived from wheat germ and E. coli have been used to express hundreds of malaria vaccine candi-
dates and a few enzymes (22–28), but the functional assessment of the protein products is mostly limited to autoradiograms, Western blots, and solubility.

With the ultimate goal of deriving a convenient, universal protocol for producing verifiable, functionally active malaria protein products, we selected 13 genes for expression tests, and we report on their expression in catalytically functional forms. The training set varied in terms of size, isoelectric point, and history of success in cell-based expression systems. We report an expression protocol which generates fully functional malaria proteins, without fail, in our test cases.

**MATERIALS AND METHODS**

All chemicals, unless noted, were purchased from Sigma-Aldrich Corp., St. Louis, MO. Salt-free-grade primers were obtained from Operon Biotechnologies (Huntsville, AL, USA). A Slide-A-Lyzer dialysis system (molecular-mass cutoff of 10 kDa) was from ThermoScientific (Rochester, NY, USA). All radiochemicals were from Moravek, Brea, CA. PCR premix was obtained from Bioline Inc., Taunton, MA.

**Construction of genes.** DNA primers (Table 2) for all of the selected test case malaria genes were designed based on PlasmoDB sequences (http://plasmodb.org/plasmo/). cDNA strands were synthesized from *P. falciparum* total RNA by using random hexamer primers and a reverse transcription kit from Life Technologies Inc., Carlsbad, CA. Double-stranded DNAs were then amplified from cDNAs using sequence-specific primers (Table 2). A typical 50-μl PCR mixture contained 25 μl of PCR premix, 0.25 μM forward and reverse primer of each respective gene, and 5 μl of cDNA. The mix was subjected to 25 cycles as follows: 98°C for 1 min, 98°C for 15 s, 55°C for 30 s, and 72°C for 1 to 5 min, depending on the size of the gene. The *PfDHFR-TS* and green fluorescent protein (GFP) were constructed as described before (20).

**Optimized codons for wheat system.** For some studies, malarial coding sequences were optimized to align with wheat codon usage and chemically synthesized (Geneart; Life Technologies). Synthetic codons of *P. falciparum* serine hydroxymethyltransferase (*PfSHMT*), *PfDHFR-TS*, and *P. falciparum* GTP cyclohydrolase I (*PfGTPCH*) were PCR amplified as before using gene-specific primers (Table 3).

**Cloning of genes into cell-free T-overhang vector.** An important component of developing a general method for expressing all malaria proteins was to have a streamlined, reliable cloning method. Cloning without using restriction enzymes was achieved by linearizing cell-free vector DNA and inserting a T overhang at the 3' end. In a 50-μl reaction mixture, 10 μg of cell-free vector, 160 units of EcoRV, 5 μl of 10X NRB buffer 3, and 5 μg of bovine serum albumin (BSA) were added, and the mixture was incubated for 18 h at 37°C. After complete digestion of the vector DNA, the restriction enzyme was heat inactivated at 80°C for 30 min, followed by purification of DNA using a PCR cleanup kit (Qiagen).
Inc., Valencia, CA, USA). A T overhang was then inserted into linearized vector DNA (29). Briefly, in a 50-µl reaction mixture, 1.5 µg of linearized vector DNA, 5 µl of 10× PCR buffer, 2 mM dTTP, 1.5 mM MgCl₂, 10 units of Taq DNA polymerase, and 0.2 mg/ml BSA were incubated at 70°C. After 150 min, salts and proteins were removed from the T-overhang DNA using a PCR cleanup kit. Freshly amplified target genes were directly ligated into a T-overhang expression vector, followed by transformation into DH5α competent cells (Invitrogen) which were spread on ampicillin-containing LB agar plates for overnight growth. Malaria gene inserts in selected colonies were confirmed by colony PCR by first using vector-specific primers. To confirm the correct orientation of the gene, the colony PCR product was subjected to a second round of PCR (round II PCR) using a vector-specific and enzyme-specific primer combination. After full DNA sequences were confirmed, plasmids were isolated using a plasmid midikit (Qiagen, Inc., USA).

Wheat germ lysate preparation. A wheat germ lysate preparation protocol was adapted from published work (30). Wheat seeds were cracked by hand mill. The germ fraction was separated from the cracked wheat by sequential sieving on 850-µm and 710-µm mesh. The portion retained on the 710-µm mesh was subjected to solvent separation using cyclohexane and carbon tetrachloride (vol/vol, 240:600). The solvent-floated germ fraction was collected and dried for 1 h in a fume hood.

Ten grams of solvent-floated germs was stirred magnetically in 1 liter of deionized water for 10 min. Germs were then sonicated for 5 min in 0.5% Igepal-CA 630 (Sigma). Detergent contaminant was removed by magnetically stirring germs in 1 liter of deionized water twice for 10 min each. Finally, germs were rinsed with 40 mM HEPES-KOH buffer, pH 7.8. Buffer-rinsed germs were crushed with mortar and pestle. To the germ paste, 10 ml of cold buffer 1 (40 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.5 mM magnesium acetate, 2 mM calcium chloride, 0.3 mM concentrations of 20 amino acids, and 5 mM dithiorthreitol [DTT]) was added, and the sample was ground. The ground germ slurry was then transferred to a fresh autoclaved centrifuge tube and spun for 30 min at 35,000 × g. After careful removal of the fat layer, the supernatant was spun again at 35,000 × g for another 30 min to repeat the fat removal step. The collected supernatant was then filtered using a Sephadex G25 column (GE Healthcare Biosciences, Pittsburgh, PA, USA), preequilibrated with buffer 1. RNA in the lysate was quantitated by measuring absorbance at 260 nm, and protein concentration was determined using the Bradford method. The concentration of lysate was adjusted to 150 units per ml of lysate. Lysates were stored at −80°C in small aliquots. One unit of wheat germ lysate (175 µg of protein) produces 8 µg of GFP in 24 h in a 25-µl dialysis-based translation reaction volume at 26°C.

Cell-free transcription. Cell-free transcription and translation reactions were carried out as reported previously (30). A typical transcription reaction was carried out in 100 µl with 10 µg of target gene carrying plasmid, 80 mM HEPES-KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 50 mM β-mercaptoethanol, 40 units of RNase inhibitor (NEB), 130 units of SP6 RNA polymerase (Epicentech Biotechnologies, Madison, WI, USA), and 3 mM (each) GTP, ATP, CTP, and UTP. The transcription mixture was incubated at 37°C for 3 h. Pyrophosphate-induced precipitate in the transcription reaction was pelleted by spinning at 8,000 × g for 2 min. The mRNA in the supernatant was precipitated by adding 20 µl of 7.5 M ammonium acetate and 300 µl of ethanol. After 10 min on ice, the mixture was spun at 21,000 × g for 20 min; the pellet was then washed with 200 µl of 70% ethanol and spun at 21,000 × g for 2 min. The pellet was dried for 10 min and dissolved in 30 µl of autoclaved deionized water. Typical mRNA concentrations were in the range of 1 to 1.5 mg per ml of transcription product.

Cell-free translation. The cell-free translation of the purified mRNA was carried out using a dialysis reaction method (31). A 100-µl reaction mixture contained 30 µl of mRNA (100 to 150 µg), 4 units of wheat germ lysate, 20 µg of creatine kinase, 40 units of RNase inhibitor, and 20 µl of 5× protein expression buffer (PEB) (1× PEB is 30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 5 mM DTT, 0.4 mM spermidine, 0.3 mM concentrations of 20 amino acids, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate). The translation reaction mixture was poured into a Slide-A-Lyzer Mini 10-kDa dialysis unit and immersed in a 1.5-ml microcentrifuge tube containing 1.2 ml of 1× PEB. After incubation at 26°C for 24 h, the malaria gene-translated lysate from the dialysis cup was collected and used for solubility analysis by spinning for 15 min at 20,000 × g and 4°C; the supernatant was used for further studies. For protein quantification experiments, [35S]GFP was added at 9.57 µCi (0.25 µCi/ml), and nonlabeled leucine concentrations were set at 108 µM during translation. Incorporation of labeled leucine into newly translated protein was quantified by trichloroacetic acid (TCA) precipitation assay (32).

For the autoradiogram experiments, a batch reaction method was used. Gel-filtrated transcripts (12 µg) served as templates for every 50-µl translation reaction mixture. The concentrations of [35S]GFP-labeled leucine and nonlabeled leucine were 153 µM (0.2 µCi/ml) and 84 µM, respectively. After a 4-h incubation at 26°C, the soluble fractions were prepared as mentioned earlier, resolved by SDS-PAGE, and exposed to a phosphor-imager screen (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Enzyme assays. In addition to assessing the expression of malaria proteins in cell-free expression systems by PAGE (see above), we tracked individual enzymatic activities corresponding to the predicted function of the P. falciparum gene to truly determine functional expression of each of the malaria proteins. For every assay, we looked at product formation as a function of time and as a function of expressed lysate. GFP-expressed lysate served as a positive control for the health of the translation system and as a negative control for expression of the target malaria enzyme. The enzymes assayed and their abbreviations are listed in Table 4.

dUTPH. The release of phosphate from dUTP by the action of dUTPase (dUTPH) and pyrophosphatase is based on capture of inorganic phosphate by malachite green and the resulting color change (33). To a 500-µl reaction mixture containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 2 mM DTT, 100 µM dUTP, and 5 units of pyrophosphatase, various quantities of 20-fold dilutions of either dUTPH-or GFP-translated lysates were added, and the mixture was incubated at 25°C. At different time intervals, 100 µl of malachite green was added, and absorbance was monitored at 600 nm against the reaction buffer and pyrophosphatase blank. A standard calibration curve for various concentrations of inorganic phosphate was prepared as per the malachite green assay kit instructions (BioAssay Systems, Inc., Hayward, CA, USA).

SAHH. Release of L-homocysteine from S-adenosyl homocysteine (SAH) was monitored based on a colorimetric reaction with dithio-bis-2-nitrobenzoic acid (DTNB) (34). To a 500-µl reaction mixture containing 50 mM potassium phosphate buffer, pH 8.0, and 50 µM SAH, various quantities of 2-fold dilutions of S-adenosyl homocysteine hydrolase (SAHH) - and GFP-translated lysates were added, and the mixture was incubated at 25°C. At various time intervals, DTNB was added to a final concentration of 100 µM and monitored at 412 nm. Reaction mixture with DTNB and without lysate served as a blank.

ADA. Deamination of adenosine was directly monitored by tracking changes in absorption at 265 nm (35). The 500-µl reaction mixture contained 50 mM potassium phosphate buffer, pH 8.0, and 2 mM DTT. Various quantities of adenosine deaminase (ADA) - and GFP-translated proteins were added, and the mixture was incubated at 25°C. At various time intervals, ADA was added to a final concentration of 100 µM and monitored at 265 nm. Reaction mixture with ADA and without adenosine served as a blank.

Expression of Functional P. falciparum Proteins

| Primer name       | Primer sequence (5’ to 3’)               |
|-------------------|------------------------------------------|
| SHMT-Forward      | CGAGATGTTCAACAGACGACC                   |
| SHMT-Reverse      | GCCGGTACAGCGGAATGG                      |
| DHFR-TS-Forward   | TCGAGATGGAGCAAGTCTGTG                   |
| DHFR-TS-Reverse   | GCCGGTACAGCGGCGCATGT                   |
| GTPCH-Forward     | GAGGTGTACCCTTGACATCTCG                 |
| GTPCH-Reverse     | GAGGTGTACAGCTACATCGC                   |
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TABLE 4 Protein quantitation and functional analyses of malarial enzymes expressed in a wheat cell-free system

| Gene identifiera | P. falciparum enzyme (abbreviation) | Mass (kDa) | pI | Total synthesized protein (µg/mg lysate) | Soluble protein (µg/mg lysate) | Activity (nmol/min/mg lysate) | Specific activity (µmol/min/mg) |
|------------------|-----------------------------------|-----------|-----|----------------------------------------|-------------------------------|-------------------------------|-------------------------------|
| PF3D7_0321000    | Dihydrofolate reductase (DHFR-TS) | 26.8      | 5.8 | 44.4                                   | 43.7                          | NA                           | NA                            |
| PF3D7_1415000    | Orotidine-5′-triphosphate decarboxylase (OMPD) | 38.8      | 7.7 | 40.2                                   | 35.4                          | 285.7                        | 8.07                          |
| PF3D7_0520900    | Dihydrofolate synthase (DHFR)     | 49.7      | 8.1 | 37                                     | 24.6                          | 23.3                         | 0.94                          |
| PF3D7_0512700    | Orotidine-5′-phosphate transferase (OPRT) | 33.0      | 7.4 | 31.8                                   | 10                            | 35.7                         | 3.57                          |
| PF3D7_1127100    | Serine hydroxymethyltransferase (SHMT) | 41.7      | 7.4 | 21.6                                   | 8                             | 1.78                         | 0.22                          |
| PF3D7_0513300    | Orotic acid dehydrogenase (OAS)   | 37.8      | 7.7 | 40.2                                   | 35.4                          | 285.7                        | 8.07                          |
| PF3D7_0513300    | GFP                                | 38.8      | 7.7 | 40.2                                   | 35.4                          | 285.7                        | 8.07                          |

a Gene identifiers are from PlamoDB.

b Putative.

c GFP was included as a control.

d NA, not applicable.

lysates were added, and the starting absorption was read at 265 nm. The deamination reaction was initiated by the addition of 100 µM adenosine, and the decrease in absorbance was monitored at 265 nm. The absorption coefficient for adenosine is 8,400 M⁻¹ cm⁻¹.

PNP. Release of the hypoxanthine base from the nucleoside substrate inosine was coupled to its oxidation of uric acid (36). To a 500-µl reaction mixture containing 50 mM potassium phosphate buffer, pH 8.0, 100 µM inosine, 2 mM DTT, and 1 unit of xanthine oxidase, various quantities of either purine nucleoside phosphorylase (PNP)-translated lysate or GFP-translated lysate were added. After an initial reading at 293 nm, the reaction was monitored by tracking the increase in absorbance at 293 nm. The absorption coefficient for uric acid at 293 nm is 12,900 M⁻¹ cm⁻¹.

OMPD. Decarboxylation of orotidine-5′-monophosphate (OMP) to UMP was monitored directly at 285 nm (37). The 500-µl reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 2 mM DTT, and 1 unit of xanthine oxidase, various quantities of either purine nucleoside phosphorylase (PNP)-translated lysate or GFP-translated lysate were added. After an initial reading at 293 nm, the reaction was initiated by the addition of 200 µM OMP to the final concentration, and the decrease in absorbance was monitored at 285 nm. The absorption coefficient for OMP at 285 nm is 1,650 M⁻¹ cm⁻¹.

DHFR-TS. The more fragile thymidylate synthase reaction in the bifunctional parasite enzyme was followed by tracking the release of tritiated water from a 5-fluoro-2′-deoxyuridine-labeled substrate (19). To a 75-µl reaction mixture containing 33 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer, pH 7.0, 113 µM tetrahydrofolate, 16 mM MgCl₂, 0.7 mM EDTA, 50 mM β-mercaptoethanol, 3.25 mM formaldehyde, 6.47 mM cold dUMP, and 0.196 µM 5-[3-H]labeled dUMP (2.66 µCi/ml), various quantities of 25-fold dilutions of DHFR-TS-translated or GFP-translated lysates were added, and the mixture was incubated at 26°C. At various time intervals, the reactions were stopped by the addition of 20 µl of stop solution (3 parts 2N TCA and 1 part 4.34 mM dUMP). Unreacted [5-3H]dUMP was then separated from tritiated water by absorption to 200 µl of 10% cold charcoal on ice for 15 min. The samples were then centrifuged for 10 min at 14,000 × g, followed by the transfer of 120 µl of supernatant to scintillation vials to determine the release of radioactive water.

SHMT. After the transfer of methylene from L-[3-3H]serine substrate to [3-3H]methylene tetrahydrofolate (MTHF), the product was captured on positively charged DE81 filter paper (6, 38). To a 50-µl reaction mixture containing 50 mM Tris-HCl buffer, pH 8.0, 5 mM β-mercaptoethanol, 2.6 mM tetrahydrofolate (THF), 0.25 mM pyridoxal phosphate, 2.5 mM EDTA, 0.9 mM L-serine, 64 nM L-[3-3H]serine (2 nCi/ml), various quantities of newly expressed SHMT- or GFP-translated lysates were added and kept at 37°C. At various time intervals, 10 µl of reaction mixture was spotted onto DE81 filter papers, which were dried and washed in deionized water. The filters were dried again, and radioactivity was determined by scintillation counting.

GAK. To track phosphorylation of [8-14C]AMP, the product [8-14C]AMP (97 µM) was added to a reaction mixture containing 110 mM potassium phosphate buffer, pH 6.0, 1.5 mM MgCl₂, 60 mM KCl, 2 mM DTT, 0.25 µM FeSO₄, 2 mM cold AMP, and various quantities of GTP:AMP phosphotransferase (GAK)- or GFP-translated lysates was kept at 25°C. At various time intervals, 2 µl of sample was transferred to a PEI-cellulose thin-layer chromatography (TLC) plate, followed by drying with hot air and separation in 0.375 M potassium phosphate solution, pH 3.5. The PEI-cellulose plate was then exposed to a phosphorimager, and an autoradiogram was developed.

OPRT. Attachment of a phosphoribosyl from phosphoribosylpyrophosphate (PRPP) to orotic acid can be monitored by direct increases in absorbance at 295 nm (15). To a 500-µl reaction mixture containing 50 mM Tris-HCl buffer, pH 8.0, 2 mM β-mercaptoethanol, 5 mM MgCl₂, 0.4 mM OMP, and 1 mM pyrophosphate, various quantities of orotate phosphoribosyl transferase (OPRT) or GFP-translated lysate were added. Initial absorption was recorded at 295 nm, and the increase in absorbance was monitored. The absorption coefficient for this reaction at 295 nm is 3,670 M⁻¹ cm⁻¹.

DH0ase. The assay method exploits the difference in the mobilities of [2-14C]dihydroorotic acid and [U-14C]-N-carboxymyl aspartate on a PEI-cellulose TLC plate (39). To a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 1.5% dimethyl sulfoxide (DMSO), 0.2 mM ZnCl₂, 5% glycerol, 2 mM DTT, and 30 µM [2-14C]dihydroorotate.
(DHO; 1.66 μCi/ml), various quantities of 14-fold dilutions of dihydro-orotase (DHOase)- or GFP-translated lysate were added and kept at 37°C. At various time intervals, 2 μl of reaction mixture was spotted onto PEI-cellulose TLC plates. The chromatogram was developed in 0.5 M LiCl₂ solution, pH 5.2. The PEI-cellulose plate was then exposed to a phosphorimager, and the autoradiogram was developed to quantitate product formation.

UDgly. The uracil DNA glycosylase (UDgly) enzyme removes a uracil residue from DNA, and the assay exploits the hydrolytic sensitivity of the resulting abasic single-stranded DNA under alkaline conditions (40). The end labeling of oligonucleotide DNA substrate was achieved by standard protocol using [γ-32P]ATP and T4 polynucleotide kinase. To a 20-μl reaction mixture containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 4 mM DTT, and 0.118 μM (14,000 cpm) 14C-labeled oligonucleotide (5‘-T GGGAAAAGGGGUTGCTGCAAGGCG-3‘), various quantities of UDgly- or GFP-translated lysates were added, and the mixture was then incubated at 26°C. At various time intervals, the reaction was stopped by the addition of NaOH to a final concentration of 50 mM, the product was separated on a 12% nondenaturing polyacrylamide gel, and an autoradiogram was developed.

GTPCH. Conversion of the purine nucleotide GTP to a pteridine precursor was tracked based on a difference in the mobilities of GTP and formic acid on PEI-cellulose under acidic conditions. To a 20-μl reaction mixture containing 100 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM EDTA, 5% glycerol, 2 mM DTT, and 0.38 μCi/ml, various quantities of GTPCH- or GFP-translated lysates were added, and the mixture was incubated at 37°C. At the end of 1 h, 2 μl of the reaction mixture was spotted on PEI-cellulose plates, followed by chromatography in a solvent with 0.375 M potassium monobasic acid, pH 3.5. The PEI-cellulose plate was then exposed to a phosphorimager, and the autoradiogram was developed to quantify product formation.

DHS-FPGS. In the folypolyglutamate synthetase (FPGS) assay, the attachment of radioactive glutamic acid residues to tetrahydrofolate substrate was followed based on the difference in the mobilities of glutamic acid and polyglutamated tetrahydrofolate on PEI-cellulose (41). To a 20-μl reaction mixture containing 50 mM Tris-HCl, pH 8.8, 30 mM KCl, 10 mM MgCl₂, 5 mM ATP, 2.0 mM β-mercaptoethanol, 50 μM tetrahydrofolate, 2.85 mM cold 1-glutamic acid, and 0.115 mM 14C-labeled 1-glutamic acid (2.5 μCi/ml), various quantities of DHS-FPGS- or GFP-translated lysates were added. After incubation at 37°C for various time intervals, the reaction was stopped by spotting 2 μl on PEI-cellulose TLC strips. The TLC plate was developed in 0.1 M potassium phosphate buffer containing 2% butanol and 0.5% β-mercaptoethanol, and the product was measured using a phosphorimager.

 Autoradiography conversions. The pixel count from visual assay data (for assay of GAK, DHOase, UDgly, GTPCH, and DHS-FPGS) was quantified by ImageJ software and converted into activity by using equation 1:

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\text{Pixel count for product} \times \text{substrate concentration (μM)} = \text{reaction volume (liters)}
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\text{Pixel count for substrate} \times \text{reaction time (min)} \times \text{lysate (ml)}
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RESULTS

Test panel for functional expression. To test the general utility of wheat germ cell-free expression systems for producing functional malaria enzymes, we sought out 13 P. falciparum genes as test cases. The choices were based on our long-term interest (6, 13, 19, 20, 38, 42–44) in studying nucleotide and DNA metabolism for malaria drug development. Enzymatic assays exist for the predicted functions of all of these proteins. The molecular masses of selected test cases ranged between 19 and 71 kDa, while the pl values ranged from 5 to 10. The selected proteins and their properties are shown in Table 4. To make sure our test system is truly versatile, we selected representatives of many enzymes which previously failed to express in the hands of other investigators and rare enzymes that do express well in traditional cell-based systems (7, 11). Some from the first group are of special interest because they have properties of good drug targets.

Expression plasmid. The plasmid for expression of malaria proteins in wheat germ systems has an SP6 bacteriophage transcription promoter and a plant-like omega sequence for initiation of translation (30). These expression controls are orthogonal to the native E. coli gene expression system; malaria genes were successfully maintained for extensive periods without rearrangements or deletions driven by toxic effects of leaky expression.

Efficient cloning. An important long-term goal of this project is to set the stage for efficient, high-throughput expression of many functional malaria proteins in parallel. To bypass potential genome annotation issues, all chosen genes were directly reverse amplified from total RNA of the parasite, and the PCR products were directly cloned into a T-overhang cell-free vector. The overall transformation efficiency for the desired gene was approximately 60%, with 50% of the inserts having the correct orientation (data not shown). The stability of the expression plasmids was verified through their repeated successful isolation from stock cultures and successful protein expression without any deletion or loss of function for the cell-free expression over 4 years (data not shown).

P. falciparum enzyme synthesis. All selected P. falciparum enzymes were first generated in a batch reactor with radioactive leucine and a native malarial coding sequence (Fig. 1A). The majority of the proteins even at the first attempt generated clean, single, new protein products, attesting to the high quality of the wheat germ cell-free lysate and the lack of significant contamination from proteases and RNases. There was good correlation between expected and observed masses of the translated enzymes (Fig. 1B).
The exceptions were the SHMT products, which had some truncated proteins, and GTPCH, which had low expression. Experimental attempts to prevent the fragmentation of SHMT during translation using a protease inhibitor cocktail or by addition of an RNase inhibitor or the missing cofactor pyridoxal phosphate (PLP) did not improve the quality of the produced protein (data not shown). As discussed below, we found other generalizable ways to remove truncated products and improve yields of the two poorer-performing proteins.

Comparison to wheat germ protein background. Scale-up cell-free protein expression was carried out from native P. falciparum coding sequences using a dialysis reaction method. The continuous addition of fresh translational substrates and removal of by-products as part of the dialysis procedure allowed the target protein to be produced continuously over a period of 24 h. The soluble proteins produced in the translated lysates were resolved by 12% SDS-PAGE and visualized directly by Coomassie staining (Fig. 2). Nine of the 13 test cases were directly and clearly visible on the Coomassie-stained gels. Quantification of the total and soluble expressed proteins was achieved by TCA precipitation of radiolabeled proteins (Table 4).Depending on the product, the dialysis reactor generated 23 to 248 mg of parasite soluble protein per 7 mg of translated lysate in a typical reaction mix.

Functional assays. Direct assay for predicted enzymatic activity served as a measure of both intact production and correct folding of the proteins. Malaria proteins were expressed using scaled-up translated lysates (Fig. 3A to M). For every malaria enzyme expressed, GFP-translated lysate served as the control to determine the background activity from the wheat germ lysate.

Individual assays were established for each of the malaria enzymes using a variety of analytical tools, including UV-visible spectrophotometry (dUTP, SAHH, ADA, PNP, OMPD, and ADA) (Fig. 3A to E and H), scintillation counting (DHFR-TS and SHMT) (Fig. 3F and G), TLC (GAK, DHOase, GTPCH, and DHFR-FPGS) (Fig. 3J, L, and M), and polycrylamide gel electrophoresis (UDgly) (Fig. 3K). The GTPCH assay was developed in-house, but all other assays were adopted from previous reports. For most cell-free-expressed P. falciparum enzymes, three different concentrations of enzyme at three different time points were tested. However, for TLC and polycrylamide gel based assays (Fig. 3I to M), data are shown only for single concentrations (remaining data for the other two concentrations are not shown). The pixel counts from visual data (Fig. 3I to M) were quantified by ImageJ and converted into activity by using equation 1. Specific activities were calculated using the quantity of enzymatic product generated from a known amount of total protein in the lysate (Table 4).

While most P. falciparum proteins expressed well in the cell-free system using native sequences from cDNA, one (SHMT) displayed truncated protein products, and another (GTPCH) showed low expression. To address these two issues, we optimized the coding sequences for wheat. Synthetic codons were generated for three of the test cases: SHMT, DHFR-TS, and GTPCH. As shown in the autoradiogram (Fig. 4), synthetic codons were advantageous since they not only eliminated premature termination of protein translation (Fig. 4A) but also improved expression of the proteins (Fig. 4B). These results show that while direct cloning gives ample activity for the discovery phase of large screening projects involving many malaria proteins, both the quality and quantity of some high-priority malaria recombinant proteins can be improved by optimizing the codons by designing away from the parasite sequences and toward the wheat germ codon usage preferences.

Overall, the results convincingly show that it is possible to express all tested malaria proteins in functional form. In all cases, including those of the poorly soluble proteins, 1 to 50 μg of the translated lysate was sufficient to demonstrate enzymatic activity. The system is very well suited for parallel production of many proteins as well as scaled-up production of individual enzymes.

DISCUSSION

Historically, enzymology has helped us understand the action of several clinically important antimetabolites against malaria parasites; the species-selective action of pyrimethamine against malarial dihydrofolate reductase described by Ferone et al. was a seminal study in pharmacology (42). Broader species-specific nucleic acid-binding properties of this parasite enzyme (which limit its expression) may also contribute to the high value of this target (13, 43).

Even with these celebrated roles of enzymology in understanding antimalariais, there have been very few new enzyme targets or inhibitors that have led to the development of preclinical leads and clinical approval of new drugs (44–46; also http://www.mmv.org/research-development). Leads based on natural products and high-throughput screening in cell-based assays continue to dominate the drug development pipeline for antimalariais (47–49). Target validation often occurs through genetics (50), without strong high-end enzymology support.

Difficulties in expressing full-length functional malaria proteins vary. We have suggested that autologous feedback loops of malaria proteins (13, 20, 43) and the resulting potential nonspecific nucleic acid binding in closed heterologous expression systems (6, 13, 20, 43) limit protein expression in cells. The toxicity from leaky expression of malaria proteins can make it difficult for host cells to maintain expression plasmids in intact forms.

These ideas have emerged from 25 years of detailed study of the malaria drug target dihydrofolate reductase-thymidylate synthase. Until recently, it was difficult to express DHFR-TS protein in its complete form, and even this was possible only when the host
E. coli was under genetic or pharmacological pressure to maintain the parasite enzyme in its functional form \(12, 13, 19\). We were pleasantly surprised at the ease with which a wheat germ cell-free system could express P. falciparum DHFR-TS protein, especially without any extraordinary efforts to maintain the full expression plasmid under genetic or pharmacological pressure during its construction or maintenance \(20\). Of course, there are no worries about the potential toxicity of the malaria protein to the wheat plant because protein production is completely uncoupled from the growth and viability of the organism. This motivated us to study whether wheat germ expression could offer a general solution to the expression of malaria enzymes in functional forms.

In the present study, 13 different genes coding for enzymes related to nucleotide, folate, and DNA metabolism from the P. falciparum genome were subjected to expression tests in the wheat germ system. All the malaria genes could be readily cloned and maintained in the plant expression plasmid. The lack of toxicity in E. coli is probably related to the tight suppression of gene expression from the orthogonal nature of the regulatory controls between E. coli and the wheat system. Even without codon altera-

FIG 3 Functional analysis of P. falciparum enzymes produced in a cell-free system. Enzyme activities were directly measured in scaled-up expressed translated lysates either by spectrophotometry (A to E and H), scintillation counting (F and G), or visible assays by polyacrylamide gel (K) and TLC (I, J, L, and M). In all cases, GFP-translated lyte served as a control when used at levels corresponding to the largest amount of lyte in experimental functional assays. In the case of the visible assays, the pixel counts for radioactive substrates and products were quantified by ImageJ and converted into moles of activity by using equation 1 (Materials and Methods). Visible assays are shown only for single concentrations (remaining data for the other two concentrations are not shown), but the quantified activity is shown for all three concentrations of translated lysates. In visible assays, substrate-alone reactions are represented as controls (C).
tions, every metabolic enzyme was produced as the major or only new product upon translation. Every enzyme expressed had catalytic activity, irrespective of the metabolic pathway, protein size, pI, or presence of low-complexity sequences involving repeated amino acids. This ability to directly clone genes from cDNA libraries to proteins opens up many new frontiers for parallel interrogation of enzymes from malaria parasites.

Although we observed clean expression of the majority of the test cases using native malarial sequences, partial truncation of SHMT and low expression of GTPCH suggested that the intrinsic nature of some malaria coding sequences can contribute to lower performance even in a cell-free system. These challenges were eliminated with newly designed genes using codon preferences for wheat and with removal of internal RNA structures. Recent low-cost chemical synthesis of genes makes it feasible to use wheat germ codon usage as a regular direct step for expression of any high-priority malaria protein.

The sequencing of the genomes for the major human malaria parasites *P. falciparum* and *Plasmodium vivax* (3, 51) created much excitement for massive parallel inquiry into parasite biology, including possibly finding new drug targets. Indeed, there have been tremendous advances in exploiting parallel inquiry of DNA (3, 52–54), RNA (55–58), and even metabolites (59), but general ways to query proteins with known functions have remained elusive. Our systematic, successful study of 13 malaria enzymes in a wheat germ cell-free protein expression system may

**FIG 3 continued**
provide a universal solution for the expression of functional *P. falciparum* recombinant proteins. Now, there is realistic hope for producing large families of functional malaria proteins in parallel and perhaps all malaria enzymes.

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