Biochemical and Genetic Analysis of the Phosphoethanolamine Methyltransferase of the Human Malaria Parasite Plasmodium falciparum

Jennifer M. Reynolds, Sachiko Takebe, Jae-Yeon Choi, Kamal El Bissati, William H. Witola, April M. Bobenchik, Jeffrey C. Hoch, Dennis R. Voelker, and Choukri Ben Mamoun

Received for publication, December 4, 2007, and in revised form, January 2, 2008. Published, JBC Papers in Press, January 2, 2008. DOI 10.1074/jbc.M709869200

The PfPMT enzyme of Plasmodium falciparum, the agent of severe human malaria, is a member of a large family of known and predicted phosphoethanolamine methyltransferases (PMTs) recently identified in plants, worms, and protozoa. Functional studies in P. falciparum revealed that PfPMT plays a critical role in the synthesis of phosphatidylcholine via a plant-like pathway involving serine decarboxylation and phosphoethanolamine methylation. Despite their important biological functions, PMT structures have not yet been solved, and nothing is known about which amino acids in these enzymes are critical for catalysis and binding to S-adenosyl-methionine and phosphoethanolamine substrates. Here we have performed a mutational analysis of PfPMT focused on 24 residues within and outside the predicted catalytic motif. The ability of PfPMT to complement the choline auxotrophy of a yeast mutant defective in phospholipid methylation enabled us to characterize the activity of the PfPMT mutants. Mutations in residues Asp-61, Gly-83 and Asp-128 dramatically altered PfPMT activity and its complementation of the yeast mutant. Our analyses identify the importance of these residues in PfPMT activity and set the stage for advanced structural understanding of this class of enzymes.

About 41% of the population of the world lives in malaria endemic areas, and every year more than 2 million people die from the disease (1). Malaria is caused by an intraerythrocytic protozoan parasite of the genus Plasmodium. Of the four species that infect humans, Plasmodium falciparum causes the most lethal form of the disease and has developed resistance to almost all the available drugs in the antimalarial armamentarium (2). New chemotherapeutic strategies are now needed to combat this disease. One strategy is to target the metabolic pathways that the parasite uses to synthesize new membranes, which are critical for parasite development and multiplication within erythrocytes (3). Various inhibitors of lipid metabolism have been shown to inhibit P. falciparum proliferation in vitro and in vivo, and several efforts are being made to advance these compounds for treatment of malaria.

Phosphatidylcholine (PtdCho)3 composes half of the phospholipid content of the parasite membranes (4). Biochemical studies demonstrated that PtdCho synthesis occurs via two metabolic routes (5, 6). The first route is the CDP-choline pathway, which uses host choline as a precursor. In this pathway choline is first phosphorylated to phosphocholine and then converted to CDP-choline. Subsequently, CDP-choline and diacylglycerol function as substrates for PtdCho synthesis. The second route is the serine decarboxylation-phosphoethanolamine methylation pathway (6). This pathway uses serine either transported from the host or generated by degradation of host proteins as a phospholipid precursor. The serine is first decarboxylated to produce ethanolamine, by an unknown serine decarboxylase. The ethanolamine is next phosphorylated by a parasite-specific ethanolamine kinase. A SAM-dependent triple methylation of the resulting phosphoethanolamine by a plant-like phosphoethanolamine methyltransferase results in the synthesis of phosphocholine (6). This product is then integrated into the CDP-choline pathway for the synthesis of phosphatidylcholine (6, 7).

The transmethylation step of the serine decarboxylation-phosphoethanolamine methylation pathway is catalyzed by a 266-amino acid phosphoethanolamine methyltransferase, PfPMT, that shares high homology with known and predicted phosphoethanolamine methyltransferases from plants and worms (6 – 12). Biochemical studies in P. falciparum and genetic studies in yeast have demonstrated the specificity of the PfPMT enzyme for its substrate, phosphoethanolamine (6, 7). Interestingly, no PfPMT homologs could be found in mammalian databases, suggesting that this protein could be an ideal target for development of novel antimalarial lipid inhibitors.

3 The abbreviations used are: PtdCho, phosphatidylcholine; PfPMT, P. falciparum phosphoethanolamine methyltransferase; SAM, S-adenosyl-L-methionine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine.
In this paper we report the biochemical and genetic characterization of PfPMT using yeast as a surrogate system. Scanning alanine mutagenesis of 24 conserved residues was performed followed by analysis of the activity of the mutant enzymes in vitro and in vivo using a functional complementation assay in yeast. Three residues, Asp-61, Gly-83, and Asp-128, were found to play a critical role in PfPMT catalysis and substrate binding.

EXPERIMENTAL PROCEDURES

Strain Construction, Growth Conditions, and Media—The Saccharomyces cerevisiae strain pem1Δpem2Δ (Mata his3Δ1 leu2Δ0 ura3Δ0 pem1::Kan pem2::Kan) was used in this study. Standard methods for yeast culture and manipulation were used (13). Yeast was cultivated at 30 °C in yeast extract/peptone/dextrose or in synthetic minimal media containing 2% glucose (SD medium) or 2% galactose (SG medium) supplemented with histidine (30 μg/ml), leucine (100 μg/ml), ethanolamine (200 μg/ml), or choline (140 μg/ml) as required to maintain cell growth.

Site-directed Mutagenesis—To generate PfpmtD61A, PfpmtD61E, PfpmtD61N, PfpmtG83A, PfpmtD128A, PfpmtD128E, and PfpmtD128N mutants for expression in S. cerevisiae, site-directed mutagenesis was performed using the GeneTailor site-directed mutagenesis system (Invitrogen) with primers described in supplemental Table S1. The site-directed mutagenesis system (Invitrogen) with primers described in supplemental Table S1 was performed on the codon-optimized version of PfPMT (PfPMTco) cloned in the pYES2.1::GAL1 promoter that enables expression in the BL21-CodonPlus strain (Stratagene) as previously described (6).

Complementation Assay—attempts were made to delete the regulatory control of the GAL1 promoter that enables expression in the pYES2.1 vector containing either wild-type PfPMTco or mutant forms of PfPMTco were pre-grown to mid-log phase at 30 °C in synthetic uracil—dropout media containing 4% galactose (SG-URA medium) supplemented with ethanolamine (2 mM) and choline (2 mM). The cells were harvested by centrifugation, washed twice with water, and diluted to an A600 = 0.03 in 50 ml of SG medium lacking uracil (SG-URA) but supplemented with ethanolamine (2 mM) and grown to an A600 ~ 1.5. The cells were harvested by centrifugation and washed twice with water. The lipids were extracted as described previously (14). The phospholipids were separated by two-dimensional thin layer chromatography (TLC) on Silica 60 plates using chloroform/methanol/ammonium hydroxide (84.5/45.6 v/v/v) followed by chloroform/acetic acid/methanol/water (90/30/6/2.6 v/v/v). Lipids were visualized with iodine vapor and six classes of phospholipids (PtdCho, PtdEtn, PtdSer, PtdIns, Ptd_Gro (cardiolipin), phosphatidic acid) were excised from the plate and quantified by measuring phosphorus (15). The results are shown as the percentage of total lipid phosphorus in each phospholipid fraction. Data are the means ± S.D. for three independent experiments.

Analysis by Circular Dichroism—The purified proteins were dialyzed against 5 mM phosphate buffer, pH 6.8, containing 100 mM sodium chloride, and just before the measurement, the buffer was exchanged with 5 mM phosphate buffer, pH 6.8, containing 100 mM sodium fluoride. The protein concentration was determined using the Edelhoch method (16). The circular dichroism spectrum of the purified recombinant protein (10 μM) was recorded between 190 and 280 nm using a Jasco J-715 spectropolarimeter as previously described (17).

Enzyme Assays—The in vitro enzymatic activities of the purified wild-type and mutant Pfpmt proteins were determined by measuring the incorporation of radioactivity from [*methyl-14C]SAM into N-methylated derivatives of phosphoethanolamine as previously described (6). The kinetic properties of Pfpmt for the phosphoethanolamine substrate were determined under saturating concentrations of the cosubstrate SAM (2 mM) and with increasing concentrations of phosphoethanolamine (30 μM to 2 mM). Likewise, the affinity of Pfpmt for its cosubstrate SAM was determined by using different concentrations of SAM (30 μM to 2 mM) and a saturating concentration of phosphoethanolamine (2 mM). Fifty to 100 μg of purified enzyme was used for the assay.

RESULTS

Mutagenesis and Functional Complementation in Yeast—We have previously shown that a codon-optimized P. falciparum Pfpmt gene complements the choline auxotrophy of the yeast pem1Δpem2Δ mutant lacking the two phospholipid methyltransferases, Pem1p and Pem2p (7). To characterize residues in PfPMT that play a critical role in the transmethylation reaction leading to the formation of phosphocholine from phosphoethanolamine, a sequence alignment of PfPMT with PMTs from other species was analyzed (Fig. 1). Twenty-four conserved residues in or near the predicted catalytic motifs of PfPMT were mutated to alanine in the pYes-PfPMTco vector, which harbors the codon-optimized PfPMTco. In this vector expression of wild-type and mutant PfPMTs is under the regulatory control of the yeast GAL1 promoter that enables expression in the

Characterization of P. falciparum PfPMT

MARCH 21, 2008 • VOLUME 283 • NUMBER 12

JOURNAL OF BIOLOGICAL CHEMISTRY 7895
Characterization of *P. falciparum* PfPMT

**FIGURE 1.** Sequence alignment of the polypeptide sequences encompassing the catalytic motifs (I, I-I, II, and III) of phosphoethanolamine methyltransferases from *P. falciparum* (PfPMT; accession number ANAY429590), *Spinacia oleracea* (SoPMT; accession number AF237633), *Arabidopsis thaliana* (AtPMT; accession number AA614121), and *Brassica napus* (BnPMT; accession number ANAY319479). The asterisk (*) denotes the mutated residues, Asp-61, Gly-83, and Asp-128.

**FIGURE 2.** Functional complementation of choline auxotrophic *S. cerevisiae* strain, *pem1Δpem2Δ*, by expression of the wild-type (WT) PfPMT, mutated forms (PpmtD61A, PpmtG83A, PpmtD128A, PpmtG63A, PpmtG153A), or the empty expression vector. The strains were grown in minimal medium containing 2% glucose (D) or 2% galactose (G) and supplemented with histidine and leucine in the presence or absence of choline (C) or ethanolamine (E), and the images taken after 3 days of culture.

Presence of galactose as the sole carbon source but is repressed when glucose is added. The resulting wild-type and mutated proteins harbor a C-terminal V5 and hexahistidine double tag, which allows monitoring of expression in yeast. Transformants were selected on choline-containing medium and then tested for growth on glucose or galactose media lacking choline and/or supplemented with ethanolamine. Of the 24 mutants tested, only three, Asp-61, Gly-83, and Asp-128, failed to complement the choline auxotrophy of *pem1Δpem2Δ* mutant (Fig. 2). The mutations in PfPMT in these three mutants reside in catalytic motifs I and P-I and outside of catalytic motif II, respectively (Fig. 1). As expected, transformants harboring an empty vector failed to grow in the absence of choline, whereas those harboring a wild-type copy of PfPMT grew normally on galactose medium lacking choline (Fig. 2). To further investigate the phenotype of these three mutants, *pem1Δpem2Δ* strains harboring PfpmtD61A, PfpmtG83A, PfpmtD128A were inoculated at 10^9 cells/ml of culture, and cell growth was followed by measuring changes in cell density over time. As a control, *pem1Δpem2Δ* strains harboring an empty vector, wild-type PfPMT, or the mutated PfpmtG63A and PfpmtG153A, which were not affected by choline deprivation, were also tested. Using this assay, no growth could be detected in *pem1Δpem2Δ* harboring PfpmtD61A, PfpmtG83A, and PfpmtD128A during 2 days of culture in galactose medium lacking choline. The phenotype of strains expressing PfpmtD61A, PfpmtG83A, and PfpmtD128A was indistinguishable from that of *pem1Δpem2Δ* harboring an empty vector (Fig. 3). By comparison, strains expressing PfpmtD61A showed a severe growth defect during the first 48 h of incubation, but a significant increase in cell density was observed after 2.5 days of incubation. The *pem1Δpem2Δ* strains harboring wild-type PfPMT or mutated PfpmtG63A or PfpmtG153A exhibited a similar growth rate in galactose medium lacking or supplemented with choline (Fig. 3). To further investigate the side chain contribution in residues Asp-61 and Asp-128 in PfPMT activity, these amino acids were also mutated to glutamate and asparagine, and the growth of the *pem1Δpem2Δ* strains harboring PfpmtD61E, PfpmtD61N, PfpmtD128E, and PfpmtD128N was monitored. Similar to PfpmtD61A and PfpmtD128A mutants, PfpmtD61E, PfpmtD61N, PfpmtD128E, and PfpmtD128N mutants did not grow in galactose medium lacking choline (Fig. 3). As expected, none of the strains grew in glucose medium lacking choline. To rule out the possibility that the lack of activity was due to lack or reduced expression of recombinant proteins in yeast, immunoblot analyses were performed on protein extracts from *pem1Δpem2Δ* strains harboring wild-type or mutated PfPMT proteins using anti-V5 antibodies. Similar PfPMT expression levels were detected in all strains, and as expected, no signal could be detected using protein extracts from *pem1Δpem2Δ* strains harboring an empty vector (Fig. 4). As a control, antibodies against the yeast hexokinase were used and showed similar levels in all strains (Fig. 4).
Alteration in PtdCho Biosynthesis in Pfpm Mutants—The inability of PfpmD61A, PfpmG83A, and PfpmD128A mutations to complement the choline auxotrophy of pem1 pem2 strains might be due to their inability to restore the synthesis of PtdCho. Phospholipids were prepared from pem1 pem2 strains harboring wild-type and mutant forms of PfPMT, grown in the absence of choline, analyzed by TLC, and quantified (Fig. 5 and Fig. 6). Whereas no differences in the levels of PtdEtn could be detected among the various strains tested, the PtdCho level was dramatically affected in the pem1 pem2 strain expressing PfpmG83A, PfpmD128A, or PfpmD61A. The PtdCho content of the pem1 pem2 mutant harboring a wild-type PfPMT was 14% that of total phospholipid. In contrast, the pem1 pem2 strain expressing PfpmD61A had PtdCho contents of ~0.5, ~2, or ~3%, respectively (Fig. 5). As expected for the pem1 pem2 strain containing PfpmG63A or PfpmG153A, the PtdCho contents were 7 and 13%, respectively, consistent with their ability to grow in the absence of exogenous choline. The PtdSer level was also significantly lower in the pem1 pem2 strain with PfpmD61A, PfpmD128A, or PfpmD61A compared with that with PfPMT, PfpmG63A, and PfpmG153A (Fig. 6). Conversely, the PtdIns level was ~3-fold higher in pem1 pem2 strain containing PfpmG83A, PfpmD128A, and PfpmD61A compared with PfPMT, PfpmG63A, and PfpmG153A (Fig. 6).

Biochemical Characterization of Recombinant Pfpm Mutants—To further examine the effect of the mutations on the folding and activity of the enzyme, recombinant wild-type (PfPMT) and mutant PfpmD61A, PfpmG83A, and PfpmD128A were expressed in E. coli, purified by affinity chromatography, and analyzed for folding properties by circular dichroism (CD) spectrophotometry (Fig. 7A). The CD profiles of PfpmD61A, PfpmG83A, and PfpmD128A were similar to that of wild-type PfPMT, with a strong peak around 222 nm indicative of extensive helix content. The molar ellipticity of each mutant is consistent with normally folded proteins when compared with wild type. The secondary structure content of PfpmG63A and PfpmG153A (Fig. 6).

Characterization of P. falciparum PfPMT
Characterization of \textit{P. falciparum} PfPMT

\textbf{FIGURE 5. Two-dimensional TLC fractionation of the phospholipid constituents of pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} strains expressing wild-type PfPMT, mutated forms (Pfpmt\textsuperscript{D61A}, Pfpmt\textsuperscript{G83A}, Pfpmt\textsuperscript{D128A}, Pfpmt\textsuperscript{G63A}, and Pfpmt\textsuperscript{G153A}), or the empty vector, grown in the absence of choline.} The lower right-most panel depicts the expected positions for the different phospholipids (PtdOH, phosphatidic acid; PtdGro, cardiolipin) fractionated by two-dimensional TLC.

\textbf{FIGURE 6. Measurement of lipid phosphorous in fractionated constituent phospholipids of pem1\textsuperscript{Δ}pem2\textsuperscript{Δ} strain expressing wild-type PfPMT, mutated forms (Pfpmt\textsuperscript{D61A}, Pfpmt\textsuperscript{G83A}, Pfpmt\textsuperscript{D128A}, Pfpmt\textsuperscript{G63A}, and Pfpmt\textsuperscript{G153A}) or the empty vector, grown in the absence of choline.} The phosphorus content in the respective phospholipids is depicted as the percentage of the total phospholipids. Data are the mean ± S.D. of triplicate assays.

\(T_m\) close to that of wild type, whereas Pfpmt\textsuperscript{D61A} and Pfpmt\textsuperscript{D128A} exhibited \(T_m\) values above and below wild type, respectively (Fig. 7B). The thermal unfolding transition for Pfpmt\textsuperscript{D128A} is broader than wild type, consistent with a less cooperative unfolding transition expected on the basis of lower helical content.

Pfpmt\textsuperscript{D61A}, Pfpmt\textsuperscript{G61E}, Pfpmt\textsuperscript{D61N}, Pfpmt\textsuperscript{G83A}, Pfpmt\textsuperscript{D128A}, Pfpmt\textsuperscript{D128E}, and Pfpmt\textsuperscript{D128N} activities were also measured \textit{in vitro} and compared with that of wild-type PfPMT to determine the kinetic parameters of the SAM-dependent methylation of phosphoethanolamine. The apparent \(K_m\) of wild-type PfPMT for SAM and phosphoethanolamine was estimated to be \(\sim 35.23 \text{ and } 66.1 \mu M\), respectively, whereas its \(V_{max}\) for SAM and phosphoethanolamine was 0.30 and 0.95 nmol/min/mg protein, respectively (supplemental Figs. S1 and S2). No activity could be detected with the mutated Pfpmt proteins.

\textbf{DISCUSSION}

The transmethylation of phosphoethanolamine by the malarial phosphoethanolamine methyltransferase, PfPMT, is critical for the synthesis of PtdCho in the human parasite \textit{P. falciparum} (5, 7). Genetic and pharmacological studies suggest that this step may also play an essential role during various stages of the parasite life cycle.\(^4\) Understanding the biochemical and structural properties of this enzyme may help in the design of specific inhibitors that could block the enzyme activity and interfere with parasite proliferation inside human red blood cells.

PfPMT is a member of an unusual class of SAM-dependent methyltransferases also found in worms and plants but absent in mammals (8–12). They share significant sequence similarity but seem to have evolved different structural and biochemical properties. The plant PMTs have two catalytic domains, with the N-terminal domain involved in the methylation of phosphoethanolamine into mono- and dimethylphosphoethanolamine and the C-terminal domains involved in the last two methylation reactions to form phosphocholine (8, 10, 11). \textit{Caenorhabditis elegans} has two PMT enzymes (Pmt1 and Pmt2) with a length similar to that of the plant PMTs but each having a single catalytic domain located in either the N-terminal domain in Pmt1 or to the C-terminal domain in Pmt2 (9, 12). Pmt1 catalyzes only the first methylation reaction, whereas Pmt2 catalyzes the last two methylation reactions. Although the malarial PfPMT shares homology with both plant and \textit{C. elegans} PMTs, it is only half the size of these proteins and catalyzes the three methylation steps (6). All PMT enzymes have similar steady state kinetic properties for SAM and phosphoethanolamine. Although the function and biochemical properties of this class of enzymes have only started to be elucidated (5–12, 18, 19), nothing is known about their structure, and the residues within these enzymes that are critical for catalysis and substrate binding are unknown.

The studies reported here provide the first structure-function analysis of the activity of a PMT enzyme. These studies

\(^4\) W. H. Witola \textit{et al.} unpublished data.
benefited from the previous genetic analysis in yeast, which showed that a codon-optimized PfpmtD61A gene complements the choline auxotrophy of a mutant pem1Δpem2Δ, lacking PEM1 and PEM2 genes encoding the methyltransferase enzymes essential for the transmethylation of PtdEtn to form PtdCho.

Twenty-four residues within and outside the catalytic core of the enzyme were mutated to alanine, and the corresponding genes were expressed in the pem1Δpem2Δ strain under the regulatory control of the GAL1 promoter. Our studies revealed that amino acids Asp-61, Gly-83, and Asp-128 play a critical role in enzyme activity. Asp-61 is located within the motif II NNFDLIYS. Its mutation to either asparagine or glutamate resulted in loss of complementation of Pptm D61A. This amount of PtdCho has been shown to allow survival but not optimal growth of pem1Δpem2Δ cells in the absence of choline (7). Although this partial complementation suggests that the enzyme might have suboptimal activity, in vitro transmethylation activity assay using recombinant PfpmtD₆₆₁₄ enzyme showed no activity. This discrepancy could be due to the differences in sensitivity between the genetic survival assay in yeast and the in vitro transmethylation assay.

Gly-83 is located within the motif P-I HGID of Pfpmt. This residue is also conserved in all PMTs, and like Asp-61, the mutation to alanine resulted in enzymatic loss of affinity for SAM and phosphoethanolamine as well as a concomitant complete loss of function in vivo. Consistent with this loss of activity, phospholipid analysis of pem1Δpem2Δ cells harboring this mutant enzyme revealed a PtdCho content that was reduced by 96% compared with that of pem1Δpem2Δ cells harboring a wild-type Pfpmt.

Asp-128 is located outside the predicted motif II NNFDLIYS. This residue is also conserved in all PMTs and is strictly essential for Pfpmt activity. Its mutation to alanine resulted in a complete loss of activity in vitro and lack of complementation in yeast. Consistent with this loss of activity, phospholipid analysis of pem1Δpem2Δ cells harboring this mutant enzyme revealed a PtdCho content of ~2%.

The molar ellipticity of the mutants relative to WT Pfpmt makes clear that the loss of function of the mutants does not result from failure to fold. The secondary structure content of D128A and D61A, which is lower and higher than WT, respectively, is consistent with the lower and higher thermal stabilities. Yet both display complete loss of enzymatic activity. We hypothesize that the side chain of Asp-128 is involved in favorable electrostatic interactions, whereas that of Asp-61 is involved in unfavorable electrostatic interactions, thus leading to the observed decrease (D128A) or increase (D128A) in stability upon mutation to alanine. NMR structural studies with the wild-type Pfpmt and point mutants are under way. The ionization states and pKₐ values of the critical aspartate residues, which can be determined by NMR, will be especially valuable for elucidating the physical basis of catalysis and binding. The mechanism underlying the loss-of-function in G83A is intriguing. Glycine is unlikely to play a direct role in catalysis or binding and yet the mutation does not appear to substantially alter the structure or stability. A potential mechanism is the loss of flexibility needed to undergo required conformational change. Such a dynamic effect could be revealed by thermodynamic or NMR relaxation studies.

In conclusion, we have identified three residues in Pfpmt that are critical for enzymatic activity. Further investigations are planned to elucidate the structural, thermodynamic, and catalytic roles played by these residues. The insights derived from these studies will enable intelligent screening of potential inhibitors, whether by NMR, high throughput screening methods, or in silico.

Acknowledgments—We are grateful to Harriet Zawistowski (General Clinical Research Center, University of Connecticut Health Center) for technical help. We thank Glenn King, Scott Robson, Li Luo, Oksana Gorbatyuk, and Iulian Rujan for technical assistance with the CD studies. The University of Connecticut Health Center General Clinical Research Center is supported by National Institutes of Health Grant M01RR06192.
Characterization of P. falciparum PfPMT

REFERENCES

1. WHO Expert Committee on Malaria (2000) World Health Organ. Tech. Rep. Ser. 892, i–v, 1–74
2. White, N. J. (2004) J. Clin. Investig. 113, 1084–1092
3. Vial, H. J., and Ben Mamoun, C. (2005) in Molecular Approaches to Malaria (Sherman, I. W., ed) pp. 327–352, American Society for Microbiology, Washington, DC
4. Vial, H., and Ancelin, M. (1998) in Malaria: Parasite Biology, Biogenesis, Protection (Sherman, L., ed) pp. 159–175, American Society for Microbiology, Washington, DC
5. Pessi, G., and Ben Mamoun, C. (2006) Future Medicine, Future Lipidology, Vol. 1, pp. 173–180, www.futuremedicine.com/loi/flp
6. Pessi, G., Kociubinski, G., and Ben Mamoun, C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6206–6211
7. Pessi, G., Choi, J. Y., Reynolds, J. M., Voelker, D. R., and Ben Mamoun, C. (2005) J. Biol. Chem. 280, 12461–12466
8. Bolognese, C. P., and McGraw, P. (2000) Plant Physiol. 124, 1800–1813
9. Brendza, K. M., Haakenson, W., Cahoon, R. E., Hicks, L. M., Palavalli, L. H., Chiapelli, B. J., McLaren, M., McCarry, J. P., Williams, D. J., Hresko, M. C., and Jez, J. M. (2007) Biochem. J. 404, 439–448
10. Charron, J. B., Breton, G., Danyuk, J., Muzac, I., Ibrahim, R. K., and Sarhan, F. (2002) Plant Physiol. 129, 363–373
11. Nuccio, M. L., Ziemak, M. J., Henry, S. A., Weretilnyk, E. A., and Hanson, A. D. (2000) J. Biol. Chem. 275, 14095–14101
12. Palavalli, L. H., Brendza, K. M., Haakenson, W., Cahoon, R. E., McLaird, M., Hicks, L. M., McCarry, J. P., Williams, D. J., Hresko, M. C., and Jez, J. M. (2006) Biochemistry 45, 6056–6065
13. Guthrie, C., and Fink, G. R. (1991) Guide to Yeast Genetics and Molecular Biology in Methods in Enzymology, Academic Press, Inc., San Diego, CA
14. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
15. Rouser, G., Siakatose, A. N., and Fleischer, S. (1966) Lipids 1, 85–86
16. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
17. Nikulina, K., Patel-King, R. S., Takebe, S., Pfister, K. K., and King, S. M. (2004) Cell Motil. Cytoskeleton 57, 233–245
18. Witola, W. H., and Ben Mamoun, C. (2007) Eukaryot. Cell 6, 1618–1624
19. Witola, W. H., Pessi, G., El Bissati, K., Reynolds, J. M., and Ben Mamoun, C. (2006) J. Biol. Chem. 281, 21305–21311