Analysis of Amino Acid Motifs Diagnostic for the *sn*-Glycerol-3-phosphate Acyltransferase Reaction[†]

Tal M. Lewin, Ping Wang, and Rosalind A. Coleman*

Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina 27599-7400

Received November 30, 1998; Revised Manuscript Received February 26, 1999

ABSTRACT: Alignment of amino acid sequences from various acyltransferases [*sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), acyl-CoA:dihydroxyacetone-phosphate acyltransferase (DHAPAT), 2-acylglycerophosphatidylethanolamine acyltransferase (LPEAT)] reveals four regions of strong homology, which we have labeled blocks I−IV. The consensus sequence for each conserved region is as follows: block I, [NX]-H-[RQ]-S-X-[LYIM]-D; block II, G-X-[IF]-F-I-[RD]-R; block III, F-[PLI]-E-G-[TG]-R-[SX]-[RX]; and block IV, [VI]-[PX]-[IVL]-[IV]-P-[VI]. We hypothesize that blocks I−IV and, in particular, the invariant amino acids contained within these regions form a catalytically important site in this family of acyltransferases. Using *Escherichia coli* GPAT (PlsB) as a model acyltransferase, we examined the role of the highly conserved amino acid residues in blocks I−IV in GPAT activity through chemical modification and site-directed mutagenesis experiments. We found that the histidine and aspartate in block I, the glycine in block III, and the proline in block IV all play a role in *E. coli* GPAT catalysis. The phenylalanine and arginine in block II and the glutamate and serine in block III appear to be important in binding the glycerol 3-phosphate substrate. Since blocks I−IV are also found in LPAAT, DHAPAT, and LPEAT, we believe that these conserved amino acid motifs are diagnostic for the acyltransferase reaction involving glycerol 3-phosphate, 1-acylglycerol 3-phosphate, and dihydroxyacetone phosphate substrates.

Acyltransferases catalyze essential reactions in the biosynthesis of all phospholipids and, in eukaryotes, triacylglycerol (*1*). Recently, several reports have shown homology among the amino acid sequences of different acyltransferases (*2*−*5*). In Table 1, we show the alignment of the homologous regions (labeled blocks I−IV) in GPAT,[1] LPAAT, DHAPAT, and LPEAT amino acid sequences from bacteria, yeast, nematodes, and mammals. We hypothesize that blocks I−IV and in particular the highly conserved residues (shown in Table 1 in boldface type) might comprise a catalytically important site in this family of acyltransferases. This hypothesis is strengthened by recent identification of nonfunctional human DHAPAT mutations which have amino acid substitutions at the invariant arginine residue in block II (*3*). In addition, early experiments showed that the arginine-modifying reagents butanedione and phenylglyoxal inhibited bacterial GPAT activity (*6*), consistent with the presence of conserved arginine residues in blocks I−IV (Table 1). The invariant histidine and aspartate in block I of bacterial GPAT have also been found to be important for activity (*5*).

To determine if the invariant amino acid residues in blocks I−IV are part of a catalytically important site in acyltransferases, we used bacterial GPAT as a model enzyme. GPAT (EC 2.3.1.15) catalyzes the initial reaction in the pathway of glycerolipid biosynthesis, the transfer of an activated fatty acyl chain to the *sn*-1 position of glycerol 3-phosphate (*1*, *7*). In *Escherichia coli*, GPAT is an integral cytoplasmic membrane protein encoded by the *plsB* gene (*7*−*9*). Although PlsB has been purified and reconstituted in phospholipid (*10*, *11*), little is known about the GPAT catalytic mechanism. PlsB exhibits a high degree of similarity both to GPATs cloned from other species and to other acyltransferases (Table 1). Working in a bacterial system provided several advantages since manipulations requiring mutagenesis and overexpression are easily accomplished. Further, functionality of mutant GPAT clones could be readily tested in a *plsB*$^-$ *E. coli* strain. We examined the role of specific amino acids residues in blocks I−IV in GPAT activity through chemical modification and site-directed mutagenesis experiments. We found that invariant amino acids in blocks I, III, and IV are important for GPAT catalysis while residues in blocks II and III play a role in binding the glycerol 3-phosphate substrate. The results are discussed in relation to the hypothesis that the mechanism of catalysis is similar for acyltransferases that have as substrates glycerol 3-phosphate, 1-acylglycerol 3-phosphate, or dihydroxyacetone phosphate.

[†] This work was supported by U.S. Public Health Service Grants HD19068 (R.A.C.) and HD08431 (T.M.L.) from the National Institutes of Health.

* Corresponding author. Tel: 919-966-7213. Fax: 919-966-7216. E-mail: rcoleman@sph.unc.edu.

[1] Abbreviations: APB, *m*-aminophenylboronate; DEPC, diethyl pyrocarbonate; DHAPAT, acyl-CoA:dihydroxyacetonephosphate-acyltransferase; DMSO, dimethyl sulfoxide; EDTA, (ethylenedinitrilo)-tetraacetic acid; GPAT, *sn*-glycerol-3-phosphate acyltransferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl *β*-D-thiogalactopyranoside; LPAAT, lysophosphatidic acid acyltransferase; LPEAT, 2-acylglycerophosphatidylethanolamine acyltransferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

10.1021/bi982805d CCC: $18.00 © 1999 American Chemical Society
Published on Web 04/15/1999

Table 1: Acyltransferase Homology Blocks[a]

	Block I	Block II	Block III	Block IV	Accession[b]
GPAT	303[c]	348	382	417	
E. coli	VPC**H**R**S**HM**D**YLLL	G**A**F**F**I**R**R	Y**F**V**E**G**G**R**S**RT**G**R	ITLI**P**IYI	130326
H. influenzae	VPC**H**R**S**HI**D**YLLL	G**A**F**F**I**R**R	Y**F**I**E**G**G**R**S**RT**G**R	ISIV**P**VYV	1172533
M. tuberculosis	AFS**H**R**S**YL**D**GMLL	G**A**I**F**I**R**R	W**S**I**E**G**G**R**T**RT**G**K	VYLV**P**TSI	2791522
C. elegans	ICL**H**R**S**HL**D**ILSM	N**T**I**F**I**R**R	F**F**L**E**G**T**R**S**RF**G**K	ISII**P**VVF	1458332
M. musculus	LPV**H**R**S**HI**D**YLLL	G**G**F**F**I**R**R	I**F**L**E**G**T**R**S**RS**G**K	ILVI**P**VGI	2498786
R. norvegicus	LPV**H**R**S**HI**D**YLLL	G**G**F**F**I**R**R	I**F**L**E**G**T**R**S**RS**G**K	ILVI**P**VGI	2444459
LPAAT					
E. coli	IAN**H**QNNY**D**MVTA	G**N**LLL**D**R	M**F**P**E**G**T**R**S**R.**G**R	VPII**P**VCV	1789395
S. typhimurium	IAN**H**QNNY**D**MVTA	G**N**LL**I**D**R**	M**F**P**E**G**T**R**S**R.**G**R	VPII**P**VCV	154236
H. influenzae	IGN**H**QNNY**D**MVTI	G**N**I**F**L**D**R	M**F**P**E**G**T**RNR.**G**R	VPII**P**VVC	1573737
M. genitalium	VAN**H**K**S**NL**D**PLVL	DCV**F**I**D**R	V**F**A**E**G**T**RIL.SN	VPIL**P**VSI	1045898
S. cerevisiae	IAN**H**Q**S**TL**D**IFML	G**T**Y**F**L**D**R	V**F**P**E**G**T**RSTSEL	IPIV**P**VVV	464422
C. elegans	ICN**H**Q**S**SL**D**ILSM	N**T**I**F**I**D**R	V**F**P**E**G**T**RNREGG	IPII**P**VVF	1403001
M. musculus	VSN**H**Q**S**SL**D**LLGM	G**I**I**F**I**D**R	V**F**P**E**G**T**RNHN**G**S	VPII**P**IVF	2467310
H. sapiens alpha	VSN**H**Q**S**SL**D**LLGM	G**V**I**F**I**D**R	V**F**P**E**G**T**RNHN**G**S	IPII**P**VVF	2155238
H. sapiens beta	VSN**H**Q**S**IL**D**MMGL	G**V**F**F**I**N**R	IYP**E**G**T**RNDN**G**D	VPIV**P**VVY	2155240
DHAPAT (*H. sapiens*)	LPS**H**R**S**YI**D**FLML	G**A**F**F**M**R**R	F**F**L**E**G**T**R**S**RSAK	TYLV**P**ISI	3258645
LPEAT (*E. coli*)	TPN**H**V**S**FI**D**GILL		I**F**P**E**G**T**RITTTG	ATVI**P**VRI	290403
GPAT consensus	φ--**H**R**S**-φ**D**φφ--	G-φ**F**I**R**R	φF**E**G**T**RSR-**G**K	φφφφ**P**φφφ	
LPAAT consensus	φ-N**H**Q**S**-φ**D**φφ--	G-φ**F**I**D**R	φF**P**E**G**T**R**---**G**-	φP**P**φφ**P**φφφ	

[a] Blocks of homology were identified on the basis of amino acid alignments performed using the CLUSTAWL algorithm. [b] Accession numbers are for the National Center for Biotechnology Information Protein Data Base. [c] Numbers indicate the amino acid residue number at the beginning of each block in *E. coli* GPAT.

EXPERIMENTAL PROCEDURES

Strains. Strains Lin 8 [*fhuA22* Δ*phoA8 ompF627* (T_2^R) *fadL701* (T_2^R) *relA1 glpD3 glpR2*(Const) *pit-10 spoT1 rrnB-2 mcrB1 creC510*] and BB26-36 [*fhuA22* Δ*phoA8 ompF627* (T_2^R) *plsX50 fadL701* (T_2^R) *relA1 glpD3 glpR2*(Const) *pit-10 spoT1 glpK14 rrnB-2 plsB26 mcrB1 creC510*] (*12*) were obtained from the *E. coli* Genetic Stock Center.

Plasmid Construction. Plasmid pTrcf1 was constructed by cloning the 401 bp *Pvu*II (Klenow filled)−*Hind*III fragment from pTrc99A (Pharmacia) into pET21a(+) (Novagen) digested with *Bgl*II, treated with Klenow, and then digested with *Hind*III. Plasmid pTrcf1 contains the IPTG-inducible *trp-lac* hybrid promoter from pTrc99a instead of the T7 promoter found in pET21a(+).

Plasmid pPlsBHis was constructed in the following manner: The open reading frame for PlsB (starting at the third amino acid) was amplified by the polymerase chain reaction (PCR) with primers PLSB5′ (5′-TCGTTT**GAGCTC**GGCTG-GCCACGGATTTAC) and PLSB3′ (5′-CTCTC**CTCGAG**C-CCTTCTCCTTGCGTCGC) using plasmid pVL1 (gift from T. J. Larson, Virginia Tech, Blacksburg, VA) (*9*) digested with *Eco*RI as template. The PCR product was digested with *Sac*I and *Xho*I (recognition sites indicated in boldface type in primers PLSB5′ and PLSB3′, respectively) and ligated into pTrcf1 digested with *Sac*I and *Xho*I. This generates plasmid pPlsBHis, which allows IPTG-inducible expression from the *trp-lac* hybrid promoter of PlsB with a C-terminal 6XHis tag. The PlsB sequence was verified at the UNC Automated Sequencing Facility.

Plasmid pCRplsB was constructed by digesting the same *pls*B PCR product described above with *Sac*I and *Xho*I. This 2.4 kb DNA fragment encoding PlsB was ligated into plasmid pCR2.1 (Invitrogen) digested with *Sac*I and *Xho*I. The PlsB sequence was verified at the UNC Automated Sequencing Facility.

Table 2: Mutagenic Oligonucleotides

Mutation[a]	Oligonucleotide Sequence[b]	Enzyme
H306G	TATGTGCCTTG**CggCCG**CAGTCACATG	*Eag*I
S308A	CCTTGCCACCGTgcT**CAtATG**GACTAC	*Nde*I
D311G	GCAGTCACATGG**ggTACC**TGCTGCTT	*Kpn*I
F351A	GTCTGGGtGCGT**TCgcgA**TTcGaCGTACG	*Nru*I
I352A	GGGCGTTCT**Tcgcga**GaCGTACGTTTAAAGG	*Nru*I
R354C	GTTCTTTATTCGC**tGTACa**TTTAAAGGCAA	*BsrG*I
R354K	GTTCTTTATTCG**ggaaACGTTc**AAAGGCAATAAAC	*Xmn*I
E385R	CGAGTACTTCGT**ccgcGG**CGGTCGTTCC	*Sac*II
G386L	GTACTTCGTG**GAgctC**GGTCGTTCCCG	*Sac*I
G386A	GTACTTCGTGGAggctGGTCGTTCCCGT	
S389A	CGTGGAAGGtGGTCGTgCgCGT**ACcGGt**CGTTTGCTG	*Age*I
P421S	CGATTACGCT**GATatc**tATCTATATCGG	*EcoRV*

[a] Numbers indicate PlsB amino acid residue. [b] To facilitate comparison with the plsB sequence, the oligonucleotide sequence from 5′ to 3′ is shown as the **complement** of the actual oligonucleotide used for mutagenesis. Lower case letters indicate nucleotides changed from the wild-type plsB sequence. Boldface type highlights restriction enzyme recognition sites.

Construction of Mutants. Each mutation was generated using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, U.S. Patent 5 780 270). Mutagenesis was performed using plasmid pCRplsB as template, the Bottom Selection Oligonucleotide provided in the kit, and each of the mutagenic oligonucleotides is shown in Table 2. The following modifications were made to the Promega mutagenesis protocol: Annealing reactions were heated to 85 °C, and only half the amount of GeneEditor Antibiotic Selection Mix was used to select mutants. The presence of each mutation was verified by diagnostic restriction digest (Table 2) and DNA sequencing at the UNC-CH Automated

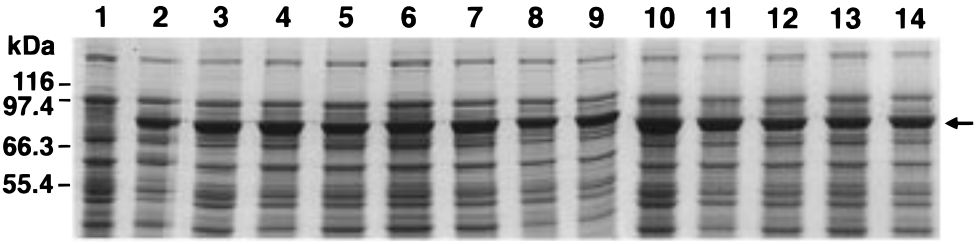

FIGURE 1: Overexpression of wild-type and mutant PlsB in BB26-36 membranes. Membranes were isolated from strain BB26-36 containing plasmids pTrcf1 (lane 1), pPlsBHis (lane 2), and pPlsBHis plus the indicated mutation (lane 3, H306G; lane 4, S308A; lane 5, D311G; lane 6, F351A; lane 7, I352A; lane 8, R354C; lane 9, R354K; lane 10, E385R; lane 11, G386L; lane 12, G386A; lane 13, S389A; lane 14, P421S) as described in Experimental Procedures. Proteins from the membrane fractions were separated by SDS−PAGE and visualized by Coomassie blue staining. Positions of the molecular mass markers are indicated in kDa on the left side of the gel. The arrow indicates the position of the overexpressed wild-type or mutant PlsB protein (~83 kDa).

DNA Sequencing Facility. Each mutant was then recloned into plasmid pPlsBHis.

Complementation of Glycerol Auxotrophy. Plasmids bearing mutations in *pls*B were tested for complementation of the glycerol requirement of strain BB26-36 (*plsB26*) (*12*). Cells were grown at 37 °C for 16 h on minimal M56LP (*12*) plates containing 0.4% glucose, 0.1% casamino acids, and 60 μg/mL ampicillin, with or without 0.1% glycerol. Growth on plates lacking glycerol indicated complementation of the *plsB26* phenotype.

Cellular Fractionation. Expression and localization of wild-type PlsB and PlsB mutants were determined in the following manner: Strain BB26-36 containing plasmid pTrcf1, pPlsBHis, or pPlsBHis plus the indicated mutation was grown at 37 °C in 200 mL of Luria−Bertani media supplemented with 0.4% glucose, 0.1% glycerol, and 60 μg/mL ampicillin. At 2.5×10^8 cells/mL, protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM. Cultures were grown for an additional 30 min and then harvested by centrifugation at 5000 rpm for 10 min in a Sorvall HS-4 rotor. Cells were fractionated using a modified procedure described previously (*13*): The cell pellet was frozen at −20 °C overnight and then resuspended in 12 mL of 10 mM HEPES, pH 7.8, and 0.5 mM EDTA (HE buffer). Lysozyme was added to a final concentration of 100 μg/mL, and the cell suspension was incubated on ice for 30 min. The lysed cells were sonicated six times with a 10 s burst followed by a 10 s rest on ice. Cellular debris was removed from the cell lysate by low-speed centrifugation. The supernatant was layered over a 2 mL cushion of 55% (w/w) sucrose topped with 0.5 mL of 5% (w/w) sucrose in HE buffer. After centrifugation in a Beckman SW41 rotor at 35 000 rpm for 3 h, 1 mL of the supernatant above the sucrose cushion was saved as the soluble fraction. The membrane band at the interface was collected with a 19 gauge needle and syringe. Proteins from the soluble and membrane fractions were separated by electrophoresis on an 8% SDS−polyacrylamide gel and visualized by staining with BLUPRINT Coomassie blue (Life Technologies).

Assay for Glycerol-3-phosphate Acyltransferase. Membranes containing overexpressed wild-type or mutant PlsB were isolated as described above. GPAT activity was assayed in 10−30 μg of isolated membrane protein with 300 μM [^{3}H]glycerol 3-phosphate and 50 μM palmitoyl-CoA as described previously (*12*). The assay was proportional to time and protein. [^{3}H]glycerol 3-phosphate was synthesized enzymatically (*14*). For aminophenylboronate (APB) inhibition studies, DMSO or APB dissolved in DMSO was added to the GPAT assay before membranes were added. APB concentrations ranged from 10 to 30 mM, and DMSO was 15% of the assay volume. For diethyl pyrocarbonate (DEPC) inhibition studies, isolated membranes (2 mg/mL) were mixed with 312.5 μM (for wild-type PlsB) or 125 μM (for H306G PlsB mutant) DEPC in acetonitrile (12.5% of total incubation volume) or with acetonitrile alone (control) in 0.1 M potassium phosphate, pH 6.5, at room temperature. Aliquots (30 μL) of DEPC-treated and control membranes were removed at indicated time points for immediate assay. After 10 min, hydroxylamine was added to the DEPC-treated and control membranes at final concentrations of 375 mM (for wild-type) or 150 mM (for H306G), and aliquots (37.5 μL) were removed for assay at the indicated time points.

RESULTS

Construction of PlsB Mutants. To determine if the amino acid motifs in blocks I−IV (Table 1) are diagnostic for the acyltransferase reaction, we generated the following amino acid substitutions in *E. coli* GPAT (PlsB): H306G, S308A, D311G, F351A, I352A, R354C, R354K, E385R, G386L, G386A, S389A, and P421S. Each mutant was generated using the GeneEditor in vitro Site Directed Mutagenesis System and expressed in strain BB26-36 (*plsB26*) as described in Experimental Procedures. Fractionation of these cells revealed that wild-type PlsB and each of the mutant enzymes were correctly localized to the membrane fraction (Figure 1, Table 3) and migrated as a band corresponding to 83 kDa, which is in agreement with previous reports (*7*, *8*, *10*). No overexpressed protein was detected in the soluble fraction (data not shown) and no accumulation of inclusion bodies was observed, indicating that overexpressed wild-type and mutant PlsB proteins are inserted into the cytoplasmic membrane. Expression levels of the overexpressed protein varied in each membrane preparation but did not correlate with GPAT activity measurements (see below).

Each mutant was also tested for its ability to complement the glycerol auxotrophy of strain BB26-36. *E. coli* strain BB26-36 (*plsB26*) requires supplementation with glycerol for growth (Table 3), because the apparent K_m for glycerol 3-phosphate of its mutant PlsB enzyme is elevated 10-fold (*12*). Addition of 0.1% glycerol to the media permits growth because the strain also has a constitutively active glycerol kinase that can synthesize excess glycerol 3-phosphate (*12*). The requirement for 0.1% glycerol was overcome by transforming strain BB26-36 with plasmid pPlsBHis (Table

Table 3: Characteristics of PlsB Mutants

mutant	BB26-36 growth[a]	membrane localization[b]	glycerol 3-phosphate K_m (μM)[c]	glycerol 3-phosphate V_{max} (nmol min^{-1} mg^{-1})[c]
Lin 8[d]	(++++)	ND	32	8.4
plsB26[e]	−	ND	407	0.1
wild type	++++	+	49	13.1
block I				
H306G	−	+	65	2.9
S308A	++++	+	35	11.9
D311G	−	+	95	0.3
block II				
F351A	−	+	152	1.1
I352A	−	+	51	1.3
R354C	−	+	>640	2.0
R354K	++++	+	21	8.2
block III				
E385R	−	+	288	0.9
G386L	−	+	51	0.3
G386A	++++	+	29	7.3
S389A	++++	+	>160	3.7
block IV				
P421S	−	+	77	1.3

[a] Strain BB26-36 (*plsB*$^-$) containing a plasmid encoding the indicated mutant was grown on minimal media without glycerol as described in Experimental Procedures. ++++ indicates growth similar to that of wild-type and complementation of the *plsB*$^-$ phenotype; − indicates no growth. [b] Cells were fractionated and analyzed by SDS−PAGE as described in Experimental Procedures and Figure 3. + indicates presence of the particular overexpressed PlsB protein in the membrane fraction. ND = not determined. [c] GPAT assays were performed on membrane fractions containing the indicated overexpressed PlsB protein as described in Experimental Procedures. K_m and V_{max} for glycerol 3-phosphate were determined by double reciprocal analysis. Regression coefficients were all greater than 0.98. Endogenous GPAT activity in BB26-36 (0.1 nmol min^{-1} mg^{-1}) was not subtracted. [d] Characteristics of PlsB protein in strain Lin 8 (parent of BB26−36). (++++) indicates that Lin 8 grows on minimal media without glycerol. [e] Characteristics of PlsB26 mutant protein in strain BB26-36 containing the empty vector pTrcf1.

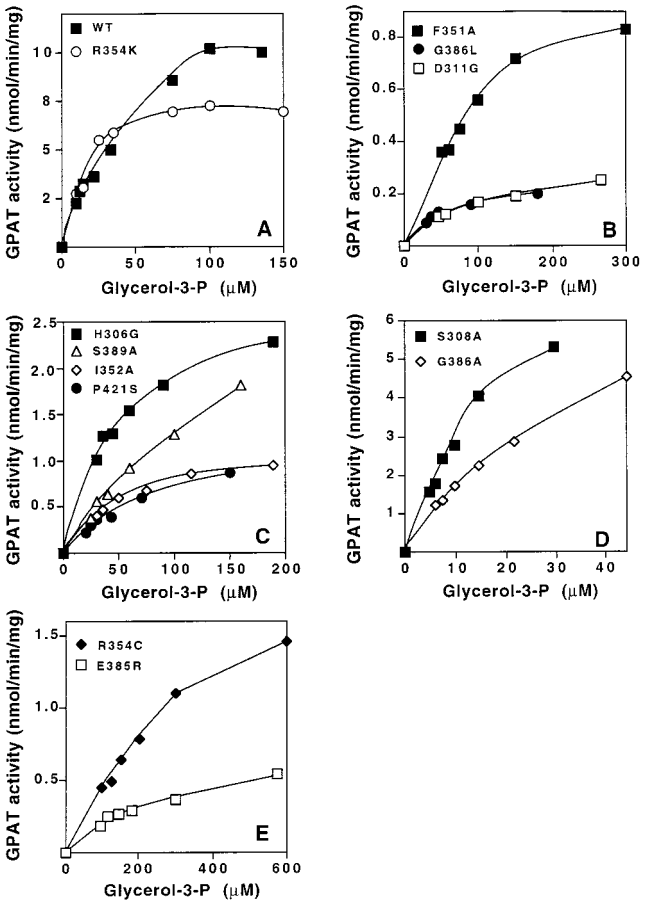

FIGURE 2: Glycerol 3-phosphate dependence of wild-type and mutant PlsB enzymes. Membranes (10−30 μg of protein) isolated from strain BB26-36 overexpressing wild-type or mutant PlsB were assayed for GPAT activity in the presence of increasing concentrations of [^{3}H]glycerol 3-phosphate as described in Experimental Procedures. The range of glycerol 3-phosphate was between 0.3 and 2.0 times the K_m for each enzyme assayed in order to accurately determine V_{max} and K_m as described previously (*15*).

3), indicating that wild-type PlsB with a C-terminal 6XHis tag is functional in vivo.

Plasmids encoding PlsB with the indicated amino acid substitution were transformed into strain BB26-36 and tested for their ability to complement the glycerol auxotrophy as described in Experimental Procedures. Strain BB26-36 that contained plasmids encoding mutations H306G, D311G, F351A, I352A, R354C, E385R, G386L, or P421S failed to grow in 16 h on media that lacked glycerol (Table 3), indicating that the mutations interfered with functional GPAT activity. After ≥24 h of growth a few colonies were observed with all amino acid substitutions except R354C. Because some of these colonies were found to harbor revertant plasmids, growth conditions for kinetic experiments (see below) were ≤16 h in the presence of glycerol. PlsB plasmids bearing mutations S308A, R354K, G386A, and S389A were able to complement the growth phenotype as well as did a plasmid carrying wild-type *plsB* (Table 3), suggesting that these amino acid substitutions do not perturb functional PlsB activity.

Although we planned to carry out kinetic measurements on PlsB enzymes (all with a C-terminal 6XHis tag) purified in detergent by Ni−NTA affinity chromatography and then reconstituted in phospholipid, the reconstitution proved to be inefficient. A previous report showed that PlsB purified in Triton X-100 and reconstituted in phospholipid has kinetic parameters different from those of the membrane-bound enzyme (*11*), suggesting that full reconstitution of all the purified GPAT did not occur. Therefore, we carried out all kinetic measurements in isolated membrane fractions from strain BB26-36 overexpressing wild-type PlsB or the indicated mutant. The endogenous GPAT activity measured in BB26-36 membranes is very low (0.1 nmol min^{-1} mg^{-1}, Table 3) and does not interfere with overexpressed PlsB activity. GPAT activity in membranes isolated from the parent strain, Lin 8 (*plsB*$^+$), was 8.4 nmol min^{-1} mg^{-1} with an apparent K_m for glycerol 3-phosphate of 32 μM (Table 3). Membranes containing overexpressed wild-type PlsB displayed high levels of GPAT activity (13.1 nmol min^{-1} mg^{-1}) and an apparent K_m for glycerol 3-phosphate of 49 μM, similar to values reported previously (*12*) (Figure 2A, Table 3). For each mutant, an optimized range of glycerol 3-phosphate was used in order to accurately determine V_{max} and K_m for glycerol 3-phosphate (*15*).

Invariant Amino Acid Residues in Blocks I, III, and IV Are Important for PlsB Catalysis. PlsB enzymes with amino acid substitutions at conserved residues in blocks I, III, and IV displayed varying amounts of GPAT activity. In block I, the amino acid substitution D311G had a profound effect on GPAT activity. The D311G mutant enzyme was non-

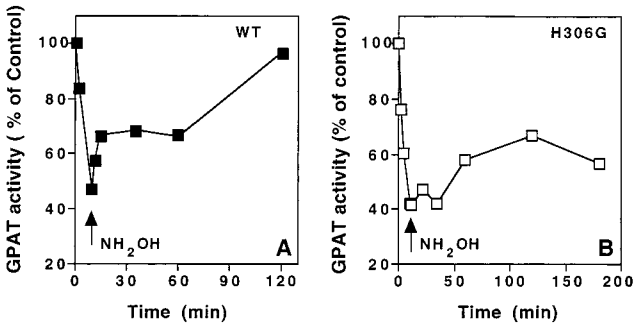

FIGURE 3: Effect of diethyl pyrocarbonate (DEPC) on GPAT activity of wild-type PlsB and H306G mutant PlsB. (A) Membranes isolated from strain BB26-36 overexpressing PlsB were mixed with 312.5 μM DEPC, and aliquots (22.5 μg of protein) were removed at the indicated time points for immediate assay. After 10 min, 375 mM hydroxylamine was added, and aliquots were removed for assay at the indicated time points. The data are presented as percent of activity in control (acetonitrile-treated) membranes (16.3 nmol min^{-1} mg^{-1} at time 0). (B) Membranes containing the overexpressed PlsB H306G mutant were mixed with 125 μM DEPC, and aliquots (30 μg of protein) were removed at the indicated time points for immediate assay. After 10 min 150 mM hydroxylamine was added to the DEPC-treated membranes. The data are presented as percent of activity in control (acetonitrile-treated) membranes (3.7 nmol min^{-1} mg^{-1} at time 0).

functional in vivo, as it was unable to complement the glycerol auxotrophy of stain BB26-36 (Table 3). Consistent with this result is the observation that the D311G mutant had a GPAT activity of 0.3 nmol min^{-1} mg^{-1}, which is only 2% of the GPAT activity measured in wild-type PlsB (Table 3, Figure 2B). The K_m for glycerol 3-phosphate in the D311G mutant was comparable to that measured in wild-type PlsB, suggesting that the loss in GPAT activity is not the result of a major change in substrate binding. Taken together, these data suggest that the invariant aspartate in block I is important for GPAT activity.

Another amino acid substitution in block I, H306G, did not have as adverse an effect on the GPAT activity of PlsB. Although the H306G mutant failed to complement the glycerol auxotrophy of strain BB26-36, in vitro assays for GPAT activity revealed that the mutant enzyme had a GPAT activity of 2.9 nmol min^{-1} mg^{-1} (Table 3, Figure 2C). This result, indicating that the H306G mutant retained 22% of the activity measured in wild-type PlsB, was surprising in light of data reported for PlsB with a H306A substitution (*5*). Although the H306A mutant was completely inactive, our results with the H306G substitution indicated that the histidine at position 306 is not essential for GPAT activity. To clarify this discrepancy, we treated wild-type or H306G mutant PlsB with DEPC which modifies histidine residues by forming an *N*-carboxyhistidyl derivative (*16*). Treatment of membranes containing wild-type PlsB with DEPC inhibited GPAT activity 50% in 10 min (Figure 3A). Subsequent incubation with hydroxylamine for 2 h restored activity to 97% of the control (Figure 4A). Hydroxylamine reversal of DEPC inhibition suggests that an essential histidine residue lies within the PlsB catalytic domain. If H306 is the histidine residue modified by DEPC in wild-type PlsB, then DEPC should not inhibit the H306G mutant. Surprisingly, treatment of the H306G mutant with DEPC inhibited GPAT activity 58% in 10 min (Figure 3B); however, the inhibition was only minimally reversed by hydroxylamine (Figure 3B). The

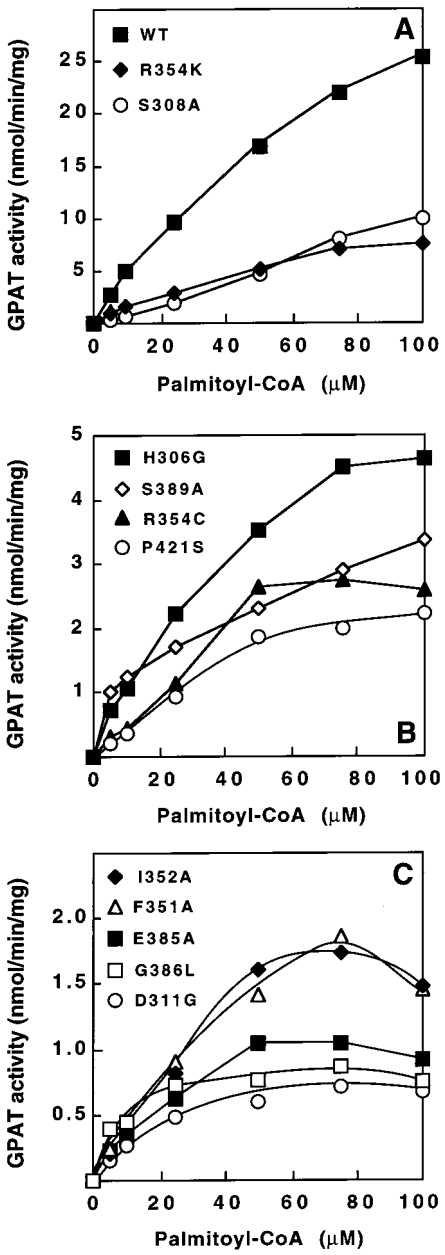

FIGURE 4: Palmitoyl-CoA dependence of wild-type and mutant PlsB enzymes. Membranes (10−30 μg of protein) isolated from strain BB26-36 overexpressing wild-type or mutant PlsB were assayed for GPAT activity in the presence of increasing concentrations of palmitoyl-CoA as described in Experimental Procedures. Each enzyme was assayed with excess glycerol 3-phosphate (see Figure 2).

inability of hydroxylamine to reverse the inactivation indicates that DEPC treatment of the H306G mutant now modifies an amino acid residue other than histidine.

Serine to alanine substitutions in block I (S308A) and block III (S389A) had only modest effects on PlsB activity. Both mutant enzymes were able to complement strain BB26-36, suggesting that they were functional in vivo. Consistent with this result, the S308A mutant had GPAT activity of 11.9 nmol min^{-1} mg^{-1} which was very similar to that measured for wild-type PlsB (Table 3, Figure 2D). The S389A mutant had 3.5-fold less activity (3.7 nmol min^{-1} mg^{-1}) than wild-type PlsB and a greater than 3-fold higher apparent K_m for glycerol 3-phosphate (>160 μM) (Table 3, Figure 2C). These data suggest that the serine at position

389 may play a role in binding the glycerol 3-phosphate substrate, although neither conserved serine is absolutely required for GPAT activity.

This result was surprising since our experiments with a competitive serine hydrolase inhibitor showed that APB inhibited the GPAT activity of membranes containing overexpressed PlsB in a dose-dependent manner (data not shown). Aromatic boronic acids inhibit serine hydrolases by interaction of the boron atom with the catalytic serine residue (*17*), suggesting that PlsB might be a member of the family of serine hydrolases (including lipases and proteases) which typically have a catalytic triad composed of histidine, serine, and aspartate (or glutamate) residues (*18*). Although we were unable to identify the -GXSXG- motif found at the active site serine of many lipases and proteases (*19*), we reasoned that one of the two conserved serines in the GPAT sequence might be reacting with APB. We treated membranes containing overexpressed S308A and S389A PlsB mutants with increasing amounts of APB. APB inhibited the S308A and S389A mutants with a concentration-dependent profile similar to that observed for wild-type PlsB (data not shown), suggesting that the boron atom does not interact with either of the two conserved serine residues found in blocks I and III.

The invariant glycine in block III was also examined for its role in PlsB activity. The G386L mutant was unable to complement the glycerol auxotrophy of strain BB26-36, and this enzyme had very low GPAT specific activity (0.3 nmol min^{-1} mg^{-1}) (Table 3, Figure 2B). In contrast, the more conservative G386A substitution generated a mutant enzyme that retained 50% of wild-type GPAT activity (7.3 nmol min^{-1} mg^{-1}) consistent with the ability of the G386A mutant to complement BB26-36 (Table 3, Figure 2D).

In block IV, the P421S mutant was unable to complement strain BB26-36, indicating that the mutant enzyme was not functional in vivo. In agreement with this result, the GPAT activity of the P421S mutant was only 1.3 nmol min^{-1} mg^{-1} or 10% of that measured for wild-type PlsB (Table 3, Figure 2C). The apparent K_m for glycerol 3-phosphate was virtually unchanged (77 μM) from wild type. Taken together, these kinetic data suggest that the invariant proline is important for GPAT catalysis.

Invariant Amino Acids in Blocks II and III Are Important for Glycerol 3-Phosphate Binding. The F351A and I352A substitutions in block II generated mutant enzymes that were unable to complement strain BB26-36. Consistent with the inability of the F351A and I352A mutants to function in vivo is the observation that the GPAT activity of these mutant enzymes was only 8−10% (1.1 and 1.3 nmol min^{-1} mg^{-1}, respectively) of that measured for wild-type PlsB (Table 3, Figure 2B,C). The F351A mutant has an apparent K_m for glycerol 3-phosphate of 152 μM, which is 3-fold higher than that measured for the wild-type enzyme (Table 3), suggesting a minor role for the F351 residue in substrate binding.

A much more striking result with respect to glycerol 3-phosphate binding was observed when the arginine at position 354 was replaced with cysteine. Cysteine was chosen because it was one of the amino acid substitutions found in the nonfunctional human DHAPAT mutants (*3*). The R354C mutant has an apparent K_m for glycerol 3-phosphate at least 13-fold higher (>640 μM) than that measured for wild-type PlsB (Table 3, Figure 2E). The more conservative R354K mutant retained 62% of GPAT activity (8.2 nmol min^{-1} mg^{-1}) and had an apparent K_m for glycerol 3-phosphate of 21 μM, consistent with the mutant enzyme's ability to function in vivo (Table 3, Figure 2A). Taken together, these data suggest that a positive charge in the last residue of block II is critical for binding glycerol 3-phosphate.

Block III may also play a role in substrate binding. Although substituting alanine for serine in block III (S389A) had only a modest effect on binding the glycerol 3-phosphate substrate (Table 3), a more profound effect was found with a glutamate to arginine substitution at position 385. The E385R mutant enzyme has a 6-fold higher apparent K_m (288 μM) for glycerol 3-phosphate, explaining its low GPAT specific activity (0.9 nmol min^{-1} mg^{-1}) and its inability to complement the glycerol auxotrophy of strain BB26-36 (Table 3, Figure 2E). These data suggest that the glutamate in block III is important for substrate binding.

Palmitoyl-CoA Dependence. Because wild-type PlsB activity was not saturable with respect to palmitoyl-CoA (Figure 4), we were unable to accurately determine its kinetic constants. However, several of the mutants displayed saturation kinetics (Figure 4), and we were able to determine the palmitoyl-CoA concentrations for maximal activity. The apparent K_m values we calculated for palmitoyl-CoA for the D311G, H306G, F351A, I352A, R354C, R354K, E385R, and S389A mutants were in the range of 10−75 μM, in agreement with previous reports (*20*, *21*). Since the V_{max} for wild-type PlsB was not saturable, it appears that palmitoyl-CoA acts as an activator of wild-type PlsB GPAT activity.

DISCUSSION

Alignment of GPAT, LPAAT, DHAPAT, and LPEAT amino acid sequences from bacteria, yeast, nematodes, and mammals reveals several regions of strong homology (labeled blocks I−IV in Table 1) (*2*−*5*). We did not include sequences from plant acyltransferases in our homology table because GPATs cloned from several plant species did not have blocks III and IV, suggesting a difference in their catalytic domain. To determine if the conserved blocks I−IV form a catalytically important site in acyltransferases, we generated amino acid substitutions of several conserved residues within blocks I−IV of *E. coli* GPAT. The ability of each mutant to catalyze the acyltransferase reaction was assessed in vivo by determining the ability of each mutant to complement a *plsB*$^-$ strain and in vitro by measuring GPAT activity in isolated membranes. We found that the histidine and aspartate residues in block I, the glycine in block III, and the proline in block IV all play a role in PlsB catalysis. The phenylalanine and arginine in block II and the glutamate and serine in block III appear to be important for binding the glycerol 3-phosphate substrate.

Our data are fairly consistent with a recently proposed model for GPAT catalysis (*5*). In this model, the invariant histidine in block I acts as a general base to abstract a proton from the hydroxyl group at the *sn*-1 position of glycerol 3-phosphate to facilitate the nucleophilic attack on the thioester of palmitoyl-CoA. The invariant aspartate in block I may act in a charge relay system with the histidine residue to increase the nucleophilicity of the glycerol 3-phosphate hydroxyl group.

The major discrepancy we find is with the essential function of the histidine in block I. Unlike the completely

inactive H306A PlsB mutant (*5*), our H306G mutant retained 22% (2.9 nmol min^{-1} mg^{-1}) of GPAT activity as compared to wild-type PlsB (Table 3, Figure 2). Although the H306G mutant fails to complement the glycerol auxotrophy of strain BB26-26 (Table 3), our GPAT activity data suggest that the histidine residue in block I is not essential for catalysis of the GPAT reaction. The discrepancy between GPAT activity in vitro and functionality in vivo could result from the PlsB activity falling below a threshold level required for cell growth [all mutants which complemented BB26-36 had GPAT specific activities $\geq$3.7 nmol min^{-1} mg^{-1} (Table 3, Figure 2)] or arise from an altered enzyme conformation in isolated membranes. The latter explanation is consistent with the inability of hydroxylamine to reverse DEPC inactivation of the H306G mutant as is normally observed for wild-type PlsB (compare panels A and B of Figure 3). These results indicate that H306 can be carbethoxylated and decarbethoxylated in wild-type PlsB and suggest that the histidine residue in block I normally participates in GPAT catalysis. In the H306G mutant, the flexible nature of the glycine residue probably allows a change in conformation so that another amino acid side chain can substitute for the histidine function as general base. The aspartate in block I is a good substitute candidate because carboxylic groups often act as general bases and can form amide bonds with DEPC (*16*). In addition, there is sufficient evidence to suggest that the aspartate in block I normally participates in GPAT catalysis.

Our D311G mutant had very low GPAT specific activity (0.3 nmol min^{-1} mg^{-1}), indicating its importance in the GPAT reaction. Our result is consistent with that of Heath and Rock (*5*), who showed that substituting aspartate with glutamate yielded a mutant PlsB enzyme which retained 8% of wild-type GPAT activity. Their mutant with the less conservative D311A substitution failed to assemble into the membrane. Assembly of our D311G mutant into the membrane may have been possible because glycine is a more flexible amino acid than alanine.

The conformation of the GPAT active site also contributes to the efficient activity of PlsB. Substitution of the invariant glycine in block III with leucine resulted in a mutant enzyme which displayed very low GPAT activity (0.3 nmol min^{-1} mg^{-1}) (Table 3, Figure 2), whereas the mutant with a more conservative glycine to alanine substitution had GPAT activity similar to that of wild-type PlsB (Table 3, Figure 2). These data suggest that the invariant glycine in block III plays a steric role within the active site. Large side chains disrupt the active site conformation, while small ones are tolerated.

The results of our APB inhibition experiments suggested the presence of a critical serine in the PlsB active site. Amino acid alignments of GPAT enzymes from various species identified only two highly conserved serine residues, one in block I and the other in block III (Table 1), however; substituting each serine with alanine in PlsB (S308A and S389A mutants) had only modest effects on GPAT activity (Table 3, Figure 2). Further, APB inhibited the S308A and S389A mutants with K_i values similar to those observed for wild-type PlsB (data not shown). These results suggest that APB is not interacting with either of the two conserved serine residues. It is possible that APB either interacts with a serine we did not identify through amino acid alignments or inhibits by some other mechanism.

We also identified four amino acid residues that appear important for binding the glycerol 3-phosphate substrate. Mutating the glutamate or serine in block III or the phenylalanine or arginine in block II resulted in mutant PlsB enzymes with 3−13-fold higher apparent K_m values for glycerol 3-phosphate (Table 3, Figure 2). The serine in block III does not seem to be essential, since the S389A mutant still functions in vivo although the mutant enzyme has a 3-fold higher K_m for glycerol 3-phosphate. However, a similar 3-fold increase in K_m was observed for the F351A mutant which is nonfunctional in vivo, suggesting that the phenylalanine in block II plays a more significant role in glycerol 3-phosphate binding.

The critical residue we identified for glycerol 3-phosphate binding was the invariant arginine in block II. Our R354C mutant had a greater than 13-fold higher apparent K_m for glycerol 3-phosphate (Table 3, Figure 2). DHAPAT mutants with cysteine or histidine substitutions at the same arginine in block II were identified in patients with rhizomelic chondrodysplasia punctata type 2, a peroxisomal disorder (*3*). When these mutant DHAPAT enzymes were overexpressed, very little DHAPAT activity was detected. Because phosphate binding is a common role for arginine residues (*22*), we propose that the invariant arginine in block II coordinates with the phosphate group in both the glycerol 3-phosphate and dihydroxyacetone phosphate substrates.

There is the possibility that there may be more than one arginine involved in glycerol 3-phosphate binding. Previous studies showed that the arginine reactive reagents butanedione and phenylglyoxal inhibit PlsB GPAT activity (*6*). Our studies with phenylglyoxal inhibition of the R354K mutant revealed the same kinetics of inhibition as for wild-type PlsB (data not shown). We can interpret this result in two ways: First, because phenylglyoxal can modify lysine as well as arginine side chains (*23*), the inhibitor may still be effective with the R354K mutant. This is a reasonable explanation since phenylglyoxal has been shown to have an especially high affinity for anion binding pockets (*22*). Second, there is a possibility that the phenylglyoxal reacts with one of the other three conserved arginine residues in the PlsB sequence (see Table 1).

Our inability to determine palmitoyl-CoA-dependent kinetic constants arose because increasing amounts of palmitoyl-CoA activated GPAT activity (Figure 4). This observation is consistent with previous reports showing that overexpressing PlsB causes inactive GPAT aggregates to form (*24*). Palmitoyl-CoA, an amphipathic molecule, might disrupt the PlsB aggregates, thereby activating GPAT activity. Further, PlsB normally exhibits sigmoidal palmitoyl-CoA dependence curves (*24*), again suggesting activation by palmitoyl-CoA. Although we were unable to accurately measure its palmitoyl-CoA dependence, the P421S mutant appears to have a 2−5-fold higher K_m for palmitoyl-CoA (data not shown) than previously reported for wild-type PlsB (*20*, *21*). This result suggests that the hydrophobic block IV may participate in binding the acyl-CoA substrate.

In conclusion, we have shown that conserved residues in blocks I−IV are involved in GPAT catalysis and glycerol 3-phosphate binding. Our results are fairly consistent with the recently proposed catalytic mechanism for GPAT (*5*), and in addition, we have identified a potential binding site for the phosphate group on the glycerol 3-phosphate sub-

strate. However, an accurate picture of the acyltransferase mechanism would best be obtained through high-resolution analysis of protein crystals. Since blocks I−IV are conserved in several acyltransferases, we believe that these motifs may be diagnostic for the acyltransferase reaction involving substrates with glycerol 3-phosphate, 1-acylglycerol 3-phosphate, and dihydroxyacetone phosphate backbones.

NOTE ADDED IN PROOF

The *plsB26* mutant has recently been sequenced (*25*), revealing a missense mutation resulting in expression of PlsB with an A349T substitution in block II. The PlsB26 mutant has an elevated K_m for glycerol 3-phosphate compared to wild-type PlsB, consistent with our hypothesis that block II is required for binding the glycerol 3-phosphate substrate.

ACKNOWLEDGMENT

We thank T. J. Larson of Virginia Tech in Blacksburg, VA, for the gift of plasmid pVL1.

REFERENCES

1. Bell, R. M., and Coleman, R. A. (1980) *Annu. Rev. Biochem. 49*, 459−487.
2. West, J., Tompkins, C. K., Balantac, N., Nudelman, E., Meengs, B., White, T., Bursten, S., Coleman, J., Kumar, A., Singer, J. W., and Leung, D. W. (1997) *DNA Cell Biol. 16*, 691−701.
3. Ofman, R., Hettema, E. H., Hogenhout, E. M., Caruso, U., Muijsers, A. O., and Wanders, R. J. A. (1998) *Hum. Mol. Genet. 7*, 847−853.
4. Neuwald, A. F. (1997) *Curr. Biol. 7*, R465−R466.
5. Heath, R. J., and Rock, C. O. (1998) *J. Bacteriol. 180*, 1425−1430.
6. Green, P. R., and Bell, R. M. (1984) *Biochim. Biophys. Acta 795*, 348−355.
7. Wilkison, W. O., and Bell, R. M. (1997) *Biochim. Biophys. Acta 1348*, 3−9.
8. Larson, T. J., Lightner, V. A., Green, P. R., Modrich, P., and Bell, R. M. (1980) *J. Biol. Chem. 255*, 9421−9426.
9. Lightner, V. A., Larson, T. J., Tailleur, P., Kantor, G. D., Raetz, C. R. H., Bell, R. M., and Modrich, P. (1980) *J. Biol. Chem. 255*, 9413−9420.
10. Green, P. R., Merrill, A. H., Jr., and Bell, R. M. (1981) *J. Biol. Chem. 256*, 11151−11159.
11. Scheideler, M. A., and Bell, R. M. (1989) *J. Biol. Chem. 264*, 12455−12461.
12. Bell, R. M. (1974) *J. Bacteriol. 117*, 1065−1076.
13. Lewin, T. M., and Webster, R. E. (1996) *J. Biol. Chem. 271*, 14143−14149.
14. Chang, Y.-Y., and Kennedy, E. P. (1967) *J. Lipid Res. 8*, 447−455.
15. Segel, I. H. (1993) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 44−48, John Wiley & Sons, Inc., New York.
16. Miles, E. W. (1977) *Methods Enzymol. 47*, 431−442.
17. Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., and Kraut, J. (1975) *J. Biol. Chem. 250*, 7120−7126.
18. Dodson, G. G., Lawson, D. M., and Winkler, F. K. (1992) *Faraday Discuss. 93*, 95−105.
19. Brenner, S. (1988) *Nature 334*, 528−530.
20. Rock, C. O., Goelz, S. E., and Cronan, J. E., Jr. (1981) *J. Biol. Chem. 256*, 736−742.
21. Ray, T. K., and Cronan, J. E., Jr. (1975) *J. Biol. Chem. 250*, 8422−8527.
22. Patthy, L., and Thesz, J. (1980) *Eur. J. Biochem. 105*, 387−393.
23. Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171−6179.
24. Scheideler, M. A., and Bell, R. M. (1991) *J. Biol. Chem. 266*, 14321−14327.
25. Heath, R. J., and Rock, C. O. (1999) *J. Bacteriol. 181*, 1944−1946.

BI982805D