The Active Site His-460 of Human Acyl-coenzyme A:Cholesterol Acyltransferase 1 Resides in a Hitherto Undisclosed Transmembrane Domain*

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Human acyl-coenzyme A:cholesterol acyltransferase 1 (hACAT1) esterifies cholesterol at the endoplasmic reticulum (ER). We had previously reported that hACAT1 contains seven transmembrane domains (TMD) (Lin, S., Cheng, D., Liu, M. S., Chen, J., and Chang, T. Y. (1999) J. Biol. Chem. 274, 23276–23285) and nine cysteines. The Cys near the N-terminal is located at the cytoplasm; the two cysteines near the C-terminal form a disulfide bond and are located in the ER lumen. The other six free cysteines are located in buried region(s) of the enzyme (Guo, Z.-Y., Chang, C. C. Y., Lu, X., Chen, J., Li, B.-L., and Chang, T.-Y. (2005) Biochemistry 44, 6537–6548). In the current study, we show that the conserved His-460 is a key active site residue for hACAT1. We next performed Cys-scanning mutagenesis within the region of amino acids 354–493, expressed these mutants in Chinese hamster ovary cells lacking ACAT1, and prepared microsomes from transfected cells. The microsomes are either left intact or permeabilized with detergent. The accessibility of the engineered cysteines of microsomal hACAT1 to various maleimide derivatives, including mPEG5000-phenylmaleimide (large, hydrophilic, and membrane-impermeant), N-ethylmaleimide, 4-acetamido-4'-maleimidystilbene-2,2'-disulfonic acid (small, hydrophilic, and ER membrane-permeant), and N-phenylmaleimide (small, hydrophobic, and ER membrane-permeant), were monitored by Western blot analysis. The results led us to construct a revised, nine-TMD model, with the active site His-460 located within a hitherto undisclosed transmembrane domain, between Arg-443 and Tyr-462.

* This work was supported by National Institutes of Health Grant HL60306 (to T.-Y. C.).
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2 The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol acyltransferase; AMS, 4-acetamido-4'-maleimidystilbene-2,2'-disulfonic acid; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; HA, hemagglutinin; IA, iodoacetamide; ME, 2-mercaptoethanol; NEM, N-ethylmaleimide; NPM, N-phenylmaleimide; PEG-mal, mPEG5000-maleimide; TMD, transmembrane domain; WT, wild type; aa, amino acid(s); PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase.

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Active Site Histidine Is located in TMD7 of ACAT1

FIGURE 1. The ACAT1 membrane topology models deduced by using various methods. A, by Lin et al. (11). Vertical green lines indicate the inserted positions of the HA tags (11). A vertical black line indicates the position of the putative active site His-460. B, by Joyce et al. (25), C, by Guo et al. (13). Blue lines indicate the positions of the free cysteines and the position of the disulfide linkage in ACAT1. The long red bar indicates region of uncertainty. D, by prediction using the TMD algorithm called TMbase (28). D also illustrates the positions of various mutants created in the current work. Red lines indicate those residues mutated to Cys using the [C92A]His-ACAT1 as the template; underlined black lines indicate those residues mutated to Cys using the [C2 (528/546)]His-ACAT1 as the template; the two black arrows indicate the positions of the two pentapeptide (SGCGS) insertions (I and II).

us to determine the sidedness of the HA tag along the ER membranes. The results obtained, summarized below in Fig. 1A, suggested that hACAT1 contains at least seven TMDs, with the N-terminal region located in the cytoplasmic side of the ER, and the C-terminal region located in the ER lumen (11). The region near the C-terminal half is very hydrophobic and may contain additional membrane embedded segment(s) (11). hACAT1 contains nine cysteines. None of the cysteines is required for catalysis (12). mPEG-maleimide (PEG-mal) is a large, hydrophilic and membrane-impermeant sulfhydryl-specific reagent. The PEG attachment causes an easily detectable shift of ACAT1 band on SDS-PAGE and Western blot (13). We used PEG-mal to map the disulfide linkage and to probe the environment of the free sulfhydryls of the enzyme. The results show that a disulfide bond, formed between Cys-528 and Cys-546, is located in the lumen of the ER membranes. Cys-92 is at the cytoplasmic side of the ER. All the other remaining free cysteines (Cys-333, Cys-345, Cys-365, Cys-387, Cys-467, and Cys-516) are not accessible to PEG-mal under native condition; thus, they are either buried within the ER membranes, or are folded within various regions of the ACAT1 protein itself (13). These results are consistent with a modified, 7-TMD model for ACAT1, as summarized in Fig. 1C. In contrast, Joyce and colleagues had proposed a 5-TMD model for ACAT1 (25). The 5-TMD model, summarized as Fig. 1B, was deduced by monitoring the sidedness of the tag at the end of the C-terminal of the protein, after successive deletions from the ACAT1 C-terminal to produce various truncated ACAT1s. Similar to the 7-TMD model (Fig. 1A and C), the 5-TMD model also proposes the existence of the first four TMDs near the N-terminal half, and the last TMD near the C-terminal. However, it predicts that no other TMD exists, and that all of the followings cysteines: Cys-333, Cys-345, Cys-365, Cys-387, and Cys-467, are located at the cytoplasmic side of the ER membranes. These predictions are incompatible with the PEG-mal modification data (13). In results not shown, we had found that even modest truncation(s) from the C-terminal of ACAT1 led to total inactivation in ACAT enzyme activity. Thus, it is possible that truncation(s) from the C-terminal of ACAT1 might have caused major structural alteration(s) within the ACAT1 polypeptide.

In addition to the experimentally derived 7-TMD model and the 5-TMD model, there is at least one TMD algorithm, called TMbase (28), which predicts that the hydrophobic segments (aa 354–493), depicted as a long red bar in Fig. 1C, contain regions of high helical propensity, and may form two additional TMDs; this prediction is summarized as Fig. 1D. The possible existence of these additional TMDs, depicted as TMD6 and #7 in Fig. 1D, could not be ascertained by methodologies previously employed.

ACAT1 is the prototypic member of a multimembrane-spanning acyltransferase family with more than 20 members that include ACAT1, ACAT2, and diacylglycerol acyltransferase 1. Within this family, an invariant His residue (His-460 in hACAT1) exists and may constitute as part of the active site of the enzyme (14). Previous work from this laboratory showed that His-434, the equivalent of His-460 in hACAT1, is essential for hACAT2 catalysis (15).

Recently, chemical modification by using PEG-mal together with Cys-scanning mutagenesis has been used successfully to investigate the membrane topology of various membrane proteins (16–18). In the current work, we first performed site-specific mutagenesis experiments to test if the conserved His-460 is a key active site residue for hACAT1. We next re-investigated the membrane topology of hACAT1 by performing Cys-scanning mutagenesis at regions of interest, and probed the environment of the engineered cysteines by using PEG-mal and three other small, ER membrane-permeant maleimide derivatives (NEM, AMS, and NPM) with different hydrophobicity. The results led us to construct a revised, 9-TMD model in which the active site His-460 is located in a hydrophobic environment, within a newly disclosed TMD, between...
Active Site Histidine Is located in TMD7 of ACAT1

Arg-443 and Tyr-462. A portion of these results described herein has been published previously in abstract form (19).

EXPERIMENTAL PROCEDURES

Materials—PEG-mal was from Watersheds; N-ethylmaleimide (NEM), N'-phenylmaleimide (NPM), and iodoacetamide (IA) were from Sigma; 4-acetamido-4′-maleimidylstilbene-2,2'-disulfonic acid (AMS) was from Molecular Probes. For modification under non-denaturing condition, stock solutions (PEG-mal at 40 mM, NEM and AMS at 100 mM) were freshly prepared in Buffer A (50 mM Tris-Cl, 1 mM EDTA, pH 7.8). NPM was not soluble in water, so it was dissolved in Me2SO as a 100 mM stock solution. For modification under denatured condition, PEG-mal or IA was prepared as a fresh stock solution in Lysate Buffer (10% SDS, 50 mM Tris-Cl, 1 mM EDTA, pH 8.7). 2-Mercaptoethanol was from Sigma. FuGENE 6 Transfection Reagent was from Roche Molecular Biology. [9,10-3H]Oleic acid was from Amersham Biosciences. PVDF membrane (Immobilon P) was from Millipore. Mouse anti-BiP antibody was from BD Transduction Laboratories. The rabbit polyclonal antibodies (DM10) generated against the N-terminal fragment (1–131) of hACAT1 were described previously (20). Goat anti-rabbit IgG(L+H)-HRP conjugate and Goat anti-mouse IgG(L+H)-HRP conjugate were from Bio-Rad. The Supersignal West Pico Chemiluminescent Substrate was from Pierce.

Cell Culture—The CHO cells were cultured in F-12/Dulbecco's modified Eagle's medium (50:50) supplemented with 10% fetal bovine serum in a 5% CO2 incubator at 37 °C. The ACAT1-deficient CHO cell line AC29 (21) was used to express the N-terminal His8-tagged hACAT1 (His-hACAT1) or its various engineered mutants. For transfections, the AC29 cells were cultured in 6-well plates to 70–80% confluency and transfection of 2 μg of pcDNA3 vectors encoding His-hACAT1 or its mutants, using FuGENE 6 Transfection Reagent according to the manufacturer’s protocols. On the second day, the cells were trypsinized, divided equally into three wells, and grown for 2 days in G-418 containing medium for ACAT enzyme activity and protein expression studies (22). Alternatively, for chemical modification studies, on the second day after transfection, the cells were trypsinized; cells from 2 to 3 wells were pooled to a single 90 mm dish, and grown for 2 days at 37 °C, with G418 (0.3 mg/ml) present in the growth medium. Cells were then harvested for chemical modification studies.

Recombinant DNA Technology—All of the ACAT1 mutants were prepared using the QuikChange mutagenesis kit from Stratagene according to the manufacturer's manual. For mutants based on His-hACAT1 or [C92A]His-hACAT1 as the template, the DNA constructs pGEM-7Z(−)/His-hACAT1 or pGEM-7Z(−)/[C92A]His-hACAT1 were used as the mutagenesis template (13). All of the expected mutations were confirmed by DNA sequencing. The mutated ACAT1 gene was transferred from pGEM-7Z(−) vector to pcDNA3 vector as follows: The 1.7-kb DNA fragment encoding ACAT1 mutants was released from the pGEM-7Z(−) vector by HindIII cleavage, filled in by T4 DNA polymerase, and then cleaved with EcoRI. Subsequently, the 1.7-kb DNA fragment was ligated into the pcDNA3 vector pretreated with EcoRI and EcoRV. For the mutants based on [C2 (528/546)]His-hACAT1 as the template (12, 13), the construct pcDNA3/[C2 (528/546)]His-hACAT1 was used as the mutagenesis template, and the expected mutations were confirmed by DNA sequencing. For the mutants based on hACAT1-HA6, containing a HA tag (with an amino acid sequence of YPYDVPDYA) between Tyr-404 and Lys-405, the construct pcDNA3/hACAT1-HA6 (11) was used as the mutagenesis template. The Cys-92 residue in ACAT1-HA6 was mutated to Ala-92 by replacing the DNA fragment (cut by KpnI and Eco47III) with the same fragment released from pcDNA3/[C92A]His-ACAT1.

ACAT Activity Assay in Intact Cells—This method measures the rate of [3H]cholesterol oleate synthesis in intact cells (23). The transiently transfected AC29 cells were cultured in 6-well plates at 37 °C as described above. The cells were given a fresh media change (1 ml/well) 2 h before the assay. Then, 20 μl of 10 mM [3H]oleate in 10% bovine serum albumin was added to the media at 37 °C for 30 min.

ACAT1 Protein Content Analysis after Transfection—The cells in 6-well plates were washed with 2 ml of phosphate-buffered saline and lysed by 240 μl of Lysate Buffer containing 10 mM IA. The cell lysates were transferred to Eppendorf tubes, 60 μl/tube of SDS-PAGE Loading Buffer (10% SDS, 20% glycerol, 0.05% bromphenol blue, 50 mM Tris-Cl, pH 6.8) was added, and the samples were mixed well by vigorous vortexing. Then, 60 μl of the sample mixture were loaded onto a 9% SDS-gel. After electrophoresis, the proteins were transferred to a PVDF membrane, and the ACAT1 protein bands were visualized by Western blot using DM10 as the primary antibodies. The relative amount of the hACAT1 mutants versus that of the WT hACAT1 was analyzed by densitometry.

Permeability Analysis of Microsomal Vesicles—The AC29 cells that stably express hACAT1 were cultured in 90-mm dishes and lysed by hypotonic shock followed by scraping according to the previously published procedure (24). After centrifugation (800 × g, 5 min) to remove the unbroken cells and nuclei, the whole cell homogenates underwent ultracentrifugation (100,000 × g, 30 min) at 4 °C. After ultracentrifugation the pellets that contain microsomal vesicles were gently resuspended in the cold buffer A. Then, the microsomal vesicles were treated with or without saponin, and/or with or without PEG-mal as indicated at 4 °C for 1 h, and then 2-mercaptoethanol (from a freshly prepared 100 mM stock solution in Buffer A) was added to the final concentration of 10 mM (to react with the excess amount of PEG-mal). The samples underwent ultracentrifugation (100,000 × g, 1 h) at 4 °C to collect the microsomal vesicles. After ultracentrifugation, the supernatants were carefully removed, and the pellets were resuspended in buffer A containing 10 mM 2-mercaptoethanol at the same volume as the supernatants. Then, Lysate buffers containing 40 mM IA were added to the supernatants and the pellets at a volume equal to that of the supernatants and the pellets. The reactions were incubated at room temp for 30 min. Next, SDS-PAGE loading buffer at 1/4 volume of the sample mixtures were added. The final supernatant and the pellet modification mixtures were loaded in equal amounts onto a SDS-gel (9%) for SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane, and the BiP and ACAT1 protein bands were visualized by Western blot using anti-BiP and anti-ACAT1 antibodies, respectively.

PEG-mal Modification under Non-denaturing Condition—The AC29 cells transiently expressing His-hACAT1 mutants were cultured in 90-mm dishes. The whole cell homogenates were prepared by the same method as described earlier. Then, the 40 mM stock PEG-mal solution dissolved in Buffer A (50 mM Tris-Cl, 1 mM EDTA, pH 7.8) was added to the cell lysates, in the absence or presence of saponin (which permeabilizes the microsomal membranes) (15, 13). The final concentration of PEG-mal was 4 mM, and the final concentration of saponin was 5 mg/ml. The modification reactions were carried out at 4 °C for 1.5 h. After incubation, 2-mercaptoethanol was added to the final concentration of 10 mM (to react with the excess amount of PEG-mal). The reactions were carried out at 4 °C for an additional 30 min. Subsequently, 40 mM IA in Lysate Buffer at volumes equal to the sample volume was added (to modify the remaining unreacted thiol groups of hACAT1 under denatured condition). Finally, one-half to one-fourth of
the total modification mixtures were used for analysis by SDS-PAGE; the proteins were transferred to a PVDF membrane after electrophoresis. The ACAT1 bands were visualized by Western blot using DM10 as the primary antibodies.

NEM, AMS, and NPM Modifications under Nondenaturing Condition—The whole cell homogenates of AC29 cells that transiently express ACAT1 mutants based on [C2 (528/546)]His-ACAT1 were prepared by the same method as described earlier. The 800 x g supernatants that contain the microsomes were treated with NEM, or AMS, or NPM as indicated at 4 °C for 1.5 h. The final concentration of NEM, AMS, or NPM was 4 mM. For parallel NEM/AMS modifications, the reactions were carried out in Buffer A; for parallel NPM/NE modification mixtures, because NPM is relatively insoluble in buffer A, the modifications with NEM or with NPM were carried out in buffer A containing 4% Me2SO. After incubation, the modification mixture was diluted 40-fold and incubated at 4 °C for 30 min (to quench the reactions between free cysteines in ACAT1 and the maleimides). For parallel NEM/AMS modification mixtures, Buffer A was used for dilution; for parallel NPM/NE modification mixtures, Buffer A containing 4% Me2SO was used for dilution. Afterward, the microsomes in the modification mixtures were separated from the maleimide used by ultracentrifuge (100,000 x g, 30 min) at 4 °C. The pellets were resuspended in 50 μl of Buffer A. Then, 50 μl of Lysate Buffer containing 10 mM PEG-mal was added per sample at room temperature for 30 min (to modify the residual free cysteines that did not react with NEM or with AMS). Finally, the modification mixtures were analyzed by SDS-PAGE, and the proteins were transferred to a PVDF membrane after electrophoresis. The ACAT1 bands were visualized by Western blot using DM10 as the primary antibodies.

RESULTS
Determining the Importance of the Conserved His-460 and the Conserved Ser-269 in hACAT1—Bioinformatic analysis suggested that for the membrane-bound O-acyl transferase superfamily, the absolutely conserved His residue (His-460 in hACAT1), located within a long stretch of hydrophobic amino acid residues, might constitute the active site (14). In contrast, Joyce and colleagues (25) proposed that a Ser residue (Ser-269 in hACAT1), conserved within ACAT1, ACAT2, and dicylglycerol acyltransferase 1, might be the active site. The proposal by Joyce and colleagues was based on the fact that when the mutant ACAT1 with Leu replacing Ser-269 was expressed in AC29 cells, it provided no measurable ACAT enzyme activity. These investigators did not report the protein expression level of the S269L ACAT1 mutant. In principle, it is possible that either Ser-269 or His-460, or both, may serve as the active site of ACAT1. For example, carnitine acyltransferase utilizes a His residue as the catalytic site (26), whereas lecithin:cholesterol acyltransferase utilizes a Ser residue as part of the Ser/His/Asp catalytic triad (27). To determine the importance of Ser-269 and His-460, we replaced His-460 and Ser-269 individually with different amino acids by site-specific mutagenesis, expressed the mutants in AC29 cells by transient transfections, and analyzed the activities and the protein expression levels of the individual mutant enzymes. As shown in Fig. 2 (lanes 6 and 7), when His-460 was replaced by Ala or by Asn, the mutant enzymes retained nearly normal protein expression levels, but they completely lost enzymatic activity. However, when Ser-269 was replaced by Ala or by Thr (Fig. 2, lanes 3 and 5), the mutant retained nearly normal enzyme activity as well as nearly normal protein expression levels. Interestingly, when Ser-269 was replaced by Leu (Fig. 2, lane 4), the mutant had no detectable enzymatic activity, nor did it exhibit any detectable protein expression. Therefore, the loss of activity of the S269L mutant was likely caused by the loss of ACAT1 protein expression in CHO cells. In results not shown, we found that the S269L mutant hACAT1 could be expressed in insect cells, and it still retained ~70% of the activity expressed by the wild-type hACAT1. Therefore, we conclude that His-460 is the active site of hACAT1. Ser-269 is not the active site, but it may play important role(s) in maintaining the structural integrity/protein stability of ACAT1 in mammalian cells. These results are consistent with our earlier findings in hACAT2: mutation in the conserved His-434 led to complete loss in enzyme activity without significant loss in protein expression, whereas mutating the conserved Ser-245 to Leu led to significant loss in protein expression in CHO cells (15).

Rationale for Developing a New Strategy to Study the ACAT1 Membrane Topology—We aimed at developing a new strategy to resolve the ACAT1 topology that covers the uncertainty region aa 354–493. We have previously used PEG-mal to probe the environment of the seven free cysteines of wild-type hACAT1 in microsomes. The structure of PEG-mal (as well as those of other maleimide derivatives used in the current work) is shown in Fig. 3A. The result showed that only one Cys, Cys-92, reacts to PEG-mal; the other six free cysteines are resistant to PEG-mal. We also showed that replacing Cys-92 with Ala in hACAT1 does not significantly alter the protein expression level or its enzyme activity; when the C92A hACAT1 mutant was examined, no Cys was available for modification by PEG-mal (13). These results were obtained whether the microsomes were treated with or without the mild detergent. In the current work, we took advantage of these observations, used the C92A hACAT1 mutant as the template, and created various single Cys replacement mutants. To minimize structural perturbation, only non-conserved amino acids were chosen as targets for Cys replacement. The resultant engineered single Cys mutants were expressed in AC29 cells by transient transfections. The microsomes that contain the engineered Cys ACAT1s are subjected to PEG-mal modification according to the procedure previously employed (13). This strategy is schematically presented in Fig. 3B. PEG-mal is a large, membrane-impermeant molecule; one PEG attachment causes an easily detectable shift of the ACAT1 band on SDS-PAGE and Western blot. The prediction is that, depending on the positions of the single Cys relative to the ER membrane bilayer, we may obtain three different outcomes: 1) if the introduced Cys is exposed in the cytoplasmic side of the ER, it would be modified by PEG-mal with equal efficiency with or without saponin treatment; 2) if the introduced Cys is exposed in the luminal side of ER, it would be relatively resistant to modification by PEG-mal without saponin, but would be modified much more efficiently with or without saponin treatment; and 3) if the introduced Cys is buried in the ER membranes, or is sterically hindered in the protein interior, it would be relatively resistant to modification by PEG-mal with or without saponin. Thus, differential sensitivities toward PEG-mal modification with or without saponin may serve to report the locations of various engineered Cys relative to the ER membranes.

Monitoring the Permeability of the Microsomal Vesicles before and after the PEG-mal Treatment—To adopt the procedure described in Fig. 3B for ER membrane protein topology studies, it is essential that the isolated microsomal vesicles remain sealed before and after the PEG-mal treatment. To test this assumption, we monitored the permeability of the PEG-mal-treated microsomal vesicles that contain the wild-type hACAT1 with or without saponin. It is known that BIP is a soluble protein present in the ER lumen. If the PEG-mal-modified microsomal vesicles remain sealed, subjecting the PEG-mal treated microsomes to ultracentrifugation will not cause BIP to appear in the supernatant after centrifugation, unless the PEG-mal treated microsomes are treated with...
saponin (which is a weak detergent known to permeabilize the ER membranes). As shown in panel a of Fig. 3C, without saponin treatment there is no detectable BiP in the supernatant after ultracentrifugation; with saponin treatment, most of the total BiP signal appears in the supernatant after ultracentrifugation. This result shows that after the PEG-mal treatment without saponin, the microsomal vesicles remain largely sealed. Additional results presented in panel b of Fig. 3C show that, after modification by PEG-mal with or without saponin treatment, ACAT1 remains in the microsomal pellet, indicating that saponin is a weak detergent and cannot solubilize the ACAT1 from the ER membranes.

Determining the Enzyme Activity of Engineered Single Cys ACAT1 Mutants—To put the new strategy described in Fig. 3B into practice, we used the C92A hACAT1 as the template, performed Cys-scanning mutagenesis, and created numerous single cysteines at non-conserved amino acid residues within the region aa 354–493. To serve as controls, we also created four additional mutants, with single cysteines located near the N-terminal half of ACAT1 (G165C, S212C, A245C, and S311C). We also created two insertion mutants, designated as Insert-I and Insert-II. Insert-I contained a hydrophilic pentapeptide (SGCGS) containing a C, and was inserted between Ala-245 and Tyr-246; Insert-II contained the same pentapeptide but was inserted between
Active Site Histidine Is located in TMD7 of ACAT1

Ty-462 and Ala-463. The locations of all the engineered Cys mutants are indicated in Fig. 1D. To monitor the effects of mutations on ACAT1, we measured the enzymatic activities of these mutants expressed in AC29 cells. The results show that after normalization based on ACAT1 protein content (Fig. 4B), all of the engineered single Cys ACAT1 mutants, except the H460C mutant, retained at least 50% or more of the parental ACAT1 enzyme activity (Fig. 4C). Based on the premise that gross structural changes in enzymes are usually accompanied by large losses in enzyme activity, these results suggest that most of the selected single Cys replacement mutations described in Fig. 4 do not cause gross structural changes of the ACAT1 protein. The complete activity loss of the H460C mutant supports the H460A and H460N results shown earlier (Fig. 2) and is consistent with the interpretation that His-460 is a key active site residue of ACAT1. The large loss of activity of the Insert-II mutant suggests that the region at the insertion site (between Tyr-462 and Ala-463), which is very close to His-460, is very critical for catalysis. In contrast, the Insert-I mutant possesses ~80% of the parental ACAT1 enzyme activity, suggesting that the region at the insertion site (between Ala-245 and Tyr-246) is not critical for enzyme activity.

Testing the PEG-mal Modification Method by Using the ACAT1 Mutants with Engineered Single Cys Located at the N-terminal Half—As indicated earlier (Fig. 1), a consistent feature emerging from previous ACAT1 topology studies is the existence and the approximate locations of the first four TMDs near the N-terminal half. We use this feature to test the utility and limitation of the new strategy for ACAT1 membrane topology study. We prepared microsomes that contain mutant hACAT1s, with engineered single cysteines located near the N-terminal half of hACAT1 (G165C, S212C, A245C, and S311C), as well as the Insert-I mutant, and subjected them to topology analysis using the strategy described in Fig. 3B. For each mutant (indicated at the top of each frame of Fig. 5), the results are presented in three columns: the first column is from untreated microsomes, the second column is from PEG-mal modified microsomes without saponin, and the third column is from PEG-mal modified microsomes with saponin. For each mutant, the intensities of the PEG-ACAT1 bands in columns 2 and 3 are measured by densitometry, and their ratio is reported as the second lane from the bottom of each frame. This ratio provides an estimate in relative efficiency of PEG-mal modification without saponin versus that with saponin. The results (Fig. 5) show that, as expected, the [C92A]His-ACAT1 (used as the template for all the single Cys mutants reported in Fig. 5) was resistant to PEG-mal modification with or without saponin treatment. For the mutant G165C enzyme, the efficiency of PEG-mal modification greatly increased with saponin: the ratio between the intensities of the PEG-ACAT1 bands in columns 2 and 3 is 1:1.2. This result implies that Gly-165 is located in an exposed position in the ER lumen. This interpretation is indicated at the bottom of each frame in Fig. 5. Because Cys-92 is located in cytoplasm (13) and Gly-165 is located in the ER lumen, a TMD exists between C92A and Gly-165 (TMD1). For the mutant S212C enzyme, the efficiencies of PEG-mal modification without or with saponin were approximately the same; the ratio between column 2 and column 3 is 1:1.1. This result implies that Gly-165 is located in an exposed position in the ER lumen. This interpretation is indicated at the bottom of each frame in Fig. 5. Because Cys-92 is located in cytoplasm (13) and Gly-165 is located in the ER lumen, a TMD exists between C92A and Gly-165 (TMD1). For the mutant A245C enzyme, the efficiencies of PEG-mal modification without or with saponin were approximately the same; the ratio between column 2 and column 3 was reported as ‘NA’ (not determined). The supernatant (S) and the pellet (P) were analyzed by SDS-PAGE, and the proteins were transferred to a PVDF membrane. The signals for BiP and for ACAT1 were detected by Western blot using anti-BiP (panel a) and anti-ACAT1 antibodies (panel b). The details are described under "Experimental Procedures." Results are representative of two independent experiments.

FIGURE 3. In A: panel a, the chemical structures and the molecular weights of various maleimide derivatives; panel b, the reaction between the sulfhydryl of Cys in a protein and the maleimide moiety. In B: schematic presentation of the PEG-mal modification procedure used to study ACAT1 membrane topology. Microsomal vesicles prepared from cells that express various ACAT1s that contain an engineered single Cys mutation were treated with or without saponin (final concentration is 5 mM) as indicated, at 4 °C for 1 h. Afterward, the mixtures were subject to ultracentrifugation at 4 °C (100,000 g, 1 h).
applicable). This result implies that A245C is located in a buried position not available for PEG-mal modification. Based on previous studies (summarized in Fig. 1, A and B), Ala-245 was predicted to be located in the ER lumen. We note that the loop between the predicted TMD3 and TMD4 is very short (Fig. 1, A and B). Thus it is possible that, being located in two adjacent TMDs, the A245C is shielded from reacting with PEG-mal by steric hindrance. To test this hypothesis, we tested the Insert-I mutant. As indicated earlier, Insert-I contains a hydrophilic pentapeptide (SGCGS) containing a C, and is inserted between Ala-245 and Tyr-246. The result showed that the efficiency of PEG-mal modification greatly increases with saponin; the ratio between column 2 and column 3 is 1:10. This result implies that Insert-I is located and is exposed in the ER lumen and supports the interpretation that Ala-245 is located within a short loop between TMD3 and TMD4. For the S311C enzyme, the result shows that it can be modified by PEG-mal with or without saponin at equal efficiency, with the ratio being 1:0.9. Thus, Ser-311 is located at an exposed position in the cytoplasm, and there should be a TMD (TMD4) between Insert-I and Ser-311. In summary, the present PEG-mal modification method suggests that there are four TMDs in the N-terminal half of ACAT1 and is consistent with the results derived from previous studies by using two different methods.

Using the PEG-mal Modification Method to Re-investigate the Membrane Topology of hACAT1 within the Region aa 354–493—We next used the PEG-mal modification method to examine the locations of the single Cys in mutant hACAT1s, with the Cys engineered at various positions within the region as 354–493. We also examined the location of the single Cys in the Insert-II mutant. The results are shown in Fig. 5. The results can be summarized in three categories: the first category includes S446C, A454C, V459C, H460C, A463C, and V477C. These cysteines are relatively resistant to PEG-mal modification with or without saponin. The second category includes Q354C, Y462C, and Insert-II. The efficiency of PEG-mal modification of these cysteines is greatly enhanced by saponin treatment. The third category includes G399C, K405C, S410C, S441C, A485C, and S493C. The efficiency of PEG-mal modification of these cysteines is not significantly enhanced by saponin treatment. To analyze these results from Gln-354 to Ser-493 sequentially: the Q354C mutant enzyme showed relative resistance to modification by PEG-mal without saponin but became much more sensitive to...
FIGURE 5. PEG-mal modification of ACAT1 mutants with single Cys replacements or with Cys-containing pentapeptide insertions. The ACAT1 mutants were created by using [C92A]His-ACAT1 as the template. The modified mixtures were analyzed by SDS-PAGE, and the ACAT1 bands were visualized by Western blot using anti-ACAT1 antibodies at 0.5 μg/ml. The details are described under "Experimental Procedures." The results are the composite of several experiments performed at different times. The exposure time for Western analysis ranged from 30 s to 2 min. For each construct, the results shown are representative of at least two independent experiments.

Active Site Histidine Is located in TMD7 of ACAT1

modification with saponin, suggesting that Gln-354 is located at an exposed position in the ER lumen. Because Ser-311 is located in the cytoplasm, this result suggests that a TMD may exist between Ser-311 and Gln-354. This interpretation is consistent with our recent result indicating that the two native cysteines, Cys-333 and Cys-345, are both located in certain buried positions, enabling them to be resistant to PEG-mal modification with or without saponin (13). This TMD is designated as TMD5; shown in Fig. 10. The existence of TMD5 had previously been reported by our group (11). When the G399C, K405C, S410C, and S441C mutants were tested, the results showed that each was equally susceptible to PEG-mal modification with or without saponin; these results are consistent with the existence of a long hydrophilic loop, located in the cytoplasm and containing all of these residues. Because Gln-354 is in the ER lumen, whereas the long hydrophilic loop is in the cytoplasm, a TMD may exist between them (designated as TMD6, Fig. 10). The existence of TMD6 is inconsistent with results reported previously (11, 25), but is consistent with the TMD prediction by using the TMbase algorithm (Fig. 1D). It is also consistent with our recent result indicating that the two native cysteines, Cys-365 and Cys-387, are both resistant to PEG-mal modification with or without saponin (13). When the S446, A454, V459, and the H460C mutants were tested, the results showed that each was relatively resistant to PEG-mal modification with or without saponin; these results are consistent with the existence of a long hydrophilic region that contains all of the residues. However, when the Y462C mutant, with Y462 located only 2 aa away from H460, was tested, the results showed that the efficiency of PEG-mal modification was moderately stimulated (the ratio is 1:3.5), suggesting that Y462 might be located at a partially exposed position in the ER lumen. To test the validity of this interpretation, we tested the Insert-II mutant. As indicated earlier, Insert-II contains the hydrophilic pentapeptide (SGCGS) inserted between Y462 and A463. The result showed that the efficiency of PEG-mal modification of the Cys within the engineered pentapeptide is greatly stimulated (the ratio is 1:12). These results support the interpretation that the region that contains Y462 is partially exposed at the ER lumen. Thus, it is likely that the long hydrophilic region that precedes Y462, and contains residues S446, A454, V459, and the H460C forms a TMD (designated as TMD7, Fig. 10). The possible existence of TMD7 could not be revealed by the methodologies employed in previous studies, but is consistent with the TMD prediction by using the algorithm TMbase (Fig. 1D). When the mutant enzymes A463C and V477C were tested, the results showed that each was relatively resistant to PEG-mal modification with or without saponin. Consistent with these results, we had previously shown that the native Cys-467, at the vicinity of Ala-463, was also relatively resistant to PEG-mal modification with or without saponin (13). When the A485C mutant and the S493C mutant were tested, the results showed that each mutant could be modified with or without saponin at approximately equal efficiency (the ratio was 1:1.6 or 1:1, respectively). Together, these results suggest the existence of another TMD between Tyr-462 and Ala-485, designated as TMD8, which includes Ala-463 and Val-477. The existence of TMD8 had previously been reported by our group (11). The disulfide linkage near the C-terminal (between Cys-528 and Cys-546) is located in the ER lumen (13). Because Ser-493 is located in the cytoplasm, whereas the C-terminal is located in the ER lumen, there should be another TMD between them, designated as TMD9. The existence of TMD9 is consistent with results of previous studies (11, 25). In summary, our current work revealed the probable existence of two additional TMDs: one is located between Cys-365 and Cys-387 (TMD6, Figs. 1D and 10), and the other is located between Arg-443 and Tyr-462 (TMD7, Figs. 1D and 10).

Testing the Probable Existence of TMD6 and #7 by Using the C2 (528/546) His-ACAT1 as the Template for Mutagenesis—We used the C92A ACAT1 mutant as the template to create the single Cys mutants described in Fig. 5. The C92A mutant contains 6 free cysteines, all are resistant to PEG-mal modification under non-denaturing condition. When the engineered single Cys mutants were subjected to PEG-mal modification, one assumes that the 6 native cysteines present in the template remain silent toward PEG-mal modification. Although this is a reasonable assumption, the possibility exists that introduction of an engineered single Cys mutation may alter the accessibility of the 6 native free cysteines to PEG-mal. To safeguard against this possibility, we created 9 selected single Cys mutants (Q354C, K405C, S441C, S466C, K405C, G410C, S441C, S466C, A454C, and V459C) to test the possible existence of a TMD between them (designated as TMD6, Fig. 10). The possible existence of TMD6 could not be revealed by the methodologies employed in previous studies, but is consistent with the TMD prediction by using the algorithm TMbase (Fig. 1D). When the mutant enzymes A463C and V477C were tested, the results showed that each was relatively resistant to PEG-mal modification with or without saponin. Consistent with these results, we had previously shown that the native Cys-467, at the vicinity of Ala-463, was also relatively resistant to PEG-mal modification with or without saponin (13). When the A485C mutant and the S493C mutant were tested, the results showed that each mutant could be modified with or without saponin at approximately equal efficiency (the ratio was 1:1.6 or 1:1, respectively). Together, these results suggest the existence of another TMD between Tyr-462 and Ala-485, designated as TMD8, which includes Ala-463 and Val-477. The existence of TMD8 had previously been reported by our group (11). The disulfide linkage near the C-terminal (between Cys-528 and Cys-546) is located in the ER lumen (13). Because Ser-493 is located in the cytoplasm, whereas the C-terminal is located in the ER lumen, there should be another TMD between them, designated as TMD9. The existence of TMD9 is consistent with results of previous studies (11, 25). In summary, our current work revealed the probable existence of two additional TMDs: one is located between Cys-365 and Cys-387 (TMD6, Figs. 1D and 10), and the other is located between Arg-443 and Tyr-462 (TMD7, Figs. 1D and 10).
A454C, V459C, H460C, Y462C, and V477C), using the C2 (528/546) hACAT1 (designated as the C2ACAT1) as the template. The C2ACAT1 retains the disulfide linkage near its C-terminal but is completely devoid of the 6 native free cysteines. Our previous data showed that when expressed in AC29 cells, the C2ACAT1 remains mainly localized in the ER and exhibits 70% of the parental hACAT1 activity; enzyme kinetic analysis showed that the C2ACAT1 exhibits a 2-fold increase in the $K_m$ value for oleyl coenzyme A ($14 \mu M$, versus $7 \mu M$ for the parental hACAT1) and exhibits the same sigmoidal cholesterol saturation curve as the parental hACAT1 (12).

The single Cys mutants produced in the C2ACAT1 template were expressed in AC29 cells by transient expression; their activities and expression levels were analyzed and subjected to PEG-mal modification studies. For activity studies, the results show that after normalization based on ACAT1 protein content, five of these nine mutants (Q354C, K405C, S446C, A454C, and V477C) retained at least 50% or more of the parental ACAT1 enzyme activity; the sixth mutant enzyme H460C, exhibited no enzyme activity as expected (Fig. 6).

The PEG-mal modification results (Fig. 7) show that the A454C, H460C, and V477C mutants were relatively resistant to PEG-mal modification with or without saponin. For the Q354C mutant, the PEG-mal modification was greatly enhanced by saponin. For the K405C mutant, it was modified by PEG-mal with or without saponin at approximately equal efficiency. These results corroborated with the results obtained when the corresponding mutants were constructed by using the C92AHisACAT1 as the template. For the S446C mutant, the Cys was resistant to PEG-mal without saponin, but became moderately sensitive to PEG-mal modification with saponin. This result slightly deviated from the previous result: when the S446C was present in the C92A ACAT1 background, the Cys was resistant to PEG-mal modification with or without saponin.

The three remaining mutants (S441C, V459C, and Y462C) exhibit poor protein expression and/or poor ACAT enzyme activity, as shown in Fig. 6 (B and C). The S441C mutant only retained 10% of the parental enzyme activity. The PEG-mal modification analysis of this mutant suggests that S441C is in the cytoplasm, consistent with the result obtained when the corresponding mutant was present in the C92A ACAT1 background. For the V459C mutant and the Y462C mutant, the relative protein expression levels of these mutants were much lower than any other mutants tested (Fig. 6B), which caused the evaluation of their enzyme activities to be unreliable. The PEG-mal modification analysis (Fig. 7) shows that for Y462C, its PEG-mal modification is much stimulated with saponin; this result is consistent with the result obtained when the corresponding mutant was present in the C92A ACAT1 background.
The V459C mutant is relatively resistant to modification without saponin but becomes moderately sensitive to modification after saponin treatment. This result slightly deviates from the previous result, which showed that when the V459C mutant is in the C92A ACAT background, it is relatively resistant to modification with or without saponin.

In summary, the PEG-mal modification results obtained from seven of these nine additional mutants in the C2ACAT1 background corroborate with the results using the corresponding mutants in the C92A ACAT1 background. Two anomalies exist: S446C and V459C. When present in C2ACAT1, the cysteines in both mutants respond to saponin in their susceptibility to PEG-mal modification. Instead, when present in C92A ACAT1, both exhibit resistance to PEG-mal modification with or without saponin. We note that the Ser-446 and Val-459 are located near the two opposite ends of the proposed seventh TMD (Figs. 1D and 10). These results suggest that TMD7 in C2ACAT2 may not be as efficiently packed within the ER lipid bilayer as it is in the C92A ACAT1.

Comparing NEM, AMS, and NPM Modifications of Single Cys Mutants in C2ACAT1—The results described above show that the single cysteines engineered in the A454C, H460C, and V477C mutants are resistant to modification by PEG-mal with or without saponin, suggesting that these residues are located in a sealed region within the ER membrane bilayer. PEG-mal is a hydrophilic molecule with large molecular weight. Therefore, it is possible to explain these data in the following alternative manner: these single Cys mutants are resistant to PEG-mal modification, because they are located within the ACAT1 protein interior, such that they are sterically shielded from reacting with PEG-mal, a relatively large molecule. To test this alternative interpretation, we first compared the reactivity of each of the single Cys mutants described in Fig. 6 toward two smaller, hydrophilic thiol-specific modification agents, NEM (molecular mass, 125 Da) and AMS (molecular mass, 490 Da) (their structures are shown in Fig. 3A). The NEM can cross both the plasma membranes and the ER membranes, whereas AMS cannot cross the plasma membranes but can cross the ER membranes (29). Both NEM and AMS are small molecules, so their reaction to the single Cys in ACAT1 cannot cause a detectable band shift of ACAT1 on SDS-PAGE. Thus, we used a two-step modification procedure to detect the NEM/AMS modification: the ACAT1 mutants were first treated with NEM or with AMS under native condition (without saponin and without SDS). Afterward, they were treated with PEG-mal under fully denatured condition (5% SDS (13)). If the introduced single Cys of the ACAT1 mutant is sensitive to modification by NEM or by AMS, it will be much less available for further modification by PEG-mal, and there will be little or no band-shift on SDS-PAGE, and

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vice versa. The results show (Fig. 8A) that the Q354C, K405C, S441C, and V462C mutants can all be easily modified either by NEM or by AMS. These results are consistent with the previous results obtained by using the PEG-mal modification alone (Fig. 7) and support the interpretation that these residues are located in exposed region(s). For the S446C and V459C mutants, the results show that either NEM or AMS can also easily modify them. Previous results showed that, in the C2ACAT1 background, these two cysteines are only partially sensitive to PEG-mal modification. Together, these results suggest that both the S446C and the V459C are located in a sterically hindered region where large molecules cannot readily gain access, but small molecules can. These results are consistent with the interpretation that in C2ACAT1, S446C and the V459C are probably located in region(s) that are loosely packed within the ER lipid bilayer, where they are partially exposed to small molecules. For the V459C mutant and the Y462C mutants, the results show that they are more susceptible to modification by NEM than by AMS (the ratio being 1:6 or 1:5, respectively, Fig. 8A). Both NEM and AMS are hydrophilic molecules; the molecular weight of AMS is larger than that of NEM (Fig. 3A). These results suggest that, in the C2ACAT1 background, V459C and Y462C are located in the sterically hindered region(s) such that they are more accessible to reagents of smaller size.

In contrast, the A454C, H460C, and V477C mutants demonstrated resistance to modification either by NEM or by AMS (Fig. 8A). These results are consistent with the interpretation derived from previous results, i.e., A454C, H460C, and the V477C are tightly packed within the membrane bilayer, where the concentration of the hydrophilic modification agent is much lower than that in the aqueous phase.

We next compared the reactivities of various single Cys mutants in C2ACAT1 toward NEM and NPM, which is a hydrophobic maleimide (structure shown in Fig. 3A). As shown in Fig. 8B, those cysteines (Q354C, K405C, S441C, V459C, and Y462C) that can be modified by NEM can also be modified by NPM. For the A454C, H460C, and V477C mutants, the results show that they are more susceptible to modification by NPM than by NEM (the ratios being 1:0.5; 1:0.5; or 1:0.2, respectively). In results not shown, we had performed additional experiments on the A454C, H460C, and V477C mutants, by restricting the NEM/MPM reaction time to 30 min (instead of 90 min). The results of these experiments again showed that the A454C, H460C, and V477C mutants were more susceptible to NPM modification than NEM modification (results not shown). Together, these results support the interpretation that A454C, H460C, and V477C are located within the lipid bilayer; their topological locations in the ER membrane render them to be more susceptible to modification by the hydrophobic maleimide (NPM) than by the hydrophilic maleimide (NEM). These results are not compatible with the alternative interpretation that the resistance of these single Cys mutants to PEG-mal modification is because they are located within the ACAT1 protein interior, and are sterically shielded from interacting with PEG-mal. For the V459 and Y462C mutants, they exhibited approximately equal sensitivity toward the modifications by either NEM or NPM. These results support the previous results obtained by using the NEM/AMS pair, and suggest that in the C2 ACAT2 background, these residues are located in the environment partially susceptible to small molecules.

Resolving the Discrepancy between the Immunofluorescence Data and the PEG-mal Modification Data—Our previous immunofluorescence analysis suggested that the HA tag (amino acid sequence: YPYDVPDYA) inserted between Tyr-404 and Lys-405, designated as HA6 (Fig. 1A) of hACAT1 is located in the ER lumen (11). In contrast, the current PEG-mal modification data (Figs. 5 and 7) suggest that a long hydrophilic stretch exists between residues 399 and 441; this region is located in the cytoplasm and includes the HA6 insertion site. To investigate the cause for this discrepancy, we first used the HA6 HisACAT1 construct employed in our previous work (11) as the template and created the C92A mutant (designated as C92A HA6 HisACAT1). We then produced two additional mutants using the C92A HA6 HisACAT1 as the template: in the first mutant (designated as C92A, HA6, K405C HisACAT1), the Lys-405 was mutated to C; in the second mutant (designated as C92A, HA6, V-C HisACAT1), the Val residue within the HA tag sequence was mutated to C. These mutants and their template were expressed in AC29 cells and were employed to perform the PEG-mal experiments. As shown in Fig. 9A, the Cys present in both mutants can be modified by PEG-mal with or without saponin, whereas the template C92A, HA6 HisACAT1 could not. This result suggests that the HA tag inserted between Tyr-404 and Lys-405 is located on the cytoplasmic side of ER membrane. In a separate experiment, we performed immunofluorescence analysis of cells that express these mutants or their template. The HA1 HisACAT1 construct described in our previous work (11) was also used to serve as a control. This construct contains the HA

![Figure 9](image-url)
tag near the ACAT1 N-terminal, which is known to be located in the cytoplasm (Fig. 1A). After selective permeabilization by digitonin or by Triton X-100, double immunostainings using antibodies against the HA tag and against the antigenic sites near the ACAT1 N-terminal were performed according to procedures previously described (11). The signals were monitored under a fluorescence microscope. The results show that in all the parental and mutant ACAT1s examined, the N-terminal ACAT1 antigenic sites react to the anti-ACAT1 antibodies after cells are exposed to digitonin or to Triton X-100. Thus, the ACAT1 N-terminal segment is located in the cytoplasm. Likewise, the HA tag located in HA1 HisACAT1 also reacts to the anti-HA antibody after cells are exposed to either digitonin or to Triton X-100. In contrast, the HA tag in the C92A, HA6 HisACAT1, or in the HA6 HisACAT1 can only react to the HA antibody after cells are exposed to Triton X-100, but not when exposed to digitonin. These results reproduce our previous results using the HA1 HisACAT1 or the HA6 HisACAT1 constructs (11). Additional results using cells that express C92AHA6 V-C HisACAT1 (Fig. 9B, last row) show that the Val to Cys mutation in the HA tag sequence destroys the antigenicity of the HA tag.

The findings based on the immunofluorescence data had led us to conclude that the HA6 tag is located at the luminal side of the ER (11). In light of the current PEG-mal modification data (Figs. 5, 7, and 9), the immunofluorescence data are more consistent with the alternative interpretation, not considered previously: the HA6 tag is within a folded peptide domain of ACAT1; this domain is located in the cytoplasm but is sterically hindered from exposing to the bulky HA antibody; and treating fixed cells with Triton X-100 alters the conformation/configuration of this domain such that the HA tag sequence becomes exposed to the HA antibody. In contrast, when a Cys is inserted within this domain, its reactivity toward PEG-mal is not affected by treating the microsomal vesicles with or without detergent. We attribute the difference in reactivity of this domain toward the anti-HA antibody and toward PEG-mal to the difference in size between these two reagents, with PEG-mal being the much smaller one.

**DISCUSSION**

In the current report, using a site-specific mutagenesis approach, we first presented evidence to show that His-460 is a key residue involved in ACAT1 catalysis. Using a newly developed chemical modification method coupled with Cys-scanning mutagenesis, we next elucidated the probable locations of two TMDs, TMD6 and TMD7, whose existence had not been revealed previously. We showed that His-460 is probably located within TMD7. These new results led us to propose a nine-TMD topology model for ACAT1 (Fig. 10). To relate our findings with two structurally well characterized proteins: the cytochrome bc₁ is an integral membrane protein complex that catalyzes the electron transfer from ubiquinol to cytochrome c. X-ray crystallographic evidence shows that a His residue, which plays a key role in binding the membrane-embedded substrate ubiquinol, is located within the lipid bilayer of the inner mitochondrial membrane (30). In addition, the cytosolic enzyme cholesterol sulfotransferase (SULT2B1b) can use both cholesterol and pregnenolone as its substrates. X-ray crystallographic evidence shows that a His residue, which plays a key role in interacting, through hydrogen bonding, with the 3-β-OH moiety of the steroid, and serves as a general base in catalysis (31). Thus, it is possible that His-460 in ACAT1 may interact with the 3-β-OH moiety of cholesterol and serve as a general...
Active Site Histidine Is located in TMD7 of ACAT1

base to facilitate catalysis in a very hydrophobic environment. The membrane acyltransferase superfamily includes enzymes that catalyze the transfer of organic acids, such as fatty acids, onto the -OH group of membrane-embedded substrates, such as cholesterol or dicetylpyridinium. It is conceivable that the conserved His may form a hydrogen bonding with the -OH moiety, which is part of the membrane-embedded sub-

Acknowledgments—We thank Xiaohui Lu, Jay Liu, and Ruhong Dong for sharing reagents and for discussions, and we also thank Helina Josephson for careful editing of the manuscript.

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